Regulation of Arachidonic Acid Pathway and Eosinophilic Inflammation in Chronic Rhinosinusitis/ Nasal Polyposis

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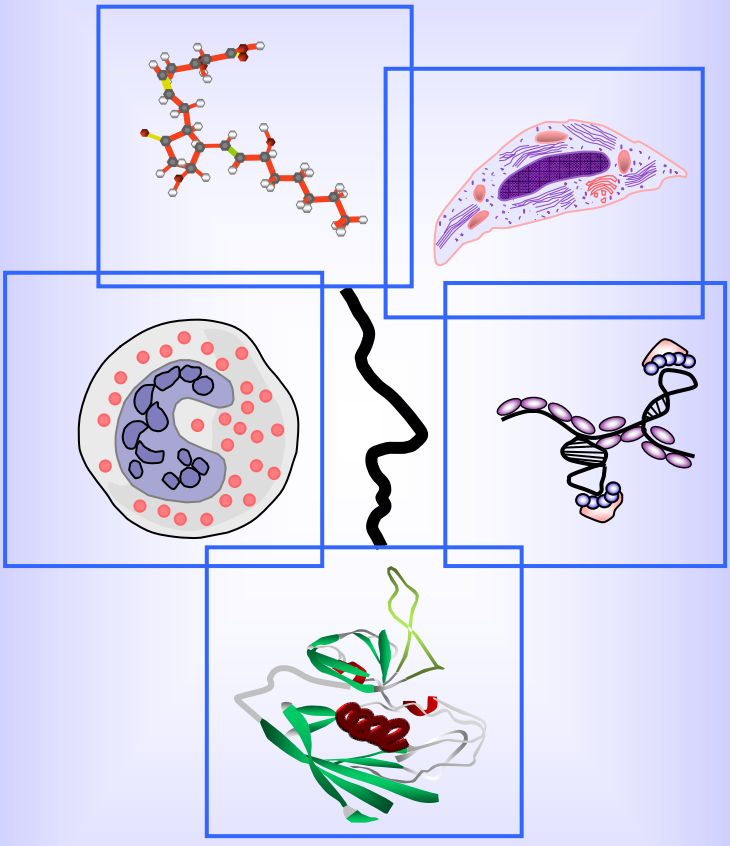

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2006

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A mi madre querida,

A mi hermana, mi padre y mi abuela,

A mi abuelo que aunque no esté, siempre vive en mi corazón,

A mi esposo David y mi querida hija Anna,

A mis angeles del alma, sin ellos esta obra no hubiera culminado,

Al entendimiento cuando creo que lo tengo totalmente entre mis manos, miro al
frente y me doy cuenta que sólo tomé un grano de arena en
una playa de dimensiones infinitas

When I believe that I have the understanding totally in my hands, I look at the front
and realize that I only took a grain of sand in
a beach of infinite dimensions

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Con amor,
With love,

Claudina

LIST OF PUBLICATIONS

1) Quantitative real time polymerase chain reaction for measurement of human interleukin-5 receptor alpha spliced isoforms mRNA. Pérez C, Vandesompele J, Vandenbroucke I, Holtappels G, Speleman F, Gevaert P, Van Cauwenberge P, Bachert C. *BMC Biotechnology* 2003, 30 (3): 1- 17.

2) Impact of RNA quality on reference gene expression stability. Pérez- Novo CA, Claeys C, Speleman F, Van Cauwenberge P, Bachert C, Vandesompele J. *Biotechniques* 2005, 39 (1): 52- 56.

3) Prostaglandin, leukotriene and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis. Pérez- Novo CA, Watelet JB, Claeys C, Van Cauwenberge P, Bachert C. *Journal of Allergy and Clinical Immunology,* 2005, 115 (6): 1189- 1196.

4) Expression of eicosanoid receptors subtypes and eosinophilic inflammation: implication on chronic rhinosinusitis. Pérez- Novo CA, Claeys C, Van Cauwenberge P, Bachert C. *Submitted (in revision process) to Respiratory Research, 2005.*

5) Aspirin sensitivity and IgE antibodies to *Staphylococcus aureus* enterotoxins in nasal polyposis: studies on the relationship. Pérez- Novo CA, Kowalski ML, Kuna P, Ptasinska A, Holtappels G, van Cauwenberge P, Gevaert P, Johannson SG, Bachert C. *International Archives of Allergy and Immunology* 2004, 133 (3): 255- 260.

6) Eicosanoid metabolism and eosinophil inflammation in nasal ployp patients with immune response to *Staphylococcus aureus* enterotoxins. Pérez- Novo CA, Claeys C, Van Cauwenberge P, Bachert C. *Accepted by the American Journal of Rhinology, October 2005*.

7) *S. aureus* enterotoxin B regulates PGE_2 synthesis, growth and migration in human nasal tissue fibroblasts. Pérez- Novo CA, Claeys C, Waeytens A, Watelet JB, Van Cauwenberge P, Bachert C. *Submitted to American Journal of Respiratory Cell and Molecular Biology, January 2006.*

LIST OF ABBREVIATIONS

15*(S)*-HETE	**1**5(*S*) - hydroperoxyeicosatetraenoic acid
5*(S)*-HETE	5(*S*)- hydroperoxyeicosatetraenoic acid
15-LO	15- lipoxygenase
5- LO	5- lipoxygenase
AA	arachidonic acid
AAM	arachidonic acid metabolism
ALX	lipoxin receptor
ATL	aspirin triggered lipoxins
BLT_1	leukotriene B_4 receptor 1
BLT_2	leukotriene B_4 receptor 2
COX-1	cyclooxygenase- 1
COX-2	cyclooxygenase- 2
CysLTs	cysteinyl leukotrienes
$CysLT_1$	cysteinyl leukotriene receptor 1
$CysLT_2$	cysteinyl leukotriene receptor 2
$cPLA_2$	cytosolic phospholipase A
IFN-γ	interferon gamma
IL-1β	interleukin-1 beta
IL-3	interleukin-3
IL-5	interleukin-5
IL-8	interleukin-8
IL-5R	interleukin-5 receptor
IL-5Rα	interleukin-5 receptor alpha subunit
ECP	eosinophil cationic protein
EP	prostanoid E
FLAP	lipoxygenase activating protein
LTA_4	leukotriene A_4
LTA_4H	leukotriene A_4 hydrolase
LTB_4	leukotriene B_4
LTC_4/ D_4/ E_4	leukotriene C_4/ D_4/ E_4
LTC_4S	leukotriene C_4 synthase
LTs	leukotrienes
LXA_4	lipoxin A_4
LXs	lipoxins
MHC-II	major histocompatibility complex class II
NSAIDs	non- steroidal anti- inflammatory drugs
PBM	peripheral blood monocytes
PGE_2	prostaglandin E_2
PMNs	polymorphonuclear cells
TGF-1β	transforming growth factor-beta 1
SAgs	superantigens
SEA	*S. aureus* enterotoxin A
SEB	*S. aureus* enterotoxin B
SEs	*S. aureus* enterotoxins

TABLE OF CONTENT

SUMMARY

Eosinophilic inflammation, imbalance of eicosanoid production and bacterial infection has been considered crucial factors in the pathophysiology of upper airway diseases. The aim of this work was to study the regulation of the eicosanoid pathway and its relation with the eosinophilic inflammatory process in chronic rhinosinusitis/nasal polyposis. Subsequently we investigated the possible role of an immune response against *Staphylococcus aureus* in the regulation of these inflammatory mechanisms in nasal tissue and structural cells.

Our first objective was to develop a reliable and accurate real-time PCR methodology for mRNA quantification of the two spliced receptor variants of the IL-5 receptor alpha. Extensive work has covered the regulation of the IL-5 receptor at protein level. However, the lack of a sensitive gene expression assay difficult the detection of small changes at its transcription level. We developed an assay with efficiency higher than 95% and a coefficient of variation less than 25% that allowed us to study the regulation of the splice variants of this receptor and confirm previous results from protein analysis.

Furthermore, we analyzed the expression stability of internal reference genes and the influence of tissue degradation in their expression profile in chronic rhinosinusitis/ nasal polyp tissue. This work showed that the stability of reference genes is different in chronic rhinosinusitis and nasal polyp mucosa. We were able to select adequate internal reference genes for accurate and reliable gene expression studies in these tissues.

Once the previous objectives were achieved, we study the eicosanoid and eicosanoid receptors regulation in chronic rhinosinusitis/ nasal polyposis and their possible role in aspirin intolerance. Here we showed that CysLTs and CysLTs receptor expression increased with severity of inflammation, in contrast to PGE_2 that was down-regulated and inversely related to inflammation. Results of these studies suggest that changes in eicosanoid production may occur in chronic rhinosinusitis in absence of aspirin sensitivity syndrome and that they may not be specific but a parallel phenomenon linked to inflammation. Furthermore, EP_2 and EP_4 receptor expression was increased in chronic rhinosinusitis and nasal polyp subjects in contrast to EP_1 and EP_3, which were down regulated in the polyp group, suggesting a distinctive role of these receptors in the pathophysiology of nasal polyposis.

Finally, based on our previous findings and parallel work of our group, we studied the influence of *S. aureus* enterotoxins in the regulation of both eosinophilic inflammatory and eicosanoid pathways. We first show that nasal polyposis/aspirin intolerance was associated with increased concentrations of eosinophil-related mediators, as well as of IgE antibodies to *S. aureus* enterotoxins. Furthermore, eicosanoid release evaluated in nasal polyp patients showed an increase of leukotrienes and lipoxin A_4 in patients with immune response

against *S. aureus* enterotoxins, which seem to be correlated to the enterotoxin-derived inflammatory reaction and unrelated to asthma and allergy conditions.

These findings were extended to structural cells (fibroblasts). These *in vitro* experiments demonstrated that *S. aureus* enterotoxin B could regulate the production of PGE_2 and influence important cell physiological mechanisms like growth and migration. The results of our studies provide new insights about the molecular interactions and the role of bacterial infection in the regulation of two crucial inflammatory mechanisms operating in airway human diseases, the eicosanoid biosynthetic and the eosinophilic pathways

SAMENVATTING

Eosinofiele inflammatie, verstoord evenwicht in de productie van eicosanoïden en bacteriële infectie worden beschouwd als cruciale factoren in de pathofysiologie van aandoeningen van de bovenste luchtwegen. Dit werk had als doel de regulatie van de eicosanoïde pathway en zijn relatie met het eosinofiele inflammatoire proces bij chronische rhinosinusitis/nasale polypose te bestuderen. Verder onderzochten we ook de mogelijke rol van de immuunrespons tegen *S. aureus* in de regulatie van deze inflammatoire processen in nasaal weefsel en structurele cellen.

Ons eerste doel was het ontwikkelen van een betrouwbare en accurate real-time PCR methodologie voor mRNA kwantificering van de twee spliced varianten van de IL-5 alfa receptor. Intensief onderzoek ging uit naar de regulatie van deze receptor op proteïne niveau. Het gebrek aan een gevoelige gen-expressie analysemethode bemoeilijkte echter de detectie van kleine veranderingen op transcriptie niveau. We slaagden er uiteindelijk in een methode te ontwikkelen met een efficiëntie groter dan 95% en een variatiecoëfficiënt lager dan 25%, die ons eveneens toeliet de vroegere resultaten van proteïneanalysen te bevestigen.

Bovendien analyseerden we de expressiestabiliteit van interne referentiegenen en de invloed van weefseldegradatie op hun expressiepatroon in chronisch rhinosinusitis-/neuspoliepweefsel. Dit werk toonde aan dat de stabiliteit van referentiegenen verschillend is in de mucosa van chronische rhinosinusitis en nasale polypose. Bij deze studies konden we de beste interne referentiegenen selecteren voor accurate en betrouwbare gen-expressies in deze weefsels.

Eens deze doelstellingen bereikt werden, gingen we over tot onderzoek naar de regulatie van eicosanoïden en eicosanoïdreceptoren bij chronische rhinosinusitis/nasale polypose en hun mogelijke rol bij aspirine-overgevoeligheid. We toonden aan dat de expressie van CysLTs en CysLTs receptoren toeneemt met de ernst van inflammatie, in tegenstelling tot PGE_2, dat was down-gereguleerd en omgekeerd gerelateerd aan de ernst van inflammatie. De resultaten van deze studie suggereren dat veranderingen in eicosanoïdproductie kunnen voorkomen bij chronische rhinosinusitis in afwezigheid van aspirinegevoeligheid en dat zij geen specifiek maar een parallel fenomeen zouden zijn gelinkt aan eosinofiele inflammatie. Bovendien was de expressie van de EP_2 en EP_4 receptoren verhoogd bij chronische rhinosinusitis, in tegenstelling tot EP_1 en EP_3, die down-gereguleerd waren bij patiënten met nasale polypose. Dit suggereert een specifieke rol van deze receptoren in de pathophysiologie van nasale polypose.

Op basis van deze bevindingen, en in de lijn van het onderzoek van ons labo, gingen we ook de invloed na van *S. aureus* enterotoxines op de regulatie van eosinofiele inflammatoire en eicosanoïde pathways. We

toonden aan dat nasale polypose/aspirine-overgevoeligheid geassocieerd is met verhoogde concentraties van eosinofiel-gerelateerde mediatoren, alsook van IgE antilichamen tegen *S. aureus* enterotoxines. We konden echter geen directe impact aantonen van deze enterotoxines op de manifestatie van aspirine-overgevoeligheid. Daarnaast konden we vaststellen dat de release van eicosanoïden bij patiënten met nasale polypose en immuunrespons tegen *S. aureus* enterotoxines, zich kenmerkt door een toename aan leukotrienen en lipoxin A_4. Die zijn op hun beurt gecorreleerd aan de enterotoxine-gemedieerde inflammatoire reactie, maar niet gerelateerd aan astma en allergie.

We breidden onze bevindingen uit naar structurele cellen (fibroblasten). Met een *in-vitro* experiment toonden we aan dat *S. aureus* enterotoxine B de productie van PGE_2 kan reguleren en belangrijke cellulaire fysiologische mechanismen zoals groei en migratie kan beïnvloeden. De resultaten van onze studies brengen nieuwe inzichten in de moleculaire interacties en in de rol van bacteriële infectie in de regulatie van twee cruciale inflammatoire mechanismen bij humane luchtwegpathologie: de eosinofiele en de eicosanoïde biosynthetische pathway.

RÉSUMÉ

L'inflammation éosinophilique, un déséquilibre dans la production d'éicosanoïdes et l'infection bactérienne ont été considérés comme des facteurs cruciaux dans la physiopathologie des maladies des voies respiratoires supérieures. Le but de ce travail était d'étudier la régulation des voies des éicosanoïdes et de sa relation avec les processus d'inflammation éosinophilique dans la sinusite chronique/polypose naso-sinusienne. Par la suite, nous avons investigués la réponse immunologique possible contre *S. aureus* dans la régulation des mécanismes inflammatoires des tissus nasaux et des cellules structurelles.

Notre premier objectif était de développer une méthode reproductible et précise de real time PCR pour la quantification d'ARNm de deux variantes scindées d'un récepteur. Un travail important a décrit la régulation de ce récepteur au niveau protéïque. Cependant, le manque de test sensible pour l'expression génique a rendu difficile la détection de petits changements au niveau transcriptionnel. Nous avons développé un test avec une efficacité supérieure à 95 % et un coefficient de variation inférieur à 25 % qui nous permet de confirmer les résultats précédents de l'analyse protéique.

De plus, nous avons analysé la stabilité d'expression des gènes de référence internes et l'influence du dommage tissulaire sur leur profil d'expression dans la sinusite chronique et la polypose nasosinusienne. Ce travail a démontré que la stabilité des gènes de référence est différente dans la sinusite chronique et la polypose naso-sinusienne. Nous sommes capables de sélectionner les meilleurs gènes de référence internes pour des études d'expression géniques reproductibles et précis dans ces tissus.

Une fois les premiers objectifs atteints, nous avons étudié la régulation des éicosanoïdes et de leur récepteurs dans la sinusite chronique /polypose naso-sinusienne et de leur rôle possible dans l'intolérance à l'aspirine. Nous avons démontré ici, que l'expression des CysLTs et de leurs récepteurs CysLTs augmentait avec la sévérité de l'inflammation, au contraire de la PGE_2 qui était sous exprimée et inversément corrélée à l'inflammation. De manière intéressante, l'expression des récepteurs EP_2 et EP_4 était augmentée dans la sinusite chronique à la différence des récepteurs EP_1 et EP_3 qui sont sous exprimés chez les patients polypeux. Les résultats de ces études suggèrent des changements dans la production d'éicosanoïdes dans la sinusite chronique en absence d'intolérance d'aspirine et qu'ils ne sont pas spécifiques mais seraient un phénomène parallèle lié à l'inflammation.

Enfin, basés sur nos résultats précédents et sur des travaux parallèles dans notre groupe, nous nous sommes posés la question de l'influence des entérotoxines de *S. aureus* dans la régulation, d'une part, de l'inflammation éosinophilique et, d'autre part, de la cascade éicosanoïque. Nous avons d'abord montré que la polypose naso-sinusienne/ intolérance à l'aspirine était associée à des concentrations plus élevées de médiateurs éosinophiliques, ainsi que des anticorps IgE contre les entérotoxines du *S. aureus*. Cependant,

nous n'avons pas trouvé d'impact direct de ces entérotoxines sur la manifestation de l'intolérance à l'aspirine. De plus, la libération d'éicosanoïdes évaluée chez des patients polypeux montrait une augmentation de leucotriènes et de LXA_4 chez les patients ayant une réponse immunitaire contre les entérotoxines de *S.aureus* qui semble corrélée à la réaction inflammatoire entérotoxine-dérivée et non relatée à l'asthme ou des conditions allergiques.

Ces éléments furent étendus aux cellules structurelles de type fibroblaste. Cette expérimentation *in vitro* a démontré que l'entérotoxine B du *S. aureus* peut réguler la production de PGE_2 et influencer des mécanismes importantes telles les croissances et la migration. Les résultats de nos études proposent une vision nouvelle des interactions moléculaires et du rôle de l'infection bactérienne dans la régulation de deux mécanismes inflammatoires cruciaux intervenant dans les maladies respiratoires humaines: la biosynthèse des éicosanoïdes et la marche éosinophilique.

PART- I

INTRODUCTION

CHAPTER 1

CHRONIC RHINOSINUSITIS AND NASAL POLYPOSIS, DEFINITION AND IMMUNOLOGICAL ASPECTS

CLINICAL DEFINITION, HISTOMORPHOLOGY AND DIAGNOSIS

Chronic rhinosinusitis

Rhinosinusitis is a group of disorders characterized by inflammation of the mucosa of the nose and paranasal sinuses with a prevalence rate of about 10- 30% of the population in Europe and 15% of the population in the USA (Moss et al., 1986). Rhinosinusitis can be classified according to the temporal course of the disease (duration and frequency of episodes) as: *a)* acute rhinosinusitis, *b)* recurrent acute or chronic rhinosinusitis, and *c)* acute exacerbations of chronic sinusitis (Lanza et al., 1997).

Chronic rhinosinusitis has been defined as an inflammatory reaction of the paranasal mucous membrane, which might be based on viral or bacterial infections and is mainly characterized by an accumulation of inflammatory cells in the lamina propria, with some unspecific lesions such as edema, fibrosis or epithelial degradation (Van Cauwenberge et al., 2004). Diagnosis of chronic rhinosinusitis is based on the presence of typical sings such as discoloured nasal drainage arising from the nasal passages, oedema, or erythema of the middle meatus that persist longer than 12 weeks or more. This accompanied by a pathologic CT- scan showing diffuse mucosal thickening, opacification or swelling of the ethmoidal and maxillary mucosa with bilateral obstruction of the osteomeatal complex, but without polyp formation visible during nasal endoscopy or during surgery (Benninger et al., 2003).

Chronic rhinosinusitis/ nasal polyposis

Nasal polyposis is defined as a multifactorial disease consisting of tissue (nasal and sinus mucosa) infiltration by inflammatory cells, predominantly eosinophils, and the formation of pseudocysts with plasma exudation and albumin retention (Van Cauwenberge et al., 2004). Clinically, its diagnosis is based on an endoscopy and CT- scan of the paranasal sinuses showing the presence of endoscopically visible bilateral polyps growing from the middle nasal meatus into the nasal cavities, and affecting ethmoidal and maxillary sinuses (Benninger et al., 2003). The typical history of a patient having nasal polyps is a “cold” that persist over months or years, with nasal obstruction and discharge as the most prominent symptoms. After some time, hyposmia or anosmia and additional complaints appear (Drake e al., 1984).

CO- MORBIDITIES

Asthma

The association between sinusitis and asthma has been appreciated since long. Up to 70% of asthmatic patients also suffer from chronic rhinosinusitis (Annesi-Maesano et al., 1999) and there is circumstantial evidence that chronic rhinosinusitis may be linked to chronic lung disease, especially severe asthma (Rossi et al., 1994). However, the mechanisms by which sinusitis influences asthma are not well understood. This link between these diseases is supported by studies showing that abnormal sinus radiography are frequent in patients with asthma exacerbations, and that drug management of sinusitis (Rossi et al., 1994) or sinus surgery results in a significant improvement in asthma symptoms (Senior et al., 1999). In addition, nasal polyposis can also be frequently found in association with lower tract respiratory disorders, such as asthma and non-specific bronchial hyperreactivity (Lamblin et al., 1997). Up to 70% of patients with nasal polyposis have been reported to have asthma (Larsen et al., 1996). In addition, several studies have demonstrated the manifestation of bronchial hyper-reactivity in nasal polyp patients, even in those with no history of asthma (Kordash et al., 1975). Interestingly, patients with nasal polyposis and asymptomatic bronchial hyperreactivity have an eosinophilic inflammation similar to that observed in asthmatic patients with nasal polyposis, whereas patients with nasal polyposis without bronchial hyperreactivity do not have eosinophilic lower airways inflammation (Lamblin et al., 1999).

Aspirin intolerance

The aspirin intolerance triad is defined as a steroid-dependent asthma and nasal polyposis (rhinosinusitis) that starts with a prolonged episode of nasal congestion, rhinorrea, and hyposmia with persistent mucosal inflammation, followed by nasal polyposis, bronchial asthma and then aspirin intolerance after ingestion of aspirin and other non-steroidal anti-inflammatory drugs (Samter et al., 1968). About 80% of the patients registered at the European Network on Aspirin-Induced Asthma (AIANE) complain of nasal blockage accompanied by rhinorrhea and about 50% of patients are dependent on systemic and inhaled corticosteroids (Szczeklik et al., 1999). Between 40 and 80% of patients with aspirin intolerance, suffer from polyposis, and about 15% of polyp patients have been reported as aspirin intolerants (Settipane et al., 1996). Clinically, aspirin-intolerant rhinosinusitis is characterised by an increased eosinophil count in blood, nasal and bronchial mucosa (Szczeklik et al., 1999). This pathology has been often associated with allergy and high levels of local IgE (Zhu et al., 1997), however, an IgE-mediated mechanism has not been demonstrated, and atopy does not seem to influence the risk to develop the disease (Szczeklik et al., 2000). In addition, alterations in arachidonic acid metabolism have been suggested as one of the main factors driving the disease

(Szczeklik et al., 1999). The intake of aspirin inhibits the constitutively expressed cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2) enzymes. This inhibition results in a decrease of spontaneous synthesis of anti-inflammatory prostanoids like prostaglandin E_2 (PGE_2) leading to an enhanced 5- lipoxygenase (5-LOX) activity (Pavord et al., 1995). Overexpression of leukotriene C_4 synthase (LTC_4S) has been demonstrated in eosinophils and mast cells, resulting in an overproduction of cysteinyl leukotrienes (Cys-LTs), which may be released into the airways causing nasal congestion, rhinorrhoea, bronchoconstriction and induce eosinophilia (Cowburn et al., 1998). So far, due to the lack of a validated clinical laboratory test, the diagnosis of this syndrome is based on oral, bronchial, or nasal provocation tests (Schapowal et al., 1995). In addition, there are conservative treatment possibilities consisting of *1)* avoidance of aspirin and other non-steroidal antinflammatory drugs (NSAIDs), *2)* oral and/ or topical glucocorticosteroids, *3)* leukotriene receptor antagonists or leukotriene synthesis inhibitors and *4)* in selected cases, aspirin desensitization. The course of the disease is independent from aspirin intake, indicating that factors other than aspirin could be responsible for this disease, which, with a few exceptions, remains present during the whole life.

Allergy

Several hypotheses have been postulated in order to explain the possible role of allergy in rhinosinusitis and nasal polyposis. Most of them tried to explain the link between allergy, tissue edema and sinus obstruction and inflammation, but without succeeding due to the lack of presence of sinusitis in some patients with seasonal rhinitis. However, later, it was suggested that perennial allergic (and non-allergic) rather than seasonal rhinitis might predispose to sinusitis (Berrettini et al. 1999). Due to the similarity of the clinical symptoms (late-onset asthma, elevated local IgE in polyp fluid and a pronounced tissue eosinophilia) between allergic rhinitis and nasal polyposis, allergy has also been considered a possible cause of nasal polyposis. However, the low percentage of atopic compared to non-atopic patients manifesting the disease (Caplin et al., 1971 and Settipane et al., 1996) and the lack of association between nasal polyp symptoms and skin prick test positivity exclude this theory. Accordingly, so far allergy does not seem to be a predisposing factor for rhinosinusitis or nasal polyposis.

Cystic fibrosis

This is an autosomal recessive genetic disorder considered as one of the most common fatal inherited diseases. The cause of the disease is related to a mutation in the gene regulating the chloride transport in epithelial cells affecting the apical chloride transport, leading to an increase of mucus secretion (Yamaya et al., 1991). It has been suggested, that mutations in the gene responsible for cystic fibrosis may be associated

with the development of chronic rhinosinusitis or nasal polyposis (Wang et al. 2000). Approximately 10% to 20% of cystic fibrosis patients have been eventually reported to require surgical treatment of their sinuses (Batsakis et al., 1996). Additonally, sinusitis is frequently detected in radiological investigations of patients with cystic fibrosis and the incidence of nasal polyps in this group of patients range from 6% to 48% (Cepero et al., 1987). In addition, bacteriology of maxillary sinuses has demonstrated bacterial infection in 95 % of all cases. The predominant organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Hemophilus influenzae* and anaerobes (Hadfield et al., 2000). Unfortunately, the response of these patients to antimicrobial therapy often is insufficient when compared to non- cystic fibrosis patients. However, it has been showed that the size of polyp is reduced when nasal corticosteroids are used (Hadfield et al., 2000). Finally, surgery is another alternative for these patients. However, because of the persistent nature of the disease it only offers a transient effect. Surgery should only be performed in case of sufficient symptomsand followed by a post- operative follow- up.

PATHOMECHANISMS AND IMMUNOLOGICAL ASPECTS

Chronic rhinosinusitis

The mucosal lining in chronic sinusitis is characterized by basement membrane thickening, goblet cell hyperplasia, subepithelial edema, and mononuclear cell infiltration. In contrast to nasal polyposis, tissue eosinophilia is not a hallmark of chronic sinusitis without polyp formation. The chronic rhinosinusitis mucosa shows neutrophils as main inflammatory cells with only a few eosinophils, mast cells and basophils.

Studies of the cytokine profile in this disease have shown high levels of interleukin-1 beta (IL-1β) in some extravascular polymorphonuclear cells (PMNs) and mononuclear lymphocytes, and suggests that this cytokine induce the expression of ICAM-1 and E- selectin and stimulate the infiltration of PMNs in chronic rhinosinusitis (Tokushige et al., 1994). Interleukine-3 (IL-3) and interleukin-8 (IL-8) seem also to play a dominant role in this pathology. IL-3 displays multi-colony-stimulating factor activities and stimulates the differentiation and activation of macrophages, neutrophils, mast cells and eosinophils (Rudack et al., 1998). Its role in this disease may be related to local defense mechanisms and in the repair of chronically inflamed sinus mucosa by inducing the release of various mediators (Persson et al., 1997). In addition, the contribution of this cytokine to the development of fibrosis and mucosa thickening has been also suggested (Persson et al., 1997). IL-8 has been demonstrated in nasal discharge from chronic sinusitis patients (Rhyoo et al., 1999) and constitutes a potent neutrophil chemoattractant. TGF-1β mRNA and protein levels are also high and related to fibrosis in chronic rhinosinusitis patients compared to normal nasal mucosa and nasal polyp tissue (Watelet et al., 2004). Although great efforts have been done to elucidate the cytokine and molecular pattern

involved in the manifestation of chronic rhinosinusitis little has been achieved. In the future, more structural and functional studies are needed to finally define the causative factors of this disease.

Chronic rhinosinusitis/ nasal polyposis

Although a large number of studies have been focused on the pathophysiology of nasal polyposis, a full understanding of the underlying mechanisms has not been achieved yet. Recent insights suggest regulation of eosinophil chemotaxis, activation, and survival, together with histopathology changes (such as pseudocyst formation) as main factors contributing to the disease. Histomorphologically, polyp tissue reveals frequent epithelial damage, a thickened basement membrane, and edematous to sometimes fibrotic stromal tissue, with a reduced number of vessels and glands (Kakoi et al., 1987). The stroma of mature polyps is mainly characterized by edema and pseudocystic formations surrounded by fibroblasts and infiltrating inflammatory cells, predominantly activated eosinophils, which are present in about 80% of nasal polyps (Stoop et al., 1993) and are localized mainly around the vessels, glands and beneath of the mucosal epithelium (Kakoi et al., 1987). In addition, albumin and other plasma proteins have also been found deposited within the pseudocysts, adjacent to the eosinophil infiltration, which suggests a central deposition of plasma proteins, regulated by the subepithelial eosinophilic inflammation, as a pathogenetic principle of polyp formation and growth (Persson, 1991).

An increased production of cytokines/chemokines like granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), RANTES and eotaxin have been demonstrated in the nasal polyp tissue. This can actively contribute to migration and survival of eosinophils and extracellular matrix breakdown (Mullol et al., 1995; Bachert et al., 2000). IL-5 is one of the key cytokines in the pathophysiology of nasal polyposis. This cytokine is significantly increased in nasal polyp patients compared to healthy subjects and its expression has been found to correlate with eosinophilic cationic protein (ECP) in the polyp tissue suggesting its autocrine role in the activation of eosinophils (Bachert et al., 2001). This role for this cytokine was later supported by the finding that treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody, resulted in eosinophil apoptosis and decreased tissue eosinophilia (Simon et al., 1997). Furthermore, recent studies have shown that nasal polyps also express high levels of RANTES and eotaxin, which are potent eosinophil chemoattractants (Bartels et al., 1997).

Adhesion molecules like intercellular adhesion molecule (ICAM-1), E-selectin and P-selectin are also highly expressed in nasal polyp endothelium (Symon et al., 1994) and have been suggested as crucial factors implicated in the development of nasal polyps. In 1995, Jahnsen and col., reported an elevated expression of VCAM-1 in nasal polyp tissue vessels that correlated with the number of eosinophils. This phenomenon was

later observed to be decreased after treatment with topical glucocorticosteroids (Tingsgaard et al., 1999), emphasizing the importance of the adhesion molecules in the migration, activation and functionality of eosinophils (Palframan et al., 1998) and hence their potential role in nasal polyp formation.

Within the nasal polyp structure we can find also a large quantity of extracellular fluid contributing to edema formation. Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis and vascular permeability and has been found highly expressed in the polyp tissue. This factor together with transforming growth factor-beta (TGF-β) has been shown to contribute to the edema and increased angiogenesis in nasal polyps (Wittekindt et al., 2002). In addition, TGF-β can modulate fibroblast function contributing to eosinophil infiltration and stromal fibrosis (Lee et al., 2003). More, other mediators like albumin, histamine and immunoglobulins (IgE and IgG) are increased in nasal polyps (Bachert et al, 2000). Local IgE production can contribute to the increased recurrence of nasal polyps after surgical polypectomy via the IgE-mast cell-FcεRI cascade (Settipane et al., 1996). Finally, interactions between mast cells, T-cells, epithelial cells and fibroblasts play a crucial role in the persistent eosinophilic inflammation observed in polyps (Pawankar et al., 2003). In summary, although nasal polyposis has been defined as a multifactorial disease, chronic persistent inflammation seems to be the major factor driven- disease irrespective of the aetiology.

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CHAPTER 2

ARACHIDONIC ACID (EICOSANOID) METABOLISM

GENERAL OVERVIEW

The term *eicosanoid* stems from the greek word "*eikos"*, meaning twenty, and refers to a collective group of compounds that have twenty carbon atoms. Eicosanoids are a very important group of compounds regulating crucial body functions and they include the prostaglandins (PGs), prostacyclins, thromboxanes (TXs), and leukotrienes (LTs). The study of these molecules, which culminate in the discovery of one of the most important pathways for lipid mediators began in 1930 when Burr discovered the essential fatty acids (Burr et al., 1930) and Von Euler in 1934 identified a factor with fatty acid properties and vasodepressor and smooth muscle–stimulating activity that was termed "prostaglandin (Von Euler, 1935). Thirty years later Bergström and Samuelsson linked these observations to the structures of the "classical" prostaglandins and demonstrated that they were produced from an essential fatty acid: the arachidonic acid (AA) (Bergström et al., 1964), initiating in this way the era of eicosanoid research. In 1971, Vane discovered that analgesic, antipyretic and anti-inflammatory aspirin-like drugs could inhibit prostaglandin biosynthesis (Vane, 1971) and soon thereafter, the platelet pro-aggregator and vasoconstrictor molecule thromboxane A_2 was elucidated, followed by the isolation of its antagonist, a vascular wall-synthesized prostacyclin. In addition, the Nobel Prize for the identification of 100 eicosanoids was awarded in 1982, which strongly pushed forward the research in this field. Furthermore, Serhan et al. identified lipoxins, also derivatives of the arachidonic acid cascade (Serhan et al., 1984).

All mammalian cells except erythrocytes synthesize eicosanoids. These molecules function locally at the site of synthesis at minute concentrations and a receptor-mediated G-protein linked signalling pathway mediates their action. They have antagonistic roles like hormones, and regulate most of physiological functions such as blood pressure, inflammatory responses (Busse, 1998), uterine contractions during birth (Olson, 2003), pain, the release of gastric acid (Halter et al., 1991), the constriction and dilation of airways in the lungs and blood vessels (Holgate et al., 2003) and many others. Two main pathways (Figure 1) are involved in the biosynthesis of eicosanoids: *1)* the prostaglandins and thromboxanes, synthesized by the cyclic pathway and *2)* the LTs and lipoxins by the linear pathway. The cyclic pathway is initiated through the action of prostaglandin G/H synthases (also called prostaglandin endoperoxide synthetase), which possesses two activities, cyclooxygenase (COX) and peroxidase. The linear pathway is initiated through the action of lipoxygenases, with the enzyme 5-LOX giving rise to the LTs and the 15-LOX responsible for the production of lipoxins.

LEUKOTRIENES BIOSYNTHETIC PATHWAY

Leukotrienes are predominantly produced by inflammatory cells like polymorphonuclear leukocytes, macrophages, and mast cells. Immune complexes, bacterial peptides, and other stimuli may activate the

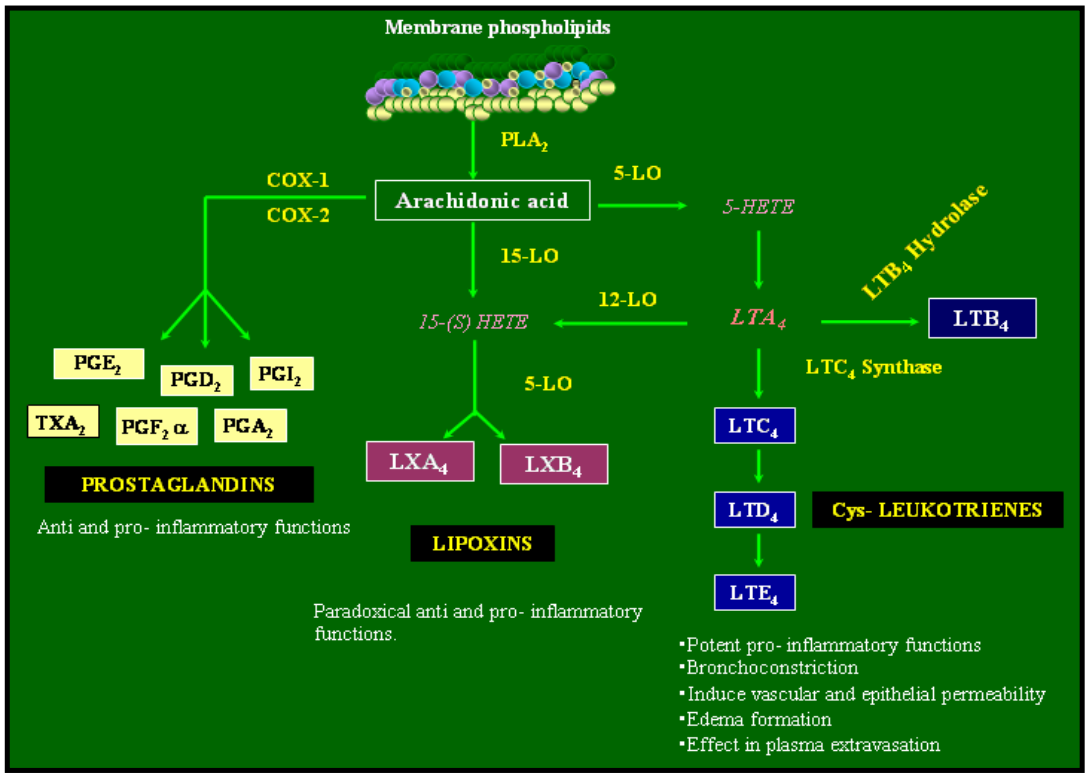

Figure 1. Arachidonic acid cascade leading to the formation of peptide (cysteinyl) leukotrienes, lipoxins, prostaglandins and tromboxanes.

biosynthesis of these compounds. Biosynthesis of LTs is initiated with the translocation of cytosolic phospholipase A_2 ($cPLA_2$) to the nuclear membrane with the consequent release of AA (Clark et al., 1991), which can be further converted to leukotriene A_4 (LTA_4) by the enzyme 5-lipoxygenase (5-LOX) (Peters-Golden et al., 2001) as shown in figure 2. 5-LOX, a non- heme iron dioxygenase, and the key enzyme in this cascade, can translocate from either the nuclear or cytoplasmic compartment depending on the cell type (Peters-Golden et al., 2001). The translocation of this enzyme seems to occur via the 5-LOX activating protein (FLAP), which is a nuclear envelope integral protein that acts as an AA transfer protein (Funk, 2001) for conversion to LTA_4 (Mancini et al., 1993; Rouzer et al., 1986). At that stage, LTA_4 can undergo 3 different types of transformation depending on the cellular context: *a)* cytoplasmic and /or *b)* nuclear hydrolytic attack by leukotriene A_4 hydrolase (LTA_4H) to yield leukotriene B_4 (LTB_4) (Radmark et al., 1984), or *c)* conjugation with glutathione to form leukotriene C_4 (LTC_4) at the nuclear envelope, reaction that is catalyzed by glutathione transferase LTC_4 synthase (LTC_4S). In this reaction, LTC_4 is metabolized extracellularly (Yoshimoto et al., 1988 and Lam et al., 1989) and cleaved by specific enzymes to produce the extracellular active metabolites LTD_4 and LTE_4 (Bergström et al., 1981), comprising the cysLTs, described many years ago as "slow-reacting substance of anaphylaxis".

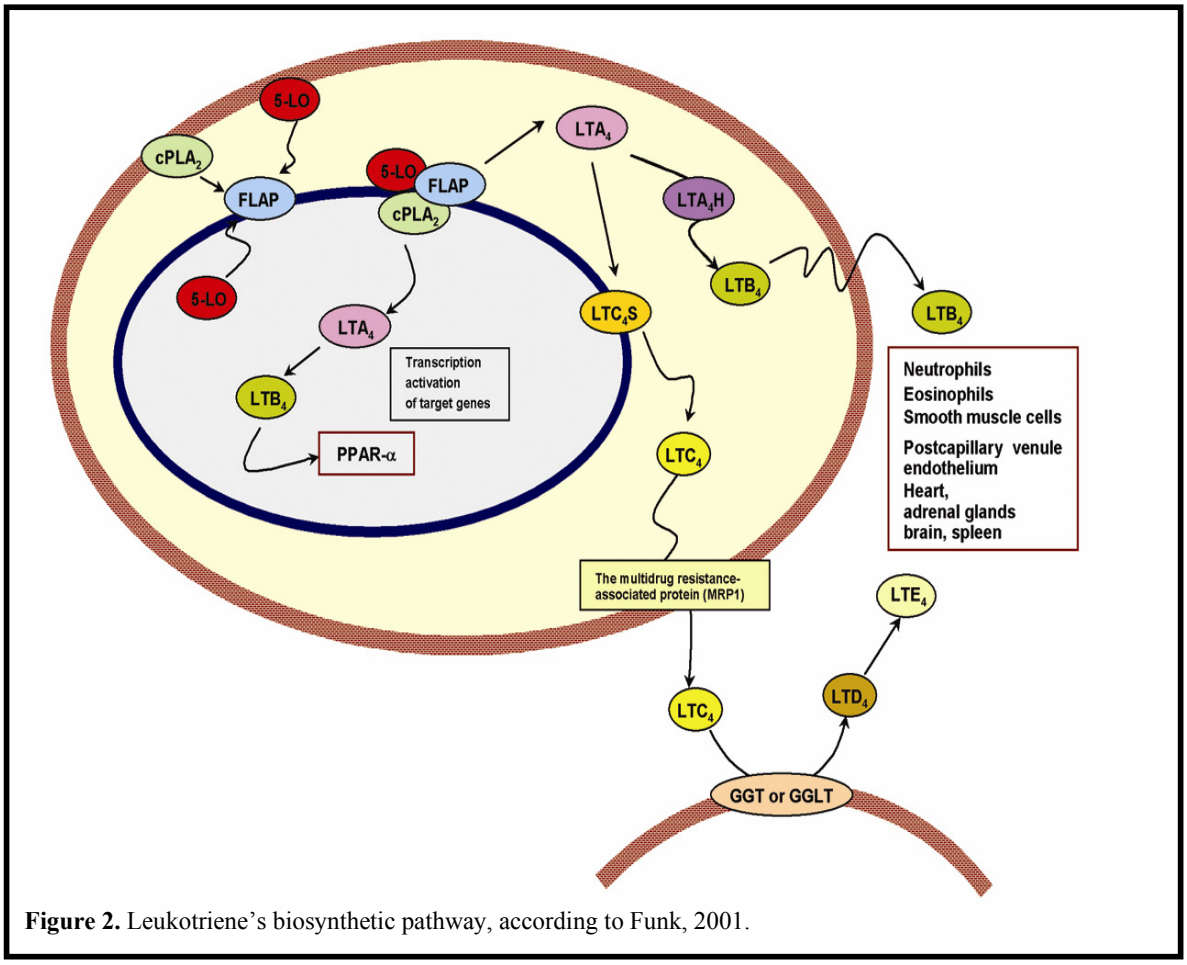

Figure 2. Leukotriene's biosynthetic pathway, according to Funk, 2001.

PROSTAGLANDINS BIOSYNTHETIC PATHWAY

Prostaglandins are formed by most cells in the body and act as autocrine and paracrine lipid mediators (i.e., they signal at or immediately adjacent to their site of synthesis) (Funk, 2001). These molecules are not stored but rather synthesized *de novo* from membrane-released AA after cell activation by mechanical trauma, cytokines, growth factors, and other stimuli.

Prostaglandin's synthesis (Figure 3) starts at the endoplasmic reticulum and nuclear membrane when AA released by $cPLA_2$ is metabolized by prostaglandin H synthase-1 or cyclooxygenase 1 (COX–1) and/ or prostaglandin H synthase- 2 or cyclooxygenase- 2 (COX– 2) to form first prostaglandin G_2 (PGG_2) and after prostaglandin H_2 (PGH_2) (Rocca et al., 2002). Then, cell-specific prostaglandin synthases catalyze the conversion of PGH_2 to biologically active end products known as prostanoids. These prostanoids include: prostaglandin E_2 (PGE_2) produced by microsomal prostaglandin E synthase (Jakobsson et al., 1999); prostaglandin F_2 (PGF_{2a}), prostaglandin D_2 (PGD_2) by PG synthases found in the uterus, prostaglandin I_2

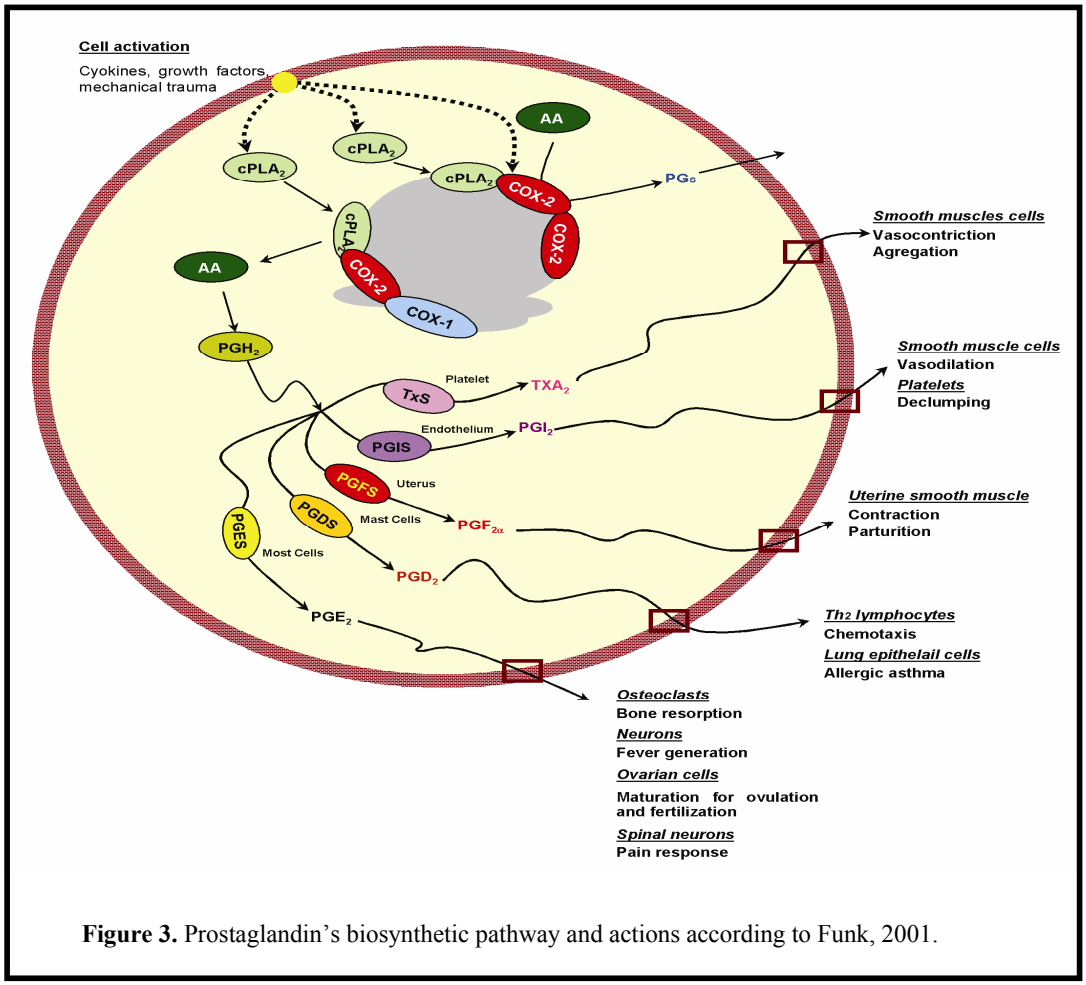

Figure 3. Prostaglandin's biosynthetic pathway and actions according to Funk, 2001.

(PGI_2) by prostacyclin synthase, located in the brain and and tromboxane A_2 (TXA_2) by tromboxane synthase located in platelets, macrophages and mast cells (Helliwell et al. 2004).

Interestingly, although both cyclooxygenases isozymes catalyze the same reactions and share approximately 60% structural and atomic homology, they are encoded by two different genes located on distinct chromosomes and have different functions even within the same cell type (FitzGerald et al., 2001). COX-1 is thought to be expressed constitutively in gastric mucosa, kidney, platelets, and vascular endothelial cells and COX-1-dependent prostaglandin production is thought to control a number of physiologic ''housekeeping'' functions in the body. The concentration of this isoform remains relatively stable, although small changes in expression can occur after stimulation with hormones, growth factors or during development (Smith et al., 2001). The expression of COX-2, in contrast, has been shown to be inducible in different cell types in response to pro-inflammatory stimuli like cytokines, endotoxins, growth factors or tumor promoters (Smith et al., 2001). Although constitutive expression of this enzyme has been found in certain regions of the brain,

reproductive tissues, kidney and thymus (Smith et al., 2001); COX-2- dependent prostaglandins play a major role in regulating inflammatory and cell proliferation events (Rocca et al., 1999).

LIPOXINS BIOSYNTHETIC PATHWAY

Biosynthesis of lipoxins (LXs) occurs via cell-cell interactions and depends on the cells and enzymes present therein and can be subjected to modulation by cytokines. In addition, these compounds can also be synthesized when the precursor molecules 15- hydroperoxyeicosatetraenoic acids (15-HETEs) accumulate within the cell membrane, in the absence of transcellular lipoxygenase activity (Brezinski et al., 1990). There are three biosynthetic pathways, which end in the formation of these molecules (Figure 4). The first of the major routes involves the activities of the 5-LOX and 12-LOX enzymes. 5-LOX, present in cells of myeloid lineage (e.g. polymorphonuclear neutrophils) generates LTA_4 from AA, which is taken up by platelets and metabolized to form lipoxin (LXA_4) through the oxygenase activity of 12-LOX present in these cells (Edenius et al., 1990; Serhan et al., 1990). A second route is via the action of 15-LOX present in epithelial cells and monocytes (Serhan et al., 1984). In this reaction, molecular oxygen is inserted into carbon 15 of AA (C20:4), predominantly in the *S* configuration to yield the 15(*S*)- hydroperoxyeicosatetraenoic acid (15*(S)*- HETE) that serve as a substrate for neutrophil 5-LOX that through the actions of epoxide hydrolases in leukocytes convert it to LXA_4 and LXB_4 (Serhan et al., 1984).

The third and additional route of LXs formation is via the aspirin-triggered 15-epi-LX (ATLs) pathway (Claria et al., 1995). In this pathway, aspirin induces acetylation of COX-2 enzyme and shifts its function as endoperoxidase to a lipoxygenase activity. This event promotes the conversion of arachidonate to 15-HETE that carries its C15 alcohol in the *R* but not in the *S* configuration (15*(R)*-HETE). This metabolite is then released from endothelial and epithelial cells and may be transformed by leukocyte 5-LOX to generate 15-epi-LXA_4 or 15-epi-LXB_4 (Claria et al., 1995). During the last 2 decades, significant efforts have been directed towards identifying the physiological actions of these molecules, and after extensive *in vivo* and *in vitro* studies, they have been generally identified as endogenous anti-inflammatory "braking signals" maybe with antagonist properties to pro-inflammatory eicosanoids (Hugh et al., 1995).

EICOSANOID RECEPTORS

Eicosanoid signalling operates through lipid G- protein- coupled receptors (GPCRs), which structures and the encoding proteins are now known for human, mouse, and rat (Brink et al., 2003). According to the International Union of Pharmacology (IUPHAR), leukotriene receptors are classified in three main groups: the BLT receptors, which biological activities are related to LTB_4 and related hydroxyacids and are classified

as chemoattractants, the CysLTs receptors related belonging to the nucleotide receptor family, the lipoxin receptors (ALX) also included in the chemoattractant receptor class along with formyl peptide receptors (FPL) and the prostanoid receptors in an apart class. (Figure 5, Table 1).

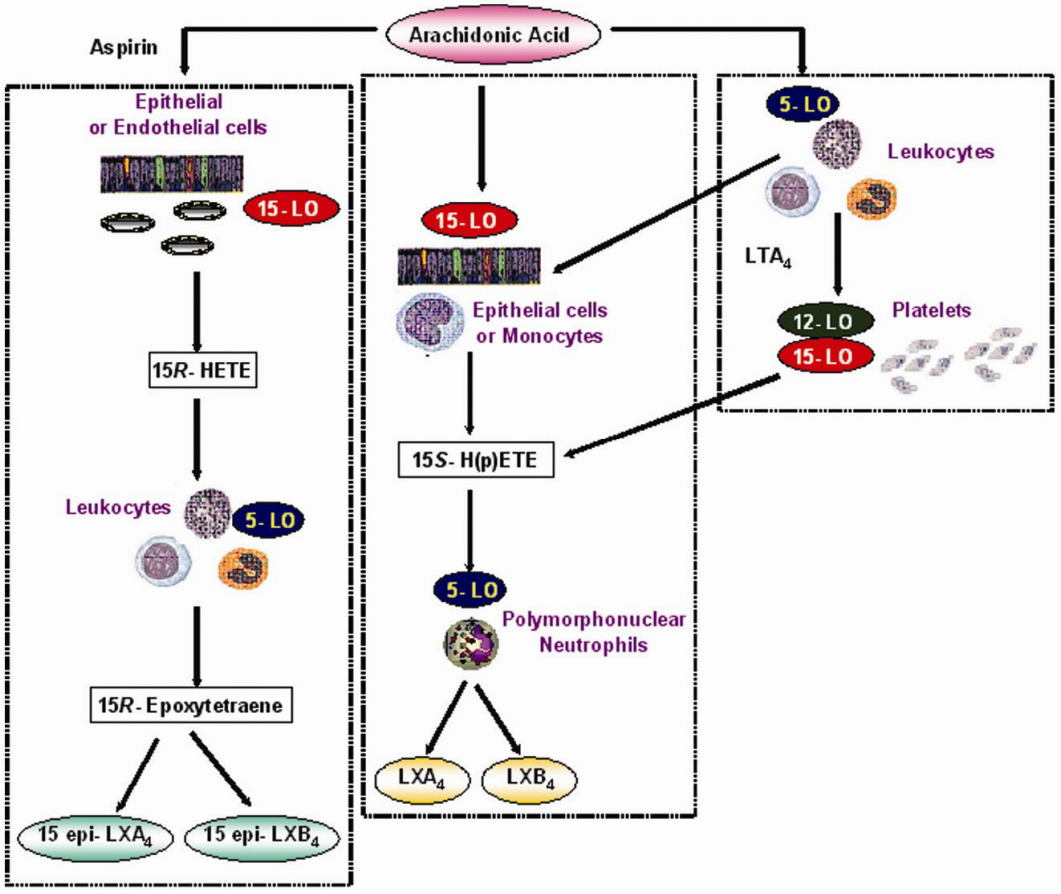

Figure 4 Transcellular biosynthesis of lipoxins and aspirin- triggered lipoxins.

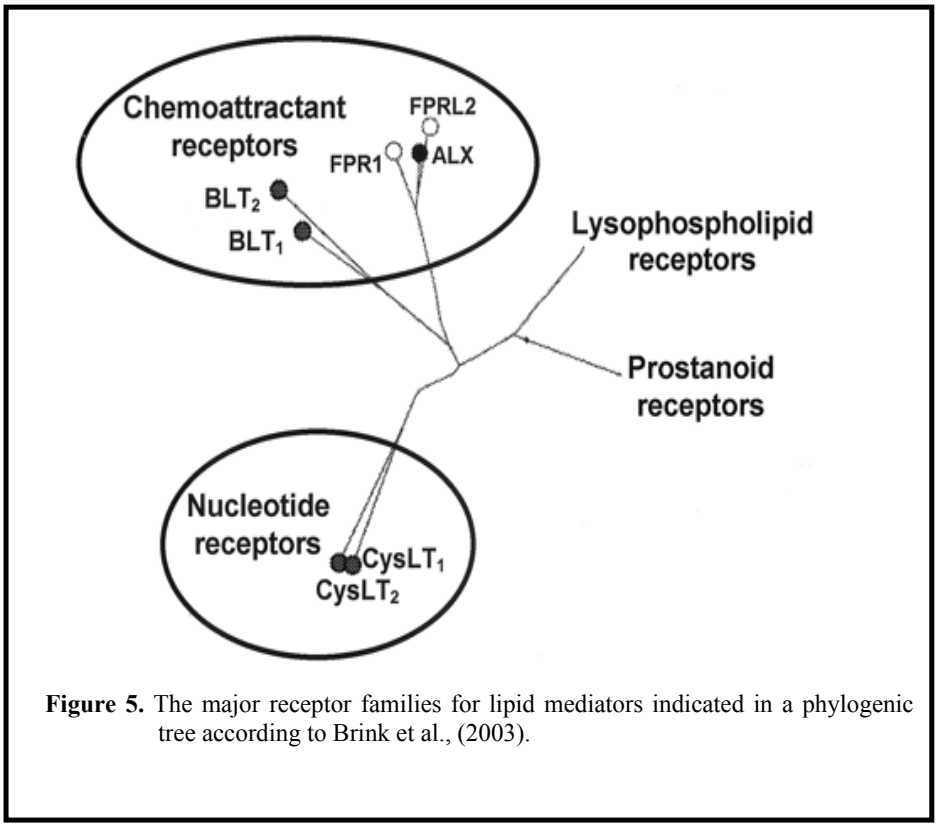

Figure 5. The major receptor families for lipid mediators indicated in a phylogenic tree according to Brink et al., (2003).

Table 1. Nomenclature of the human cloned eicosanoid receptors, according to the International Union of Pharmacology (IUPHAR).

Receptor	IUPHAR name	IUPHAR code	Non- selective ligand	Gene/ chromosome	Accession number (Swiss- Prot)
Leukotriene B_4 receptor 1	BLT_1	2.1 :BLT :1 :BLT_1	LTB_4	14q11.2-q12	Q15722
Leukotriene B_4 receptor 2	BLT_2	2.1 :BLT :2 :BLT_2	LTB_4	14q11.2-q12	Q9NPC1
Cysteinyl leukotriene receptor 1	$CysLT_1$	2.1 :CLT :1 :CLT_1	$LTC_4/LTD_4/LTE_4$	Xq13-q21	Q9Y271
Cysteinyl leukotriene receptor 2	$CysLT_2$	2.1 :CLT :2 :CLT_2	$LTC_4/LTD_4/LTE_4$	13q14.2	Q9NS75
Lipoxin receptor	ALX	2. :ALX	LXA_4/ fMLP	19q13.3	P25090

Leukotriene B_4 (BLT) receptors

The leukotriene B_4 (BLT) receptors (BLT_1 and BLT_2) are G protein-coupled seven transmembrane domain receptors (Tager et al., 2003). They have 45% amino acid identity, however, some differences in G protein-coupling mediating different cellular events, have been observed. Additionally, the affinity and specificity for LTB_4 binding is also different. BLT_1 is a high-affinity receptor specific for LTB_4, whereas BLT_2 is a low-affinity receptor that can also binds other eicosanoids including 12*(S)*- HETE, 12*(S)*- HPETE, 15*(S)*- HETE (Yokomizo et al., 2001). Northern and dot blot analyses have revealed that the distribution of BLT_1 and BLT_2 expression differs significantly. Whereas human BLT_1 is expressed primarily in leukocytes, human BLT_2 is present in most human tissues (spleen > liver, ovary, peripheral blood leukocytes, pancreas > heart, prostate, testis, small intestine, kidney, lung, colon, thymus, muscle, placenta) (Yokomizo et al., 2000; Tryselius et al., 2000; Kamohara et al., 2000; Wang et al., 2000). Further investigation of mRNA expression of these receptors in human peripheral blood leukocytes has demonstrated that neutrophils and eosinophils express significant amounts of both BLT_1 and BLT_2, whereas mononuclear cells express BLT_2 but minimal BLT_1 (Kamohara et al., 2000). Expression of BLT receptors can be up-regulated during inflammation; however, specific inflammatory stimuli responsible for their induction have not yet been fully characterized. So far, it has been demonstrated that IFN-γ and glucocorticoids can increase BLT_1 expression in human leukocytes (Huang et al., 1998); and dexamethasone can induce its transcription in peripheral blood neutrophils (Stankova et al., 2002). Finally, BLT_1 and BLT_2 may play a crucial role in host defense and in many inflammatory diseases by mediating LTB_4 activities like inflammatory cell recruitment, activation of inflammatory cell effector functions, and prolongation of inflammatory cell survival (Tager et al., 2003).

Cysteinyl leukotrienes (CysLT) receptors

Cysteinyl leukotriene (CysLTs) receptors are seven trans-membrane-spanning receptors that couple to G proteins and activate intracellular signalling pathways in response to their endogenous ligands LTC_4, LTD_4 and LTE_4 (Lynch et al., 1999; Heise et al., 2000). Biochemical studies have shown that these receptors share 38% amino acid identity and have very low homology in the extreme carboxyl termini (Brink et al., 2003). Originally, classification of CysLT receptors was based on the intolerance to the so-called "classical" antagonists including: montelukast (Singulair) (Jones et al., 1995), zafirlukast (Accolate) (Krell et al., 1990 and Garcia-Marcos et al., 2003), pranlukast (Onon, Azlaire) (Obata et al., 1992 and Keam et al., 2003), pobilukast (Hay et al., 1987) and MK571 (Jones et al., 1989). Accordingly, two main groups of these receptors have been identified: $CysLT_1$ receptor class, intolerant to the classical antagonists and $CysLT_2$ class, which mediates effects not inhibited by the classical antagonists (Tudhope et al., 1994). A few years ago, molecular biology techniques have confirmed these previous pharmacological findings and allowed the

cloning of two distinct receptors in humans, hCysLT$_1$ (Lynch et al., 1999) and hCysLT$_2$ (Takasaki et al., 2000). However, the available literature and research data suggest the existence of additional classes or subclasses for both receptors (Ravasi et al., 2002) but additional investigation is required to expand the current classification. Several studies have demonstrated the presence of intracellular CysLTs receptors (Bandeira- Melo et al., 2003). It is known that enzymes involved in leukotriene synthesis are located not only in the nuclear envelope but also inside the nucleus of many cells suggesting that lipoxygenases metabolites might not only activate cell surface receptors but also act locally at intracellular receptors such as peroxisome proliferative- activated receptors (PPARs) (Peters-Golden et al., 2001). However, although some *in vitro* studies performed with LTB$_4$ support this hypothesis, there is no information concerning the action of CysLTs on these intracellular receptors.

Expression pattern and distribution of CysLT$_1$ and CysLT$_2$ receptors vary considerably between tissues and organism species (Nicosia et al. 2000). Human CysLT$_1$ receptor is highly expressed in peripheral blood leukocytes, lung, pancreas small intestine and a few other tissues as showed in table 2. Expression of mRNA and protein of this receptor have been also detected in peribronchial smooth muscle cells ad interstitial macrophages (Figueroa et al., 2001) but also in peripheral blood monocytes, eosinophils, monocytes/macrophages, B-lymphocytes and CD34$^+$ granulocytic precursor cells. However, low or no expression has been observed in neutrophils or T-lymphocytes (Mita et al., 2001). Expression of hCysLT$_2$ receptor is also high in spleen and peripheral blood leukocytes, however differently to the hCysLT$_1$ it is also expressed in heart, adrenal glands and brain (Table 2). Eosinophils also express this receptor but in higher levels as compared to hCysLT$_1$ and expression in monocytes, neutrophils and T- cells have been also observed (Mita et al., 2001). However, in contrast to hCysLT$_1$, this receptor is weakly expressed in lung smooth muscles (Heise et al., 2000).

According to different studies it seems that both T-helper 1 (Th$_1$) and T-helper (Th$_2$) cytokines can regulate CysLTs receptor expression. This is supported by early experiments showing that receptor binding to LTD$_4$ can be enhanced by IL-5 in HL-60 cells differentiated into eosinophils (Thivierge et al., 2000). In addition, IL-4 and IL-13 were also able to induce CysLT$_1$ expression in human monocytes and macrophages (Thivierge et al., 2001) and IFN-γ was able to increase both CysLT$_1$ and CysLT$_2$ expression in human airway smooth muscle cells (Amrani et al. 2001).

However, although many efforts are being doing, research concerning the regulation of CysLTs receptor expression remains in the infancy stage. Not only pharmacological studies but also characterization of the promoter regions and transcription factors of these receptors will greatly help elucidate signalling pathways

involved in receptor regulation. That will have a big implication in the understanding of the pathogenesis involving the action of these molecules especially in airway diseases.

Lipoxin (ALX) receptors

Deduced amino acid sequences indicated that ALX belongs to the GPCR superfamily, but also on the basis of its nucleotide sequence, it is a member of the chemokine receptor superfamily, with the ability to bind both lipid and peptide ligands (Chiang et al., 2000; Christophe et al. 2001 and 2002). ALXR is the first non-prostanoid eicosanoid GPCRs receptor recognized at the molecular level (Fiore et al., 1993, 1994) and identified as the only inhibitory or anti-inflammatory receptor that acts via an agonist (LXA_4) and has a role as a "stop signal" (Fiore et al., 1994; Serhan, 1994, 1997; Takano et al., 1997). LXB_4, in contrast do not act via the ALX receptor interacts with a specific receptor present on human leukocytes (Maddox et al., 1996). Its cDNA sequence has not been cloned yet and is considered as a putative receptor. In addition to this data it has been also shown that LXA_4 can bind a CysLTs receptor (Gronert et al., 2001) and at a third type: the *aryl hydrocarbon receptor* (AhR), which is a ligand-activated transcription factor regulating genes implicated in xenobiotic metabolism and lipoxin degradation (Schaldach et al., 1999).

Table 2. Expression profile of human $CysLT_1$ and $CysLT_2$ receptors (Addapted from Hui, 2002).

Tissue	$hCysLT_1$	$hCysLT_2$
	Expression level	Expression level
Lung		
Bronchial smooth muscle cells	++	+
Interstitial macrophages	++	++
Peripheral blood leukocytes		
Eosinophils	++	++
Monocytes/macrophages	+	+
Neutrophils	±	+
B lymphocytes	+	?
T lymphocytes	±	+
CD 34^+ precursor cells	+	?
Mast cells	+	?
Spleen	++	++
Heart and coronary vessels	?	++
Brain	?	++
Adrenal glands	?	++

Expression of ALXR has been described in monocytes (Maddox et al., 1997), basolateral membrane of gastrointestinal epithelial cells (Kucharzik at al., 2003), synovial fibroblasts (Sodin-Semrl et al., 2000), bronchial epithelial cells (Bonnans et al. 2003), and mesangial cells (McMahon et al., 2002). This receptor can be regulated at the transcription level by cytokines including IL-1, IL-13 and IFN-γ (Sodin-Semrl et al., 2000). As we state before, responses evoked through ALXR have been observed in response to either lipid or peptide ligands (Chiang et al., 2000; Fiore et al., 1993) where many of them are chemotactic and elicit pro-inflammatory responses in human leukocytes. An example is that stimulation of ALXR by serum amyloid A (SAA) induces IL-8 secretion in human neutrophils (He et al., 2003).

However, LXA_4 activation of the same receptor does not induce IL-8 secretion but decreases the SAA-induced IL-8 secretion, suggesting that SAA and LXA_4 may compete for a common binding site on the ALXR (He et al., 2003). The functional significance of multiple lipid and peptide ligand binding at the ALXR remains uninvestigated. And the fact that this ALXR can switch recognition as well as function from stimulatory to inhibitory, raises the possibility that activation of this receptor by LX or ATL can protect the host from deleterious PMN-induced responses associated with innate immune responses.

Prostanoid receptors

These receptors belong to the family of G protein-coupled rhodopsin-type receptor with 7 putative transmembrane domains (Hirata et al. 1991). Eight types and subtypes of the prostanoid receptors have been until now identified (Table 3). They include the human and mouse PGD receptor (DP); the mouse, rat, and human PGE receptor EP_1 subtype; the mouse and human PGE receptor EP_2 subtype; the mouse, human, rat, rabbit, and bovine PGE receptor EP_3 subtype; the mouse, human, and rat PGE receptor EP_4 subtype; the mouse, human, bovine, rat, and sheep PGF receptor (FP); the mouse, human, and rat PGI receptor (IP) and the mouse, rat, and bovine TXA (TP) receptor (Narumiya et al. 1999). Analysis of amino acid sequences has shown that particular motifs are specifically conserved among the prostanoid receptors (Narumiya et al. 1999). However, despite the presence of these conserved sequences, overall homology among the prostanoid receptors is quite limited, ranging from ~20 to 30%. In contrast, the homology of a given type or subtype of receptor among various species is considerably higher, oscillating between 70 and 97% (Narumiya et al. 1999). With exception of the human DP and EP_2, the chromosomal localizations of the genes encoding the human prostanoid receptors have been determined and have been mapped to chromosome bands 19p13.1, 1p31.2, 5p13.1, 1p31.1, 19q13.3, and 19p13.3 (Duncan et al. 1995 and Nusing et al., 1993). Each of the prostanoid receptors shows a selective response in tissues and ligand-binding specificity that distinguishes it from the others (Narumiya et al. 1999).

Several pharmacological and biochemical studies combined with molecular biology approaches have shown that each prostanoid receptor is specifically distributed in the body and that expression levels are variable among tissues. Human DP receptor has been detected at low levels in the small intestine (Boie et al. 1995) and in specific cells in the brain (Oida et al., 1997). Among the prostanoid receptors, the EP_3 and EP_4 receptors are widely distributed throughout the human body. In contrast, the distribution of EP_1 is restricted to several organs, such as the kidney, lung, and stomach (Watabe et al., 1993). EP_2 is the least abundant among these receptors and is effectively induced in response to stimuli like LPS, IFN-γ and hormones (Katsuyama et al., 1997; Lim et al., 1997). Earlier ligand- binding studies (Powell et al., 1974 and Rao, 1973) have indicated that FP is most abundantly expressed in the corpus luteum, although also in the kidney, stomach glands and in fibroblastic cell lines such as NIH-3T3 (Arakawa et al., 1996). IP mRNA is most abundantly expressed in neurons, in which it was co- localized with the mRNA for preprotachykinin A, a precursor of substance P, indicating that this receptor may be involved in the mediation of pain. In some neurons, IP mRNA can be also co-expressed with the mRNA of EP receptor subtypes, suggesting that these receptors play overlapping roles in transmission of pain sensation. In addition, IP is also expressed in megakaryocytes and the smooth muscles of arteries, afferent arterioles of the glomerulus, in the thymus and spleen (Oida et al., 1995). Finally, northern blot analysis have shown that TP is abundantly expressed in tissues rich in vasculatures such as the lung, kidney, and heart, but also in immune-related organs such as the thymus and spleen (Namba et al., 1994). This receptor is also highly expressed by immature thymocytes such as $CD4^-$, $CD8^-$ and $CD4^+$, $CD8^+$ cells. Also it was shown that TP receptor agonist can induce apoptotic cell death of immature thymocytes in a receptor-dependent manner, suggesting its role in thymocyte differentiation and development, in addition to its well-known roles in the cardiovascular and respiratory systems (Ushikubi et al., 1993).

Table 3. Expression profile of prostanoid receptors on human immune cells.

Type	Subtype	Cells expressing the receptors
DP		Neutrophils
EP	EP_1	B lymphocytes,
	EP_2	B lymphocytes, eosinophils, neutrophils
	EP_3	B- lymphocytes, T- lymphocytes, macrophages / monocytes
	EP_4	B lymphocytes
FP		
IP		T- lymphocytes
TP		Macrophages / monocytes, eosinophils

PHYSIOLOGICAL IMPLICATIONS OF EICOSANOIDS ON CHRONIC RHINOSINUSITIS/ NASAL POLYPOSIS

In the past two decades, it has been apparent that eicosanoids play an important role in certain airway inflammatory conditions like rhinosinusitis, nasal polyposis, allergic rhinitis and asthma. The implication of LTs and prostaglandins in these diseases has been extensively investigated. Although, the regulatory mechanism governing the production of these molecules is not 100% clear, the use of anti-LTs, LTs-receptor antagonists and alternative to cyclooxygenase inhibitors has been of great help in the management of airway diseases. Interactions in eicosanoid pathway in paranasal sinus diseases and especially nasal polyposis have been demonstrated by several studies. Baenkler and col. (Baenkler et al., 1996) observed an altered pattern of the lipoxygenase and cyclooxygenase pathways when nasal tissue becomes irritated. These authors also observed a remarkable increase of CysLTs by polypous tissue upon arachidonic acid stimulation, in contrast to only slightly elevated PGE_2 release when compared to normal tissue, suggesting the involvement of these metabolites in the pathogenesis of nasal polyps. In addition, an interesting work by Klapan et al., (1995), showed that patients with post- operative recurrences had significantly lower of PGE_2 but higher TXA_2 and LTC_4 compared to subjects without polyp recurrence. In this study, LTC_4 levels were correlated with the rate of recurrence suggesting a possible prognostic value of these molecules for nasal polyp recurrence.

Differential regulation of CysLTs receptors has also been suggested as crucial factor in the pathophysiology of chronic inflammatory airway diseases. An elevated number of nasal inflammatory leukocytes bearing $CysLT_1$ has been observed in chronic rhinosinusitis-aspirin intolerant patients compared with their aspirin counterparts (Sousa et al., 2002). However, a study performed with $CysLT_1$ receptor antagonist montelukast showed that improvement of therapy was not associated with a number of clinical parameters evaluated including aspirin intolerance; introducing a controversial speculation about the role of these receptors in the development of the disease (Ragab et al., 2001). Furthermore, $CysLT_1$ receptor expression has been localized in big number of inflammatory cells within the nasal mucosa (Sousa et al., 2002) and increased levels of this receptor have been also reported on neutrophils and eosinophils on the nasal lavages from patients with allergic rhinitis (Figueroa et al., 2003).

Studies with LTs receptor antagonist have concluded that anti- leukotrienes therapy might play a significant role in controlling polyposis and symptoms secondary to sinonasal disease, and they might be an important alternative to long-term oral steroid therapy and repeated surgery (Parnes et al., 2000). In contrast to CysLTs, controversial results about LTB_4 and BLT receptors have been reported in several studies. The levels of these molecules have been found increased in nasal polyp patients with allergy in comparison to non- allergic subjects (Pinto et al., 1997); but no differences were observed between aspirin intolerant compared to

tolerant subjects (Sousa et al., 2002). Until now, no clear mechanism regulating the synthesis of these molecules in airway has been demonstrated. However, neutrophils, the major LTB_4 producing cells are able to interact with other cell types, especially epithelial cells, influencing the qualitative and quantitative profile of leukotriene production (Maclouf et al., 1988).

Furthermore, cyclooxygenase metabolic pathway has been suggested to be different in nasal polyp patients. That was first suggested by Mullol and col. (Mullol et al., 2002), who demonstrated that basal COX-1 mRNA is spontaneously up- regulated in cultured normal mucosa but not in nasal polyps. In addition, spontaneous cytokine induction of COX-2 mRNA expression was up- regulated in both cultures but delayed in nasal polyp compared to normal mucosa. Similar results were found by Gosepath et al., (2004), who observed a down- regulation of COX-2 expression in epithelial cells of nasal polyp tissue compared to normal mucosa. These authors suggested that prostanoid metabolism might play a crucial role in the pathogenesis of inflammatory nasal diseases and in the formation of nasal polyps.

Additionally, there are still molecules with a big controversial role in the pathogenesis of chronic inflammatory diseases, the lipoxins. Lipoxins are generally associated with anti-inflammatory effects and have been reported to reduce leukocyte infiltration (McMahon et al., 2004). However, certain di-hydroxyeicosatetraenoic acids (HETEs), which are precursors of these molecules, may have also pro-inflammatory effects, specifically neutrophilic and eosinophilic chemotaxis (Maddox et al., 1996). Chopped human nasal polyps have been shown to have a high capacity to produce LXA_4 after incubation with exogenous LTA_4 in presence of polymorphonuclear granulocytes (Edenius et al., 1990). In addition, severity of asthma has been associated with increased expression and activation of 15- LO enzyme, collagen deposition and eosinophil accumulation (Chu et al., 2002). Interestingly, the levels and capacity of producing these molecules are decreased in aspirin intolerant patients (Sanak et al., 2000) suggesting that maybe a particular regulation event of this pathway, operating only in these patients, is involved in the pathogenesis of this disease. Although extensive studies are going on in this field, the specific role of these molecules and their regulation in sinunasal diseases remains largely unclear.

Finally, implication of eicosanoid differential regulation in aspirin intolerance syndrome has also gained a lot of attention since 1987 when Jung and co- workers (Jung et al., 1987) showed that LTC_4 and LTB_4 concentrations were higher in nasal polyp and more in those with aspirin intolerance. In 1993, Kowalski reported an increase of LTC_4 concentration after aspirin provocation in nasal lavages of chronic rhinosinusitis aspirin- intolerant patients; suggesting that production of lipoxygenase products of arachidonic acid may induce glandular secretions contributing to the clinical symptoms observed in aspirin intolerance syndrome (Kowalski et al., 1993). Andrew Szczeklik's group showed in 1996, that aspirin challenge significantly depressed PGE_2 and thromboxane B_2 in aspirin- tolerant and intolerant patients but increased

PGD_2, $PGF_{2\alpha}$ and $PGF_{9\alpha}$ and 11β-PGF_2 only in aspirin- intolerant subjects (Szczeklik et al., 1996). Later, this group also showed that eicosanoid metabolism disturbances in terms of urinary LTE_4 and serum 9α,11β-PGF_2 after aspirin provocation, is more severe in patients with bronchial and nasal responses than in subjects with only bronchial or nasal symptoms (Swierczynska et al., 2003).

In other hand, Pinto et al. (1997) corroborated these results showing high levels of LTs in nasal polyp tissue and turbinates from patients with allergic rhinitis and a decreased cyclooxygenase/ lipoxygenase ratio in patients with aspirin intolerance. Cowburn and col. extended these observations by showing that intake of lysine-aspirin in aspirin- intolerant subjects resulted in an additional release of CysLTs into the bronchoalveolar fluid and in a bronchial responsiveness that correlated with the number of cells expressing LTC_4 synthase enzyme (Cowburn et al., 1998).

Furthermore, a significant increase of CysLTs and lower PGE_2 release after aspirin challenge was observed in nasal polyp tissue and peripheral blood from aspirin- intolerant subjects compared to normal mucosa from controls (Schmid et al., 1999). Taking this one-step further, Picado et al., (1999, 2003), looked at the regulation of the cyclooxygenase pathway in patients with rhinosinusitis and aspirin intolerance. Their results showed no differences in COX-1 mRNA expression between nasal mucosa and nasal polyps from patients with and without aspirin intolerance. However, COX-2 mRNA expression and NF-κB activity, in nasal polyp tissue from aspirin intolerant subjects, was markedly lower than in polyps from patients without intolerance, suggesting that an inadequate regulation of these inflammatory pathways may be involved in the manifestation of this syndrome. Of interest, a functional polymorphism in the promoter region COX-2 gene was observed in aspirin- intolerant patients. This polymorphism resulted in increased capacity of monocytes to produce prostaglandins and was associated with the severity of the disease and the need of corticosteroids (Szczeklik et al., 2004).

Recently, a study from our group analyzed the basal tissue levels of chronic rhinosinusitis/nasal polyp tissue in patients with and without aspirin intolerance. In this work, we showed that changes in tissue eicosanoid production may occur in chronic rhinosinusitis in absence of clinical aspirin intolerance and they may not be specific but represent a secondary phenomenon linked to the inflammatory process in these patients (Pérez-Novo et al., 2005). Summarizing all these findings, it seems that the occurrence of chronic rhinosinusitis/ nasal polyposis probably represent a result of different chronic inflammatory stimuli regulated in part by the arachidonic acid pathway.

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CHAPTER 3

MICROBIAL SUPERANTIGENS

DEFINITION

Superantigens (SAgs) are toxins of microbial or viral origin that cross-link antigen-presenting cells (APCs) and T-cells by binding simultaneously to the immunoreceptors major histocompatibility complex (MHC) class II (Dellabona et al., 1990) and the T-cell receptors (TCRs) (Choi et al., 1990). These molecules have an extreme ability to activate ($D4^+$, $CD\text{-}8^+$) T- cells polyclonally (White et al., 1989) when presented on MHC class II, and in contrast to conventional antigens, they are not processed and presented as short peptides.

IMMUNOLOGY

Conventional antigens are processed within antigen-presenting cells (APCs) into peptide fragments that are loaded into the peptide-binding groove of the major histocompatibility complex (MHC class II) class II molecule for presentation at the cell surface to T- cells. This mechanism allows that T- cells will only respond if they recognize the class II molecule through CD4 and the specific peptide being presented and only a small number of the host's T-cell repertoire (0.001 %) will be activated (Figure 5). By contrast, SAgs bind as intact proteins, directly to the immunoreceptors of MHC class II molecule outside the peptide-binding groove (Fischer et al. 1989) and T-cell receptor (TCRs), at the variable part of the TCR β-chain ($TCR\text{-}V_\beta$), which are sites away from conventional peptide-binding sites (Figure 6). SAgs binding causes a massive T-cell proliferation (up to 20% of all host' T cells), release of large amounts of the cytokines IL-2, TNF-α and IFN-γ, IL-1) (Langford et al. 1978) and cytotoxic reactions towards target cells (Dohlsten et al., 1991).

The most well characterized bacterial SAgs are the ones produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. *S. aureus* secrete more than 10 different SAgs (Peterson et al., 2004) named staphylococcal enterotoxins (SEs) A, B, C1-3, D, E, G, H, I, J, K, L, M, N, O, P, Q and toxic shock syndrome toxin-1 (TSST-1) as shown in Table 4. However, each strain of these bacteria generally produces a certain repertoire of SAgs that is all bacterial strains do not produce all different types of SAgs (Jarraud et al. 2001).

SUPERANTIGEN PRESENTATION BY MHC CLASS II MOLECULES

All structurally characterized SAgs can adopt a very similar three-dimensional fold; however they can be presented by MHC class II in three distinct ways [Li et al., 2001; Jardetzky et al., 1994). According to this ability to bind MHC class II, SAgs may be divided into three groups *1)* the single α-chain-binding SAgs (e.g. SEB), *2)* the single β-chain-binding SAgs (e.g. SEH) or *3)* the ones capable of cross-links two MHC class II

molecules by binding one α- and one β-chain (e.g. SEA) (Jardetzky et al., 1994 and Petersson et al. 2001), two α- chains (e.g. SED) or two β- chains (e.g. SPE-C) (Roussel et al., 1997 and Al-Daccak et al., 1998).

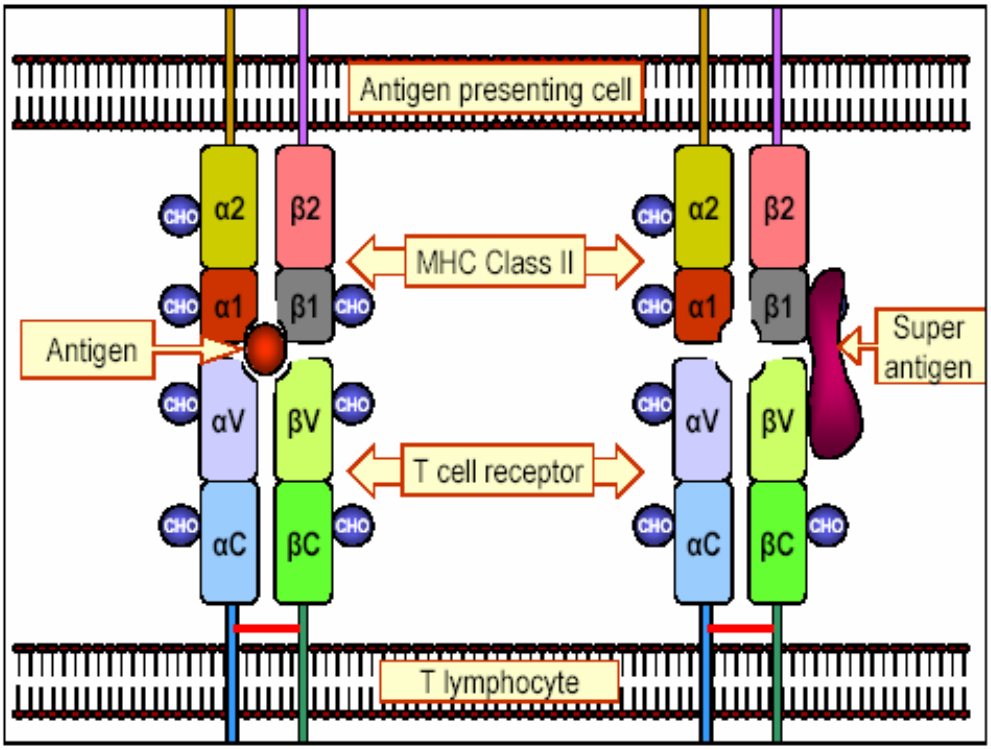

Figure 6: Differences between antigen and superantigen. Antigenic peptides are processed within the cell and presented on the cell surface in association with class II MHC molecules. They then trigger the T-cell receptor on a T lymphocyte. Superantigens are not processed but bind to the class II MHC protein and to the V beta chain of theT cell receptor. A given superantigen activates a distinct class of T cells that express a certain V beta chain.
In the case of MHC II-TCR interaction with a normally processed peptide, recognition of the peptide on theMHC molecule requires V alpha, J alpha, V beta, D beta and J beta segments of the TCR. Such an interactionoccurs at low frequency. In the case of MHC II-TCR interaction with an unprocessed superantigen, only a given Vbeta region is recognized. This clearly would occur at a much higher frequency.

SUPERANTIGEN INTERACTIONS WITH T- CELL RECEPTOR (TCR)

A hallmark for T-cell stimulation by SAgs has been the specificity for the Vβ part of the T-cell receptor, TCRV-β (Choi et al., 1990). However, SAgs possess a specific TCR-Vβ profile, that is, they specifically expand T- cells bearing some TCR-Vβs, while others will be excluded (Kappler et al., 1989). In this way, all T-cells harbouring the SAg-specific TCR-V_β will be activated. In addition, the TCR-V_α part appears to influence indirectly the specificity for SAg activation of T-cells (Seth et al., 1994) through an interaction of

the β-chain of MHC class II and the α-chain of TCR (Seth et al., 1994). The affinities for SAg- TCR-V_β interactions are relatively low, suggesting that some sort of serial triggering may also operate in T-cell activation by SAgs.

S. aureus DERIVED ENTEROTOXINS AS SUPERANTIGENS

Staphylococcal SAgs are structurally related heat-stable proteins of approximately 27 kDa molecular mass defined as a group of high-molecular weight pyrogenic proteins, which have in common an extremely potent stimulatory activity for T-lymphocytes and $\gamma\delta^+$ T-cells, B-cells, macrophages, antigen presenting cells, eosinophils and epithelial cells (Fleischer, 1994). Effect of *S. aureus* SAgs on T- lymphocytes has been mainly studied on skin inflammatory diseases. Stimulation of PBMCs from atopic dermatitis patients with SEA and SEB showed a significantly stronger response to these enterotoxins SEA or SEB compared to the response obtained from the controls subjects. In addition, this response was accompanied by an enhance of the expression of specific Vβ segments which displayed distinct patterns, suggesting a polyclonal T- cell proliferation induced by enterotoxins (Yudate et al., 1996).

Additionally, studies *in vivo* on patients with this disease, showed that application of SEB on the skin of normal individuals and affected individuals resulted in a selective accumulation of T-cells expressing SEB-reactive TCR-Vβ segments and in an inflammatory reaction; suggesting that *S. aureus* superantigen-induced T-cell activation is involved in the pathogenesis and may exacerbate and sustain the inflammation in inflammatory skin diseases (Skov et al., 2000; Strange et al., 1996). However, compared with studies on lymphocytes, just a few studies have been focused in the effects of staphylococcal SAgs on other pro-inflammatory cell types, like eosinophils, macrophages, mast cells, and epithelial cells, which are known to play crucial roles in the pathogenesis of inflammatory airway disease. Several studies performed on eosinophils have described these cells as accessory cells, which in presence of certain cytokines can increase the expression of HLA-DR (a MHC class II molecule) and a subsequent proliferation of resting ($CD4^+$) T-cells in response to enterotoxins stimulation (Mawhorter et al., 1994). In addition, it has been shown that SEs may also directly affect the eosinophil activity by inhibiting apoptosis and modulating important cell-surface antigens, including the up-regulation or down- regulation of important cell surface markers (Wedi et al., 2002). Macrophages have been also described as accessory cells for a T-cell activation / proliferation in response to staphylococcal SAgs and have been found to play a crucial role in enterotoxin- induced T cell apoptosis (Zen et al., 1996). Additionally, other studies have suggested that these cells can be activated by cross- linking both MHC-I and MHC-II molecules leading to the release of important cytokines like IL-6 and TNF-α (Wright et al., 1999). Studies with SEB have suggested that SAgs may augment antigen-specific T-cell responses by inducing IL-12 production in macrophages (Bright et al., 1999). Furthermore, mast cells

have been most widely studied in the context of allergic disease but also play a critical role in host defense against bacterial infection. These cells can also act as accessory cells in response to SAgs, similar to eosinophils and macrophages. Cord blood- derived mast cells have been shown to directly induce $CD4^+$ T-cell activation through presentation of SEB and TSST-1 SAgs; phenomenon that may be critical in subsequent cellular activation events due to the frequent co- localization of mast and T- cells at sites of antigen entry (Poncet et al., 1999). In addition, *S. aureus* Cowan 1 and the immunoglobulin (Ig)-binding protein A could induce the release of several mediators like histamine, tryptase and leukotriene C_4 from human heart mast cells by interacting with the V_H3 region of IgE, suggesting a new model for the pathogenetic link between bacterial infections, IgE-mediated activation of mast cells and cardiovascular diseases (Genovese et al., 2000).

IMMUNOLOGICAL IMPLICATION OF *S. aureus* ENTEROTOXINS ON AIRWAY DISEASES

Compared to other diseases, few studies have documented the role of *S. aureus* and its superantigens on inflammatory airway disease. Nasal carriage of *S. aureus* affect about 20% of the population and it has been identified as a risk factor for the pathogenesis of community-acquired and nosocomial infections (Kluytmans et al., 1997; Von Eiff et al., 2001). The factors determining the carrier or non- carrier status remain largely unknown. Several factors like surface glycoproteins, proteoglycans and epithelial and mucous host factors, have been shown to mediate the binding of *S. aureus*. However the molecules implicated on this interaction between host and bacteria have not been identified. The nasal vestibule has a complex epithelial surface that can be histologically classified into five distinct regions (Mygind et al., 1982). These regions range from an anterior hairy epidermis continuous with the skin to a moist pseudostratified columnar layer characterized by many ciliated cells, which have the function of transporting the mucus and entrapped particles to the pharynx, where they are swallowed. Cole et al. (2001) demonstrated that in nasal carriers, the highest concentrations of *S. aureus* are found immediately distal to the anterior hairy epidermis in a moist squamous epithelium devoid of hair, cilia, and microvilli. These authors also state that this phenomenon is due to a combination of defective nasal fluid and the lack of alternative mechanical clearance mechanisms resulting in the *S. aureus* colonization. Additionally in this study, a release of epithelial and neutrophil-derived host defense peptides into nasal secretions of carriers and acute rhinitis donor fluids (Cole et al., 2001) indicating that neutrophils are recruited in response to *S. aureus* colonization.

S. aureus is present in about 60 to 70% of cases of massive nasal polyposis (Van Zele et al., 2004). It has been extensively suggested that *S. aureus* derived enterotoxins may play an important role in the pathogenesis of nasal polyposis. This hypothesis is based on the idea that initial injury to the lateral wall of the nose may result of staphylococcal derived toxins, which may induce lymphocyte activation resulting in

the production of both TH_1 and TH_2 cytokines. This cytokines, are responsible for the massive up- regulation and activation of lymphocytes, eosinophils, and macrophages, which are the most common inflammatory cells found in nasal polyposis. Recent studies, performed in tissue from patients with bilateral nasal polyps, have demonstrated the formation of specific IgE to *S. aureus* enterotoxins, which correlated with the severity of eosinophilic inflammation and the manifestation of asthma (Bachert et al., 2001). These findings were supported by Gevaert and col. (Gevaert et al., 2005), who demonstrated an increase of tissue localized polyclonal IgE formation, which was associated with the presence of IgE specific to SEs, *S. aureus* colonization and eosinophilic inflammation. Furthermore the authors showed that these enterotoxins bind to follicular structures and lymphoid accumulations in the nasal polyp tissue; suggesting a polyclonal B-cell activation because of chronic microbial colonization and release of enterotoxins, which may result in IgE switch and formation in these patients. Additionally, aspirin intolerance has been associated with increased concentrations of eosinophil-related mediators, as well as IgE antibodies to SEs in nasal polyp tissue (Pérez-Novo et al., 2004; Suh et al., 2004). However, although a direct impact of these bacteria with this syndrome could not be established, these findings suggest that staphylococcal superantigens may drive local eosinophilic inflammation in nasal polyp tissue, and that this is exacerbated in aspirin intolerant subjects. These results were later supported by a study showing an increased *S. aureus* colonization in nasal polyposis compared to chronic rhinosinusitis patients and even more when nasal polyposis appeared in combination with asthma and aspirin intolerance (Van Zele et al., 2004).

SEs has been also suggested as a possible disease modifier for chronic obstructive pulmonary disease (COPD). This was evidenced in a study where the levels of IgE to SEs were significantly higher in patients with stable and exacerbated COPD compared to smokers and healthy subjects, and correlated with the clinical parameters of these patients (Rohde at al., 2004). In addition, adherence of *S. aureus* to components of the secreted mucus layer in cystic fibrosis patients has also been reported (Ulrich et al., 1998). However, the authors suggest that the cause for the chronic respiratory infections in CF is due to impaired mucociliary clearance present before any airway infection in these patients. This point to a secondary role of staphylococcal enterotoxins in this disease. Another evidence for the role of these enterotoxins in CF was shown by an in vitro experiment where B- cells from CF patients served as APC of staphylococcal SAgs for an immortalized T-cell line. Interestingly, this action could not be inhibited by dexamethasone, suggesting that the pulmonary response to superantigens may be poorly regulated in CF, leading to an exaggerated inflammatory response to *S. aureus* (Ben-Ari et al., 2000).

Finally, the role of staphylococcal enterotoxins in upper airway allergic diseases is getting more and more attention nowadays. Sensitization to *S. aureus* enterotoxins has been found increased in patients with allergic rhinitis, which also high total serum IgE levels and manifested polyvalent sensitization (Okano et al., 2001).

ECP levels were elevated in allergic patients with immune response to SEs compared to patients lacking specific IgE to these enterotoxins. ECP, is thought to be a reliable marker of clinical severity for allergic disease and these findings suggest sensitization to staphylococcal enterotoxins as an important prognostic factor of persistent upper airway allergic diseases (Rossi et al., 2004)

Table 4. Functional properties of superantigens and their associated diseases according to Proft et al., 2003.

SAg	MW (kDa)	Crystal structure	Zinc binding	MHC II binding α- β chain	Human TcR V_β specificity	Disease
SEA	27·1	+	+	+/+	1·1, 5·3, 6·3, 6·4, 6·9, 7·3, 7·4, 9·1, 23·1	Food poisoning
SEB	28·4	+	-	+/-	1·1, 3·2, 6·4, 15·1	Food poisoning
SEC1	27·5	-	-	+/-	3·2, 6·4, 6·9, 12, 15·1	Food poisoning
SEC2	27·6	+	-	+/-	12, 13, 14, 15, 17, 20	Food poisoning
SEC3	27·6	+	-	+/-	5·1, 12	Food poisoning
SED	26·9	+	+	+/+	1·1, 5·3, 6·9, 7·4, 8·1, 12·1	Food poisoning
SEE	26·8	-	+	+/+	5·1, 6·3, 6·4, 6·9, 8·1	Food poisoning
SEG	27·0	-	?	?	3, 12, 13·1, 13·2, 14, 15	Food poisoning
SEH	25·2	+	+	-/+	?	Toxic shock syndrome
SEI	24·9	-	?	?	1·1, 5·1, 5·3, 23	Food poisoning
SEJ	28·5	-	?	?	?	?
SEK	25·3	-	?	?	5·1, 5·2, 6·7	?
SEL	24·7	-	?	?	?	?
SEM	24·8	-	?	?	?	?
SEN	26·1	-	?	?	?	?
SEO	26·7	-	?	?	?	?
SEP	26·4	-	?	?	?	?
SEQ	26·0	-	?	?	2·1, 5·1, 21·3	?
TSST	21·9	+	-	+/-	2·1	TSS

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HYPOTHESIS OF WORK

We hypothesize that alterations in the eicosanoids and their receptors expression are related to the degree of eosinophilic inflammation in chronic rhinosinusitis/ nasal polyposis, and *Staphylococcus aureus* enterotoxins may contribute to the pathogenesis of the disease by (directly or indirectly) affecting the eicosanoid metabolism.

RESEARCH OBJECTIVES

The aim of this research work was to investigate the regulation of arachidonic acid metabolism and eosinophilic inflammation in chronic rhinosinusitis/nasal polyp patients and the possible role of *S. aureus* superantigens in the balance of these important pathways.

As primary objective we aimed to develop an accurate and sensitive real-time PCR technique that allows quantifying the expression level of the two splice variants (membrane anchored and soluble) isoforms of IL-5Rα, a molecule playing a crucial role in the regulation of eosinophil migration and activation in chronic inflammatory airway diseases. As second, we wanted to select a proper set of reference genes for accurate normalization in further gene expression studies in chronic rhinosinusitis and nasal polyp tissues. In parallel, we evaluated the influence of RNA degradation on the stability and expression pattern of these internal control genes.

Once this methodology was established, we proceed to study the gene and protein expression of eicosanoids and eicosanoid receptors in sinonasal tissue and evaluated their possible correlation with severity of eosinophilic inflammation and co-moborbidities like asthma and aspirin intolerance.

Furthermore, based on the results of the previous study together with parallel data obtained from our group related to *S. aureus* colonization and the possible role of its enterotoxins in the pathogenesis of chronic rhinosinusitis; we decided to evaluate the link between eosinophilic inflammation and the manifestation of aspirin intolerance with the immune response to staphylococcal enterotoxins. This research work showed that although staphylococcal enterotoxins are not directly implicated in the clinical manifestation of aspirin intolerance syndrome they may crucially contribute to the exacerbation of eosinophilic inflammation observed in these patients.

These findings allowed us to investigate the relationship between immune response to *S. aureus* enterotoxins (in terms of specific IgE formation), leukotrienes and lipoxins synthesis in chronic rhinosinusitis/ nasal polyp tissue. In this study, we found an up-regulation of eicosanoid levels in subjects with enterotoxin immune response that lead to the question of how these molecules could induce changes in this inflammatory pathway.

Accordingly, the previous study was then extended to investigate the role of these enterotoxins (specifically enterotoxin B) on the regulation of eicosanoid metabolism on nasal tissue fibroblasts. After stimulation of these cells with SEB, we found that the enterotoxin differentially influence COX-2 and prostaglandin E_2 synthesis and basic cell function like cell growth and migration.

PART- II

RESEARCH WORK

CHAPTER 4

INTERNAL CONTROL GENES AND REAL-TIME PCR IN THE STUDY OF AIRWAY INFLAMMATORY PROCESS

Introduction

Eosinophils are one of the crucial cells regulating the chronic inflammatory process in inflammatory airway diseases like nasal polyposis and asthma. Activation of these cells is mainly mediated by the interaction of the cytokine IL-5 and its receptor IL-5R. This receptor consists in an alpha and a beta chain, which play different roles in the inflammatory process. The alpha chain of this receptor consists in two isoforms (the transmembrane and the soluble forms). Alternative splicing of the gene for IL-5Rα regulates production of these forms. Although several studies have been evaluated the differential regulation of these receptor forms at the protein level, the lack of a sensitive and accurate gene expression method difficulted the study of its transcriptional regulation. That is why we decided to apply a novel technology: real-time PCR, to study the differential mRNA expression of these isoforms in chronic rhinosinusitis and nasal polyposis. The method established allowed us to quantify the number of molecules for each spliced variant in nasal tissue and peripheral blood by using a standard curve based methodology and SYBR– green as detection strategy..

Furthermore, we proceed to evaluate a set of internal control genes that could be used in posterior gene analysis performed in this research work. Although most of researchers known the importance of normalizing gene expression data, only recently it has been really demonstrated the need of analyzing the stability of these genes prior gene analysis experiments. In chronic rhinosinusitis and nasal polyposis this analysis has not been performed. In this study we demonstrated that stability of internal control genes vary in normal nasal mucosa, chronic rhinosinusitis and nasal polyp tissue and this variation greatly depends of the degradation profile of the samples. Our results showed the importance of controling RNA integrity before PCR based studies and provide, for the first time, a suitable set of internal control genes that can be used in gene expression analyses in chronic rhinosinusitis and nasal polyp tissues.

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Quantitative Real Time Polymerase Chain Reaction for measurement of human Interleukin – 5 receptor alpha spliced isoforms mRNA

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Abstract

Background: Expression of human Interleukin-5 receptor alpha (hIL-5Rα) is controlled by alternative splicing, which generates two different transcripts encoding a membrane-anchored and a soluble form of the receptor, respectively. Although the study of the expression and regulation of hIL-5Rα is of crucial importance in the field of immunological processing, methods and techniques until now described lack sufficient sensitivity for detection of small differences in the expression of these isoforms. The aim of this study was to develop a reliable and sensitive real-time quantitative PCR assay to analyse the expression level of each isoform.

Methods: For the quantitative real-time PCR assay, two standard curves specific for each splice variant were constructed. PCR amplifications were performed on CDNA from peripheral blood, eosinophilic chronic rhinosinusitis and normal nasal tissue using a common forward and two specific reverse primers, in combination with SYBR Green I as the detection format.

Results and conclusion: We have developed an accurate and reliable assay for quantification of interleukin-5 receptor alpha mRNA isoforms over a broad dynamic range of input molecules. Importantly, excess of one isoform did not influence accurate quantification of the other isoform. Quantification of hIL-5Rα variants in human samples demonstrated an overexpression of both membrane-anchored and soluble encoding variants in eosinophilic chronic rhinosinusitis tissue and peripheral blood in patients with eosinophilic chronic rhinosinusitis compared to healthy subjects. The implementation of this assay will allow a better understanding of the regulatory mechanisms of the hIL-5Rα gene and hence its role in the pathogenesis of chronic inflammatory diseases.

Background

Immune responses are mediated by a large group of peptides known as cytokines. These molecules play an important role in promoting cell growth, differentiation, activation and regulation of human inflammatory responses. Human interleukin-5 (hIL-5), a haemopoietin that belongs to the alpha-helical group of cytokines, plays an essential role in the induction and maintenance of eosinophilic airway infiltration [1–3]. It has been shown that this cytokine is linked to the occurrence of chronic inflammatory diseases such as asthma and eosinophilic chronic rhinosinusitis [4–6]. The action of the hIL-5 is mediated by interaction with its receptor, the human IL-5 receptor. This receptor belongs to the class I cytokine receptor family together with receptors for IL-3 and GM-CSF [7,8] and consists of a heterodimer containing a unique α-subunit required for ligand-specific binding [9], and a β-subunit involved in binding affinity and signal transduction events [9]. Expression of the α-subunit has been described, *in vitro*, in eosinophils and basophils, whereas the β-subunit is expressed in eosinophils, B cells, and basophils, but also in type II pneumocytes [10–12].

The gene for the human interleukin 5 receptor alpha subunit (hIL-5Rα) is present in a single copy on chromosome 3 (band 3p26) of the human genome [13] and is composed of 13 introns and 14 exons [13].

Recent studies have shown that function and expression of hIL-5Rα can be regulated through splicing events and by ligand (hIL-5) stimulation [14]. Splicing of the hIL-5Rα gene can generates two different transcripts: one encoding a membrane-anchored protein through alternative splicing, and a second one encoding a soluble form of this receptor, by normal splicing events [15].

Although both receptor isoforms bind to hIL-5 with equal affinity [15], different responses are generated. The membrane-anchored receptor interacts with the β-subunit, increasing the affinity for hIL-5 and activating specific signal transduction pathways, such as cellular proliferation, maturational responses and inhibition of cell apoptosis [16,17]. The soluble isoform competes with the membrane-anchored receptor for hIL-5 binding [18], and therefore this variant is considered to be a potential natural negative regulator of hIL-5 function *in vivo* [19].

Knowledge of the regulatory mechanisms of hIL-5Rα expression is of utmost relevance for the development of future therapeutic strategies to control eosinophil activation mechanisms. However, although the groundwork for such strategies is currently being laid [16,20,21], regulation of hIL-5Rα gene transcription still remains largely unknown.

Reverse transcriptase PCR (RT-PCR) is a technique that is increasingly used to quantify physiological changes in gene expression. However, this method has the limitation that accurately quantification is in most of the cases not possible [22]. To circumvent this problem, several RT-PCR techniques have been developed during the last decade with the real-time PCR being the most accurate and straightforward. This methodology consists in the continuous monitoring of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product formed in a reaction. Quantitative real-time PCR has the advantage of a large dynamic range of quantification, no requirement for post-PCR sample handling and extremely good sensitivity [23]. Several fluorescent detection strategies for this technique have been developed, including the SYBR Green I DNA binding dye [24,25] and the use of specific fluorescently labelled hybridization or hydrolysis probe(s) [26,27].

The aim of this study was to develop a reliable and accurate real-time PCR method using SYBR Green I technology that allows cost effective measurements of the expression levels of the hIL-5Rα splice variants in human tissue and peripheral blood. As a model system, we use eosinophilic chronic rhinosinusitis, a sinus disease associated with severe local and systemic eosinophilic inflammation.

Table 1: Primer sequences used for template generation of the standard curves and real-time PCR amplification of hIL-5Rα and beta-actin genes

	mRNA target	Forward primer	Reverse primer	Amplicon size
Primer pair 1	Membrane-anchored hIL5Rα gemplate standard curve	5'-GTGTCTGCTTTTCCAATCCATTG-3'	5'-TGCTGGAATTGGAAACAACT3'	347 bp
Primer pair 2	Soluble hIL5Rα template standard curve	5'-GTGTCTGCTTTTCCAATCCATTG-3'	5'-AATCTGCTATCCCTGCTGTTGTT-3'	294 bp
Primer pair 3	Membrane-anchored hIL5Rα	5'-GCAGCAGTGAGCTCCATGTG-3'	5'-AGGGCTTGTGTTCATCATTTCC 3'	89 bp
Primer pair 4	Soluble hIL5Rα	5'-GCAGCAGTGAGCTCCATGTG-3'	5'-TGGATGTTATCTCCTTTATCTTGAGAA-3'	95 bp
Primer pair 5	Beta-actin Template standard curve	5'CCAAGGCCAACCGCGAGAAGATGAC-3'	5'-AGGGTACATGGTGGTGCCGCCAGAC-3'	588 bp
Primer pair 6	Beta-actin	5'-CTGGAACGGTGAAGGTGACA-3'	5'-AAGGGACTTCCTGTAACAATGCA-3'	140 bp

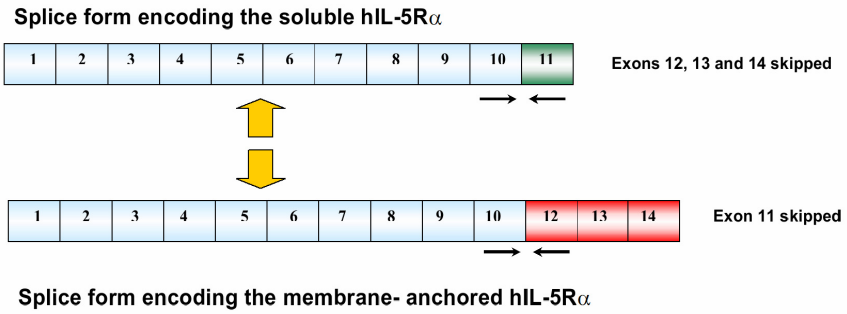


Figure 1
Alternative splicing of the human Interleukin 5 receptor alpha gene (hIL-5Rα) generating two transcripts encoding the soluble and membrane-anchored hIL-5Rα isoforms. Skipping of exons 12, 13 and 14 generate the soluble encoding form, whereas for the membrane-anchored encoding variant, only exon 11 is skipped. Arrows indicate the region amplified by the primer pairs 3 or 4.

Results and Discusion

Experimental validation

A common forward primer and two exon specific reverse primers were used for the quantification of the soluble and membrane-anchored hIL-5Rα encoding transcripts by real-time PCR amplification (Fig. 1). The forward primer is located in exon 10, whereas the reverse primer for the transcript encoding for the soluble form hybridises to exon 11, which is specific for this isoform [15]. The reverse primer for the membrane-anchored encoding variant is positioned in exon 12, which is also specific for this splice form [15]. Specific amplification was verified by agarose electrophoresis (4% in TAE), which resulted in one specific band of the expected size (membrane-anchored = 87 bp, soluble = 95 bp). These data were also confirmed in a melting curve analysis performed on the GeneAmp 5700 Sequence Detection System. Dissociation curves showed a single peak corresponding to a melting temperature of 80.2 °C for the soluble and 81.6 °C for the membrane-anchored hIL-5Rα encoding splice form, demonstrating specific amplification and the absence of primer dimers.

To exclude the possibility of coamplification of contaminating genomic DNA during RT-PCR, we performed a PCR run with either cDNA or genomic DNA extracted from eosinophilic chronic rhinosinusitis tissue samples. The amplicons were analysed by 2% agarose electrophoresis and the absence of a specific band for the genomic DNA sample confirmed the cDNA specificity of the primers. In addition, all samples were treated with DNase during RNA purification as described by the manufacturer (Qiagen, USA).

To quantify the number of molecules of each hIL-5Rα splice form, we constructed two different standard curves. The template of these standards consisted of PCR fragments obtained from two plasmids containing the specific cDNA sequence for each splice variant as explained in Materials and Methods. Analytical sensitivity in the GeneAmp 5700 Sequence Detection System was determined by using a ten-fold serial dilution of the standards for the soluble and membrane-anchored encoding transcripts as template for amplification. Amplification with the SYBR Green I Master mix and primer pair 3 or 4, resulted in sensitive standard curves where a minimum of 5 molecules of each splice variant could be detected. A high linearity (expressed as correlation coefficient R^2) was observed over a dynamic range of at least 4 orders of magnitude. The maximum amount that could be quantified to keep the standard curve's linearity was 5×10^5 molecules for the soluble and 5×10^4 molecules for the membrane-anchored encoding transcripts.

In all cases, PCR efficiency ranged between 0.95 and 0.97 for both splice variants. Accuracy of the standard curves was evaluated by analysing each standard dilution point as unknown as previously described [29]. Coefficients of variation (C.V.) less than 2% for C_T and 25 % for calculated quantities demonstrate the accuracy of the standard in all dilutions tested (Table 2).

Table 2: Accuracy of the standard curve for hIL-5Rα spliced forms. Coefficients of variation for C_T values and calculated quantities.

	C_T standard	C_T unknown	Qty standard	Qty unknown	C.V. (C_T)	C.V. (Qty)
Membrane-anchored hIL-5Rα	21.86	21.75	3.00×10^4	3.02×10^4	0.94	2.20
	24.96	24.92	3.00×10^3	3.12×10^3	0.35	4.08
	28.02	28.09	3.00×10^2	3.37×10^2	0.34	7.39
	31.88	31.92	3.00×10^1	2.79×10^1	0.60	9.19
Soluble hIL-5Rα	22.44	22.05	3.00×10^4	4.49×10^4	1.19	23.91
	24.71	24.65	3.00×10^3	2.94×10^3	0.59	2.02
	27.09	27.17	3.00×10^2	2.80×10^2	0.32	4.00
	31.60	31.15	3.00×10^1	2.61×10^1	0.83	9.29

Mean of C_T values and quantities (number of molecules) after amplification of the transcripts encoding for the soluble and membrane-anchored hIL-5Rα as standards and as unknowns. *C.V*(C_T): coefficient of variation in % for C_T values of standards and unknowns. *C.V (Qty):* coefficient of variation in % between input (Standards) and obtained quantities (unknowns).

Quantification of either soluble or membrane-anchored hIL-5Rα encoding transcripts in the presence of the alternative form was tested by mixing an excess (5×10^5 molecules) of one transcript form with a dilution series of the other. As a control, we used a standard curve containing only a single splice variant. In absence of inhibition, both dilution series should give equal C_T values and quantities for each dilution point. The low coefficient of variation values obtained in this experiment indicate that accurate and specific quantification of hIL-5Rα splice variants is possible up to 5 molecules (equivalent PCR product), in presence of excess of the alternative splice form (Figure 2).

Expression of hIL-5Rα splice variants in biological samples

Quantities of the transcripts encoding the soluble and the membrane-anchored forms of hIL-5Rα are expressed as relative number of molecules normalized to the number of molecules of internal control gene ACTB. Analysis of mRNA levels showed a significantly higher expression of both splice forms in eosinophilic chronic rhinosinusitis tissue (CRS) compared to normal nasal mucosa (Fig. 3). In addition, in peripheral blood from CRS patients compared to control subjects, both transcripts were also overexpressed, reaching statistical significance however only for the soluble variant (Fig. 3).

These data demonstrate that quantification of the hIL-5Rα splice isoforms by real-time quantitative PCR is feasible in human peripheral blood and eosinophilic chronic rhinosinusitis tissue, showing an up-regulation of both isoforms in nasal tissue and blood from eosinophilic chronic rhinosinusitis patients. In view of the crucial role of hIL-5 in the terminal differentiation of eosinophils and the involvement of these cells in severe airway and skin diseases, tools to investigate the regulation of the IL-5 receptor expression *in vivo* are mandatory to understand the pathomechanisms involved as well as to design future therapeutic approaches [3,30–33]. Treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody (mAb) resulted in eosinophil apoptosis and decreased tissue eosinophilia *in vitro* [29], but antagonizing IL-5 activity in asthma patients with humanized anti-IL-5 mAbs was largely unsuccessful [33] [34]. A reassessment of the *in vivo* regulation of the soluble and membrane anchored hIL-5Rα expression may help to understand the role of the soluble variant, which has antagonistic properties *in vitro*, and a possible role in failures of anti-IL5 treatment.

Conclusion

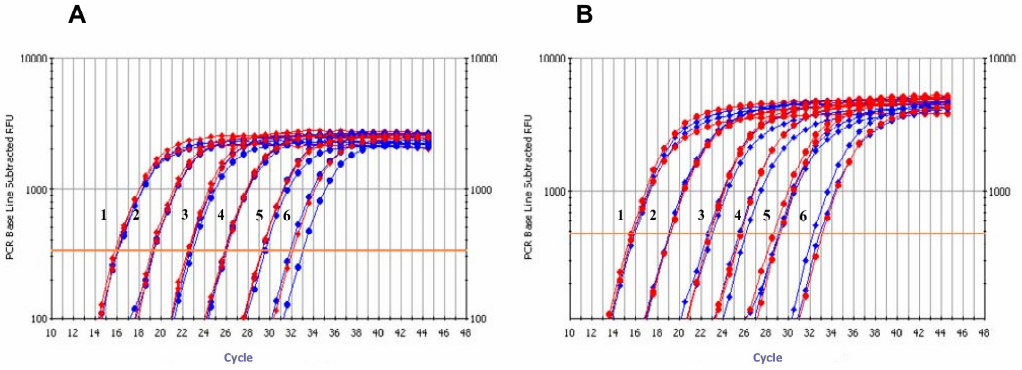
We have established a fast, accurate and reliable assay for mRNA quantification of the hIL-5Rα splice isoforms over a broad range of input molecules. The assay was applied on eosinophilic chronic rhinosinusitis tissue and human peripheral blood and demonstrated an overexpression of both soluble and membrane-anchored encoding splice variants of hIL-5Rα in both tissue and peripheral blood of patients with eosinophilic chronic rhinosinusitis compared to healthy subjects. The development of this assay will greatly help in the study of the regulatory mechanisms of hIL-5Rα. It will also allow investigations of relative expression of this receptor in other eosinophil-related diseases such as asthma, atopic dermatitis and hypereosinophilic syndrome, and hence will contribute to the development of future therapeutic strategies for eosinophil inflammatory diseases.

Methods

Sample Preparation

Samples from normal nasal mucosa and eosinophilic chronic rhinosinusitis biopsies were collected, frozen in liquid nitrogen and thoroughly grinded with a cooled mortar and pestle (Fisher Scientific, UK). 30 mg of tissue were then resuspended in 0.6 ml of lysis (RLT) buffer (Qiagen, USA) and stored at -20°C until RNA extraction.

Peripheral blood (5 ml) from subjects was collected in tubes containing EDTA (Terumo, Leuven, Belgium) and



Dilution	Standard curve (number of molecules)	Qty of membrane-anchored hIL5Rα encoding mRNA (diluted in $5x10^5$ molecules of the soluble variant)	C.V (%) for the membrane-anchored splice form	Qty of soluble hIL5Rα encoding mRNA (diluted in 5 $x10^5$ molecules of the membrane- anchored variant)	C.V (%) for the soluble splice form
1	5×10^5	5.5×10^5	7.54	5.5×10^5	7.06
2	5×10^4	5.5×10^4	7.49	5.2×10^4	3.45
3	5×10^3	5.7×10^3	9.82	4.2×10^3	11.97
4	5×10^2	5.2×10^2	2.69	5.7×10^2	10.20
5	5×10^1	5.2×10^1	2.43	6.0×10^1	13.73
6	5	7.38	63.38	3.44	26.60

Figure 2
PCR amplification plots of each hIL-5Rα splice variant in the presence of excess of the alternative splice form. PCR amplification plots of the membrane-anchored **(A)** and soluble **(B)** encoding splice variant in presence of the alternative splice variant (data generated on iCycler iQ Real-Time PCR Detection System, BioRad Laboratories, USA). Curves in blue indicate the standards diluted in water; curves in red represent standards diluted in 5×10^5 molecules of the alternative splice form. The table indicates the quantities for both standard curves and the coefficient of variation (C.V) between the quantities (molecule number) obtained for each dilution point.

centrifuged at 300 g for 5 minutes. Leukocytes were collected and red blood cells were hemolysed in 25 ml (5 ml per 1 ml of leukocytes suspension) of cold isotonic NH_4Cl-EDTA lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) during 5 minutes at 15°C. Cell suspensions were then centrifuged at 300 g for 6 minutes at 4°C and washed in PBS. Finally, cells were resuspended in 0.6 ml of RLT buffer (Qiagen, USA) and stored at -20°C until RNA preparation.

RNA isolation and reverse transcription

For extraction of total RNA, peripheral blood leukocytes, eosinophilic chronic rhinosinusitis and normal nasal mucosa tissue, (all present in RLT buffer), were first homogenized with QIAshreder homogeniser (Qiagen, USA) as described by the manufacturer. RNA purification was performed with RNeasy mini Kit (Qiagen, USA). RNA was quantified using the RiboGreen kit (Molecular Probes, Leiden, The Netherlands) on a TD-360 fluorometer (Turner Design, USA) and reverse transcription was performed for 1 μg of total RNA in a 20 μl reaction volume. Briefly, 1 μl of Oligo $(dT)_{12-18}$ (500 μg/ml) (Invitro-

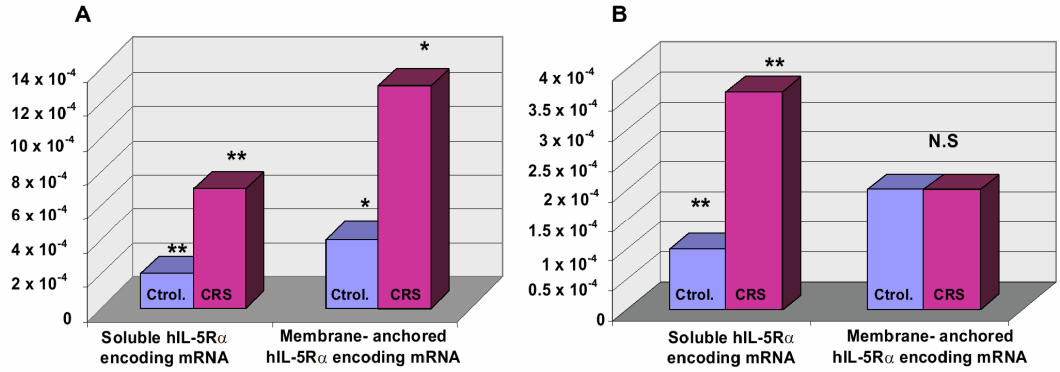


Figure 3
Relative number of molecules of the transcripts encoding the soluble and membrane anchored of hIL-5Rα after normalization to beta-actin (ACTB) gene in eosinophilic chronic rhinosinusitis and normal nasal mucosa (**A**) and in peripheral blood from eosinophilic chronic rhinosinusitis patients and healthy subjects (**B**). **: $p < 0.01$; *: $p < 0.05$; *N.S.*: non significant differences, *CRS*: eosinophilic chronic rhinosinusitis patients; *Ctrol.*: healthy subjects.

gen, USA) and 200 ng of Random Primers (Invitrogen, USA) were added to each RNA sample and incubated at 70°C for 10 min. Then, the samples were incubated with 4 μl of 5 × First-Strand Buffer (Invitrogen, USA), 1 μl of 10 mM dNTP mix (Pharmacia Biotech, USA), 2 μl of 0.1 mM dithiothreitol (Invitrogen, USA) and 200 units of Superscript RNase H- Reverse Transcriptase (Invitrogen, USA), first at 25°C for 10 minutes and subsequently at 42°C during 50 minutes. The reaction was stopped by heating at 90°C for 5 minutes. Finally, RNA complementary to the cDNA was removed by adding 1 μl (2 units) of *E. coli* Ribonuclease H (Invitrogen, USA) and incubated for 30 minutes at 37°C. All cDNA samples were stored at -20°C until analysis.

Primer design

Different primer pairs were designed for generation of standard curve template and for the actual quantification of the hIL-5Rα splice variants (Table 1), based on published cDNA sequences (GenBank accession nos. M75914 and M96652). Primer pairs 1, 2 and 5 were designed using Primer 3 software (Rozen et al., 2000) using the following parameters: primer size between 20 and 27 base pairs, primer Tm range between 60 and 66°C, GC content between 45 and 50%, and sequences containing no runs of four or more identical nucleotides.

Primer pairs 3, 4 and 5 were designed in Primer Express Software version 1.5 (Applied BioSystems, USA) using the sequences flanked by the first set of primers 1, 2 and 5 respectively. Default TaqMan parameters were used with a restriction of amplicon length between 80 and 100 bp.

Generation of gene-specific real-time PCR standards

Two plasmids containing the cDNA sequences for the soluble or membrane-anchored encoding transcripts of hIL-5Rα, kindly provided by Prof. Dr. Jan Tavernier, were used to prepare the template for the standards. A cDNA fragment from each isoform was amplified by mixing 1 × Taq Polymerase Master mix (Invitrogen, USA) with 200 nM of primer pair 1 or 2, 20 ng of plasmid DNA and nuclease-free water to a final volume of 50 μL. The PCR conditions were: 95°C for 10 minutes followed by 35 cycles at 95°C for 30 seconds and 64°C for 1 minute and a final cycle at 72°C for 5 minutes in an ICycler thermal cycler (BioRad Laboratories, USA). The PCR products consisted of a DNA fragment of 294 bp for the soluble and 347 bp for the membrane-anchored encoding transcripts. PCR fragments were run on a 2% agarose gel, excised and eluted using the QIAquick PCR purification kit (Qiagen, USA).

PCR fragments were quantified using the PicoGreen kit (Molecular Probes, The Netherlands) on a TD-360 fluorometer (Turner Design) and the molar concentration of each PCR product was calculated on the basis of the mass concentration and the length in base pairs of each fragment as previously described [29]. Equimolar quantities of both standards were 10-fold serially diluted and used to generate standard curves. The generation of a standard curve, based on serial dilutions of fluorometrically quantified PCR products has been shown to be very reliable

[29]. Template for the standard curve for the internal control gene ACTB was prepared following the same procedure described above, using primer pair 5 (Table 1).

Real-time quantitative PCR using SYBR Green I

Real-time PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied BioSystems, USA). In each experiment, duplicates of a standard dilution series of specific PCR fragments for each hIL-5Rα transcript variant and 25ng cDNA (total RNA equivalent) of unknown samples were amplified in a 25 μl reaction containing 1x SYBR Green I Master mix (Qiagen, USA) and 300 nM of primer pair 2, 3 or 4 for the membrane-anchored or soluble receptor encoding transcripts, respectively, and nuclease-free water. The thermal profile consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute. Real-time PCR efficiencies for each reaction were calculated using the formula: $Efficiency\ (E) = \left[10^{(1/slope)}\right] - 1$, from the slope values given in the GeneAmp 5700 Sequence Detection System.

Quantification and data analysis

For each run, data acquisition and analysis was done by the 5700 Sequence Detection System software (version 1.3, Applied Biosystems, USA). The relative number of molecules of each transcript was determined by interpolating the C_T values of the unknown samples to each standard curve and the obtained values were normalized with respect to the ACTB number of molecules. Statistical tests were performed using the MedCalc program version 6. The *Mann Whitney U-test* (unpaired) was used for comparison between the groups and p values < 0.05 were considered statistically significant.

Acknowledgments

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Impact of RNA Quality on Reference Gene Expression Stability

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Gene expression quantification methods are important tools in the understanding of the molecular events underlying human diseases and in the identification of diagnostic and therapeutic targets. Generally, the messenger RNA (mRNA) used for these analyses is derived from human biopsies obtained after surgery. As a consequence, several steps during tissue handling have to be carefully controlled in order to preserve the quality and integrity of the RNA material. It is well known that RNA is sensitive to degradation by postmortem processes and inadequate sample handling or storage (1). However, RNA integrity control is often not systematically performed prior to (PCR-based) downstream analyses. While in the past, RNA quality could often not be assessed due to the limited availability of the precious sample (e.g., from microdisected cells or small biopsies), the advent of capillary gel electrophoresis and (sample retention) spectrophotometry technologies (e.g., NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, SA) has addressed this issue, allowing quality estimations using only nanograms (or even picograms) of total RNA (2). In addition, amplification of RNA is now an alternative method to obtain sufficient amounts to conduct gene expression studies when postmortem tissues are scarce; however, assessment of RNA quality based on the 18S and 28S ribosomal RNA bands is often not possible anymore after amplification.

Furthermore, it remains to be determined whether the amplified mRNA can faithfully be used to assess RNA quality of the starting material. Apart from RNA quality, the choice of a proper set of reference genes for accurate normalization is another crucial factor with a profound impact on the reliability of the obtained gene expression levels (3). Reference genes are expressed constitutively in every cell; however, their expression can be regulated with diseases state, during cellular proliferation, due to cellular composition and by mitogenic stimuli (e.g., growth factors) (4, 5). Furthermore, it is now known that life styles and genetic make-up of individuals can influence mRNA expression (6). That is why the validation of the expression stability of reference genes remains an important step to ensure the accuracy and reliability of gene expression studies. The objective of this study was to analyze the influence of RNA degradation on the stability and expression pattern of different internal control genes. To this purpose, 10 commonly used reference genes were quantified in both intact and degraded RNA from clinical specimens obtained from ethmoidal and maxillary sinuses collected from patients with nasal polyposis (NP) and chronic rhinosinusitis (CRS).

Sixteen clinical tissue samples (30 mg) were homogenized in Tri-reagent buffer (Sigma, St. Louis, MO, USA) (1 mL/50–100 mg of tissue) in a chilled pestle mortar. Total RNA isolation and cDNA synthesis were performed as described previously (7). RNA quality and percent of degradation were assessed with the Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA); for representative results, see Supplementary Figure S1, which can be found online at www.BioTechniques.com. Real-time PCR amplifications were performed in an iCycler iQ® real time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) in a 25-µL volume containing 20 ng cDNA (total RNA equivalent) of unknown samples, 1× SYBR® Green I Master mix (Bio-Rad Laboratories), and 300 nM specific primer pairs for 10 references genes (*GAPD*, *UBC*, *SDHA*, *HPRT1*, *B2M*, *ACTB*, *YWHAZ*, *HMBS*, *RPLI3A*, and *TBP*); see Supplementary Table S1 for more information. Primer sequences are reported previously (3) and are available in RTPrimerDB, the real-time PCR primer and probe database (medgen.ugent.be/ rtprimerdb/) (8). The amplification protocol consisted of 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and at 60°C for 1 min. Gene expression level normalization and stability analysis was performed using the Microsoft® Excel® Visual Basic application geNorm as described previously (3). Our results show that the stability of reference genes not only is different in CRS and NP ethmoidal and maxillary sinus tissues (as can be expected due to differences in cellular origin), but also varies within the same tissue type according to the degradation status of the samples (Figure 1). The fact that highly stable genes in intact RNA samples rank among the most unstable genes in degraded samples (e.g., *ACTB* in NP tissue) and vice versa (e.g., *GAPD* in CRS and *B2M* in NP) suggests that at least some genes show different sensitivity to RNA degradation.

Step*	Degraded RNA (CRS samples)	Intact RNA (CRS samples)	Degraded RNA (NP samples)	Intact RNA (NP samples)
1	HPRT1	GAPD	HPRT1	YWHAZ
2	YWHAZ	YWHAZ	ACTB	B2M
3	B2M	RPL3IA	RPL3IA	RPL3IA
4	TBP	B2M	GAPD	UBC
5	RPL3IA	UBC	TBP	GAPD
6	UBC	HPRT1	YWHAZ	HMBS
7	ACTB	TBP	HMBS	HPRT1
8	GAPD	ACTB	SDHA	SDHA
9	HMBS- SDHA	HMBS- SDHA	B2M- UBc	ACTB- TBP

Reference genes ranked in order of their expression stability increasing from top to bottom; the two most stable genes in each sample series; for example ACTB and TBP in intact RNA from nasal polyp tissue, cannot be ranked in order because of the required use of gene ratios for gene-stability. *: *The step number at which the least stable gene is excluded, see right panel.*

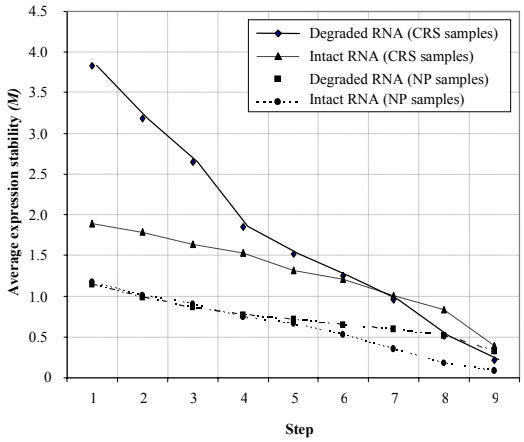

Figure 1. Average expression stability values of remaining control genes. Expression stability (*M*) was analyzed during stepwise exclusion of the least stable control gene in the different tissue samples (the different steps match those in the table). CRS, chronic rhinosinusitis; NP, nasal polyp.

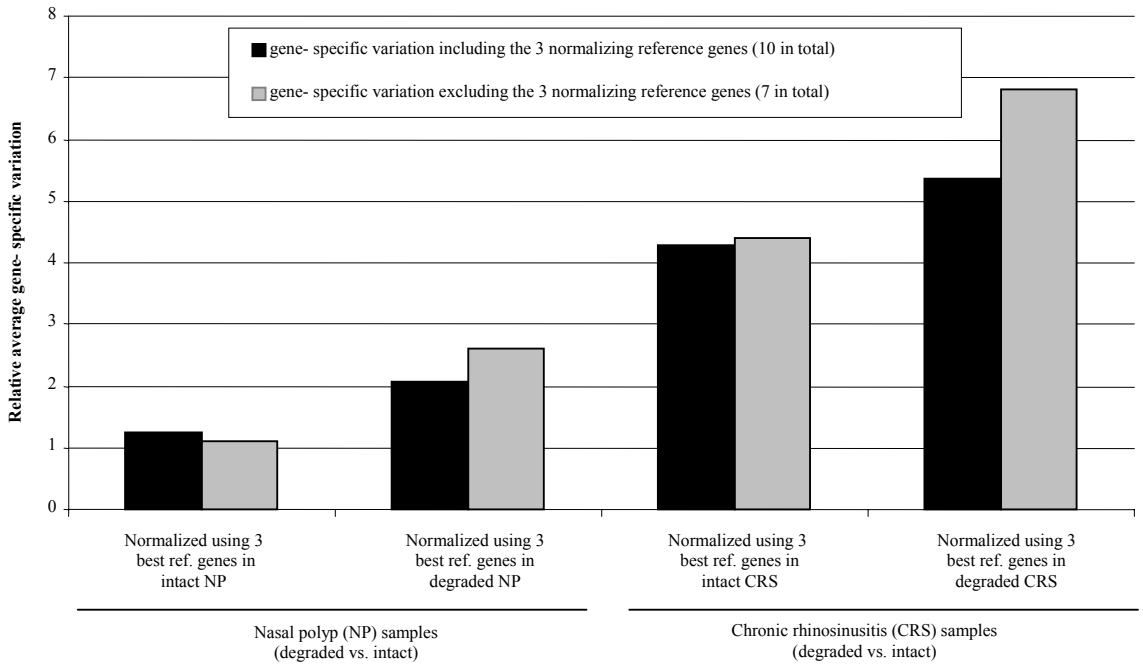

Figure 2. Relative average gene-specific variation in degraded versus intact RNA samples. Gene-specific variation in expression was calculated after normalization using the three most stable reference genes according to geNorm analysis. Bars represent the ratio of the average standard deviation of normalized gene expression levels in degraded samples versus nondegraded samples. From left to right, nasal polyp (NP) and chronic rhinosinusitis (CRS) tissues samples, normalized using 2 different normalization factors.

The bottom line is that different reference genes appear to be suitable for normalization in degraded versus intact RNA samples. When one has no prior knowledge of the RNA degradation status, incorrect conclusions could thus be drawn for the selection of proper reference genes. Furthermore, once a suitable set of reference genes has been selected (even based on only intact samples), the use of these genes to normalize mRNA content in (partially) degraded samples could lead to significant errors and misinterpretation of target gene expression levels information.

The purpose of normalization is to remove the sampling differences (such as RNA quantity and cDNA synthesis efficiency) in order to identify real gene-specific variation that, for proper internal control genes, should be minimal. Following the approach outlined in Reference 3, we have calculated the gene-specific variation for each reference gene as the standard deviation of normalized expression levels. To this end, the raw expression values were divided by two different normalization factors, calculated as the geometric mean of the three most stable control genes (as determined by geNorm) in degraded or intact RNA samples. We

subsequently determined the average gene-specific variation of all reference genes (either excluding or including the three normalizing reference genes) for each normalization factor, and within each tissue type, and plotted the ratio of the variation in degraded versus intact samples (Figure 2).

It is clear that the gene-specific variation is always higher in degraded versus intact samples, with more pronounced differences in CRS compared to NP. Furthermore, normalization using the so-called best reference genes in degraded samples systematically resulted in higher gene-specific variation than using the best reference genes identified on the basis of intact samples. In addition, in degraded samples, relative gene specific variation was also higher when the three most stable genes (used for normalization) were excluded (Figure 2, grey bars) than when they were included (Figure 2, black bars). In contrast, in non-degraded samples, the inclusion or exclusion of these genes seems not to significantly affect gene specific variation. Using a normalization factor based on the proper internal reference genes should result in the removal of nonspecific variations. However, unstable reference genes cannot completely remove variation instead, they add more, resulting in larger so-called gene-specific variation for the tested reference genes.

This analysis clearly demonstrates that most nonspecific variation was removed when the most stable control genes in intact RNA samples (as determined by geNorm) were used for normalization and that the gene expression variation in degraded RNA samples is inherently higher. While thorough RNA quality control is routinely being performed prior to microarray-based gene expression profiling, the same quality control is often considered not required or simply not performed for PCR-based quantification methods. Indeed, even on degraded RNA samples, a nice amplification curve can be obtained. However, in view of the observed difference in reference gene expression stability between intact and degraded RNA samples from the same tissue and the higher gene-specific variation in degraded samples, we propose performing RNA quality control prior to downstream quantification assays and discarding degraded samples, especially if one aims to accurately quantify small expression differences. Indeed, as it is of utmost importance to normalize samples using the same set of reference genes, our results suggest that it is inappropriate to compare degraded and intact samples.

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CHAPTER 5

REGULATION OF EICOSANOID METABOLISM IN CHRONIC RHINOSINUSITIS/ NASAL POLYPOSIS

Introduction

Arachidonic acid cascade is one of the most controversial pathways regulating the balance of pro- and anti-inflammatory events during immune response. Arachidonic acid released after cell stimulation, can be metabolized by three different ways yielding to the production of leukotrienes, prostaglandins and lipoxins, which can up- or down-regulate the expression of their receptors in combination with other stimulus like cytokines and growth factors. Implication of this pathway in chronic rhinosinusitis/ nasal polyposis and especially in aspirin intolerance syndrome has been suggested; however, the precise mechanisms operating in these diseases remain unclear. In this chapter, we demonstrated that changes in arachidonic acid metabolism may occur in absence of the clinical manifestation of aspirin intolerance in patients with chronic rhinosinusitis and they are mainly related to the eosinophilic inflammation developed in these patients. In addition, we also show that the expression of CysLTs and especially prostanoid receptors follow a different pattern in chronic rhinosinusitis patients with and without nasal polyps, which can have a big implication in the attenuation of the inflammatory responses in these patients.

Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis

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Background: Upper airway diseases and especially the aspirin hypersensitivity syndrome have been linked to changes in the arachidonic acid cascade; however, the specificity of these changes and their relation to inflammatory reactions in these diseases still remain controversial.
Objective: We aimed to study the tissue eicosanoid production in 3 subgroups of patients with chronic rhinosinusitis (CRS) and control subjects and to correlate it with the severity of inflammation and clinical manifestation of aspirin sensitivity.
Methods: Samples were prepared from sinonasal tissue of patients with CRS with (CRS-NP group, n = 13) and without nasal polyposis (CRS group, n = 11), sinonasal tissue of patients with nasal polyposis and aspirin sensitivity (CRS-ASNP group, n = 13), and normal nasal mucosa from healthy subjects (NM group, n = 8). Real-time PCR was applied for mRNA quantification of COX-2, 5-lipoxygenase, leukotriene C_4 synthase, and 15-lipoxygenase. Enzyme immunoassays were used to measure IL-5, eosinophil cationic protein, and eicosanoid (leukotriene [LT] C_4, LTD_4, and LTE_4; lipoxin A_4; and prostaglandin E_2 [PGE_2]) concentrations.
Results: COX-2 mRNA and PGE_2 concentrations were similar in the CRS and NM groups but significantly decreased in nasal polyp tissue, especially in the CRS-ASNP group. LTC_4 synthase, 5-lipoxygenase mRNA, LTC_4, LTD_4, and LTE_4 concentrations increased with disease severity among the patient groups. 15-Lipoxygenase and lipoxin A_4 concentrations were increased in all CRS groups compared with in the NM group but were significantly downregulated in the CRS-ASNP group when compared with the CRS-NP group. IL-5 and eosinophil cationic protein were increased in both groups of nasal polyp tissue compared with in the NM and CRS groups and correlated directly with LTC_4, LTD_4, and LTE_4 concentrations and inversely with PGE_2 concentrations.
Conclusion: Changes of tissue eicosanoid metabolism do occur in CRS, even in the absence of clinical aspirin sensitivity, and these changes appear to be related to the severity of eosinophilic inflammation. (J Allergy Clin Immunol 2005;115:1189-96.)

Key words: *Chronic rhinosinusitis, nasal polyposis, aspirin sensitivity, inflammation, leukotrienes, lipoxins, prostaglandins, cyclooxygenase, lipoxygenase*

Chronic rhinosinusitis (CRS) is an inflammatory reaction of the paranasal mucous membrane, mainly characterized by an accumulation of inflammatory cells in the lamina propria, with some nonspecific lesions, such as edema, fibrosis, or epithelial degradation.[1,2] Rhinosinusitis might be present in conjunction with nasal polyposis, which is a multifactorial disease consisting of tissue (nasal and sinus mucosa) infiltration by inflammatory cells, predominantly eosinophils, and the formation of pseudocysts with plasma exudation and albumin retention.[2] Nasal polyposis can be frequently linked to other conditions, such as asthma and aspirin sensitivity. About 15% of patients with polyps have aspirin sensitivity.[3] Sensitivity to aspirin consists of steroid-dependent asthma and nasal polyposis (rhinosinusitis) that begin with a prolonged episode of nasal congestion, rhinorrhea, and hyposmia with persistent mucosal inflammation, followed by nasal polyposis, bronchial asthma, and aspirin sensitivity.[4,5]

In the early and chronic phases of inflammation, excessive amounts of lipid mediators can be released. Cysteinyl leukotrienes (cysLTs; leukotriene [LT] C_4, LTD_4, and LTE_4) have profound effects on airway function[6-8] by inducing airway smooth muscle contraction, vasodilatation, and vascular permeability[9] and altering the remodeling process in asthma.[10] Increased concentrations of cysLTs have been observed in patients with nasal polyps and aspirin sensitivity.[11-13] An association between polymorphisms in the gene encoding the leukotriene C_4 synthase (LTC_4S) enzyme and aspirin sensitivity has also been suggested in several studies; however, no direct relationship between these 2 factors has been demonstrated thus far.[14-16] In addition, urine LTE_4 concentrations have been observed to increase after oral aspirin provocation in aspirin-sensitive patients, suggesting a role for these molecules in the pathogenesis of these conditions.[17]

Lipoxins (lipoxin [LX] A_4 and LXB_4), arachidonic acid (AA) metabolites of the 12/15-lipoxygenase (12/15-LO)

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Abbreviations used
5-LO: 5-Lipoxygenase
15-LO: 15-Lipoxygenase
AA: Arachidonic acid
CRS: Chronic rhinosinusitis
CRS-ASNP: Chronic rhinosinusitis–nasal polyps–aspirin sensitivity
CRS-NP: Chronic rhinosinusitis–nasal polyps
CT: Computed tomography
cysLT: Cysteinyl leukotriene
ECP: Eosinophil cationic protein
LT: Leukotriene
LTC_4S: Leukotriene C_4 synthase
LX: Lipoxin
PGE_2: Prostaglandin E_2

enzymes, are generally associated with anti-inflammatory effects.[18] These molecules have been demonstrated to serve as stop signals for leukocyte trafficking, facilitating the resolution of acute inflammatory responses.[19] It has been shown that nasal polyp tissue has a high capacity to produce lipoxins.[20] Furthermore, *in vitro* studies have found that cultured epithelial cells and peripheral blood from aspirin-sensitive patients have a decreased capacity of lipoxin production compared with those from aspirin-tolerant patients.[21-23] However, in epithelial cells from aspirin-sensitive patients, this capacity is increased after aspirin stimulation.[21]

Prostaglandin E_2 (PGE_2) plays a crucial role in the maintenance of local homeostasis and regulates a broad range of physiologic activities, including immune responses.[24] PGE_2 and COX-2 enzyme have been found to be decreased in peripheral blood cells and nasal polyp tissue from patients with aspirin sensitivity.[25] In addition, the cyclooxygenase/lipoxygenase ratio is downregulated in the nasal tissue from aspirin-sensitive patients compared with nonsensitive and control subjects,[26] suggesting that a deficit of this prostanoid might be responsible for the chronic inflammatory reactions in these diseases. The aim of this study was to analyze the local tissue eicosanoid production in 3 subgroups of patients with CRS and to evaluate the possible correlation with the severity of eosinophilic inflammation and the clinical manifestation of aspirin sensitivity.

METHODS

Patients

Study subjects were selected on the basis of a documented medical history for CRS; a pathologic endoscopy, computed tomographic (CT) scan, or both; and the failure of 3 weeks of drug therapy (combined administration of antibiotics and oral glucocorticoids, 32-mg down to 8-mg daily dosage) to persistently relieve symptoms of this condition. Samples from ethmoidal and maxillary sinuses were collected from patients during functional endoscopic sinus surgery procedures in the Department of Othorinolaryngology at the Ghent University Hospital. The patients with CRS were classified in 3 groups: subjects with CRS with nasal polyps (CRS-NP group), subjects with CRS without nasal polyps (CRS group), and subjects with CRS with nasal polyps, bronchial asthma, and aspirin sensitivity (CRS-ASNP). Patients undergoing septoplasty or rhinoseptoplasty because of anatomic variations and not having from any sinus disease were considered control subjects (NM group). All patients provided informed consent before their participation, and the study was approved by the ethical committee of the Ghent University Hospital. Clinical data of the patients are summarized in Table I.

TABLE I. Patients' clinical data

	Control group	CRS group	CRS-NP group	CRS-ASNP group
No. of subjects	8	11	13	13
Sex (female/male)	2/6	3/8	3/10	4/9
Age (y)	20-50	21-53	30-54	31-60
No. of patients with bronchial asthma	0	1	2	13
No. of patients with aspirin sensitivity	0	0	0	13
No. of patients with positive skin prick test results	0	1	3	10
Inhaled steroids	0	11	13	13

Clinical diagnosis of CRS–nasal polyposis

Clinical diagnosis followed the guidelines proposed by the American Academy of Otolaryngology–Head and Neck Surgery.[27] In the CRS group patients experienced typical symptoms, such as headache, nasal obstruction, and discolored nasal drainage arising from the nasal passages, that persisted for 12 weeks or longer and showed edema or erythema of the middle meatus, as identified by means of nasal endoscopy. Furthermore, a pathologic CT scan showed diffuse mucosal thickening, opacification, or swelling of the ethmoidal and maxillary mucosa with bilateral obstruction of the osteomeatal complex but without polyp formation. The diagnosis of CRS-NP was based on a documented medical history, typical symptoms (nasal congestion, loss of smell, changes in sense of taste, and persistent postnasal drainage), and the presence of endoscopically visible bilateral polyps growing from the middle nasal meatus into the nasal cavities and affecting the ethmoidal and maxillary sinuses according to a CT scan of the paranasal sinuses. The diagnosis of asthma was based on clinical history, typical symptoms, and lung (pulmonary) function tests (spirometry and peak expiratory flow) and was performed in the Department of Pneumology at Ghent University Hospital according to the Global Initiative for Asthma guidelines. The use of any oral medication with a possible effect on measurements of enzymes or mediators, including systemic glucocorticoids and antileukotrienes, was stopped for all subjects 4 weeks before surgery. The use of topical glucocorticoids was interrupted 2 weeks before surgery.

Diagnosis of aspirin sensitivity

Diagnosis of aspirin sensitivity (CRS-ASNP group) was based on a medical history reporting symptoms as described above; reactions, including asthma exacerbation, rhinorrhea, and nasal congestion, after the ingestion of aspirin or other nonsteroidal anti-inflammatory drugs; and a positive bronchial aspirin provocation test result.[28] Briefly, 3 different concentrations of lysine-aspirin (Aspisol; Horby Bayer AG, Leverkusen, Germany) solution (2, 1, and 0.1 M) were administrated, and varying the number of tidal breaths (1 to 23)

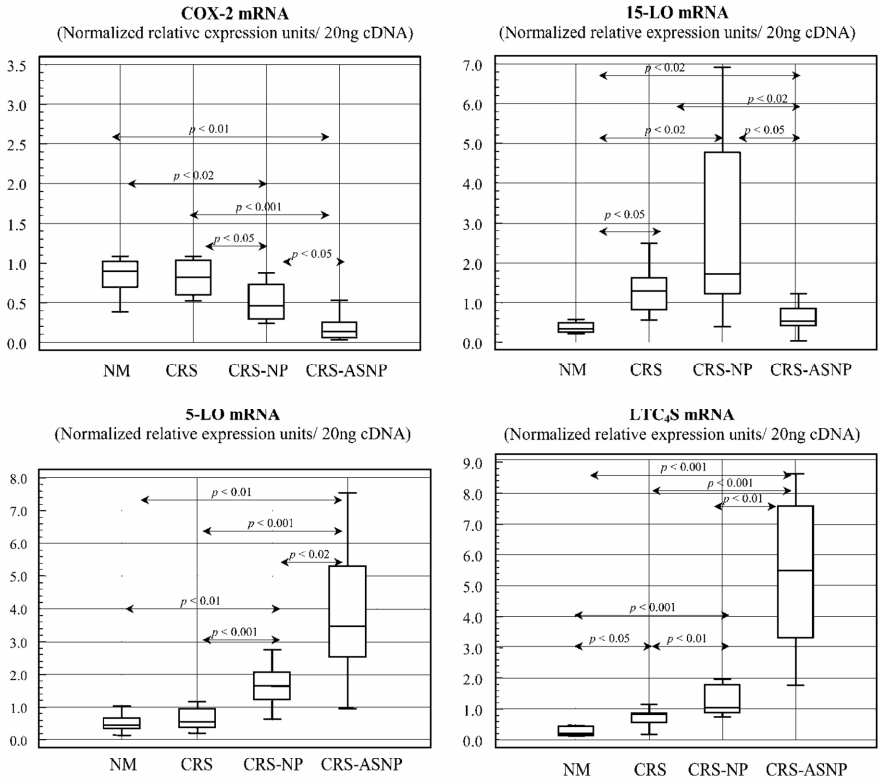

FIG 1. Relative mRNA expression of COX-2, 15-LO, 5-LO, and LTC_4S measured in cDNA equivalent to 20 ng of RNA extracted from sinonasal tissue of patients with CRS without nasal polyps (CRS group), patients with CRS with nasal polyps (CRS-NP group), patients with CRS with nasal polyps and aspirin sensitivity (CRS-ASNP group), and inferior turbinate tissue (normal mucosa) from healthy subjects (NM group). *P* values after the unpaired Wilcoxon test are shown.

TABLE II. Primer sequences used for real-time PCR amplifications

	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon size (bp)	Genbank accession no.
Enzymes				
COX-2	GCTGGAACATGGAATTACCCA	CTTTCTGTACTGCGGGTGGAA	88	XM_051899
5-LO	CACATGTTCCAGTCTTCTTGGA	ATGACCCGCTCAGAAATAGTGT	86	J03571
LTC_4S	ACGGTACCATGAAGGACGAG	CACCTGCAGGGAGAAGTAGG	86	NM_145867
15-LO	AAGCTGGAGCCTTCCTAACC	GCATCTTGGGCATAGAAGGA	100	NM_001140
Internal control genes				
β-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	139	NM_001101
Hydroxymethyl-bilane synthase	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	154	NM_00319

resulted in stepwise increments in the dose of inhaled lysine-aspirin. Baseline FEV_1 was measured as the best of 3 efforts before (prediluent baseline) and 20 minutes after (postdiluent baseline) the inhalation of 7 breaths of 0.9% sodium chloride. FEV_1 values were obtained at 10, 20, and 30 minutes after the last inhalation of each dose. The lysine challenge was completed when FEV_1 decreased by 20% or more compared with the postdiluent baseline value or the maximum dose of aspirin had been reached (540 μmol cumulated dose). A lysine-aspirin challenge test was only performed if, on the day of challenge, the patient's FEV_1 was greater than 60% of predicted value.

mRNA expression by means of quantitative real-time PCR

Snap-frozen samples from nasal or sinus tissue were homogenized in Tri-reagent buffer (Sigma-Aldrich, St Louis, MO) in a chilled

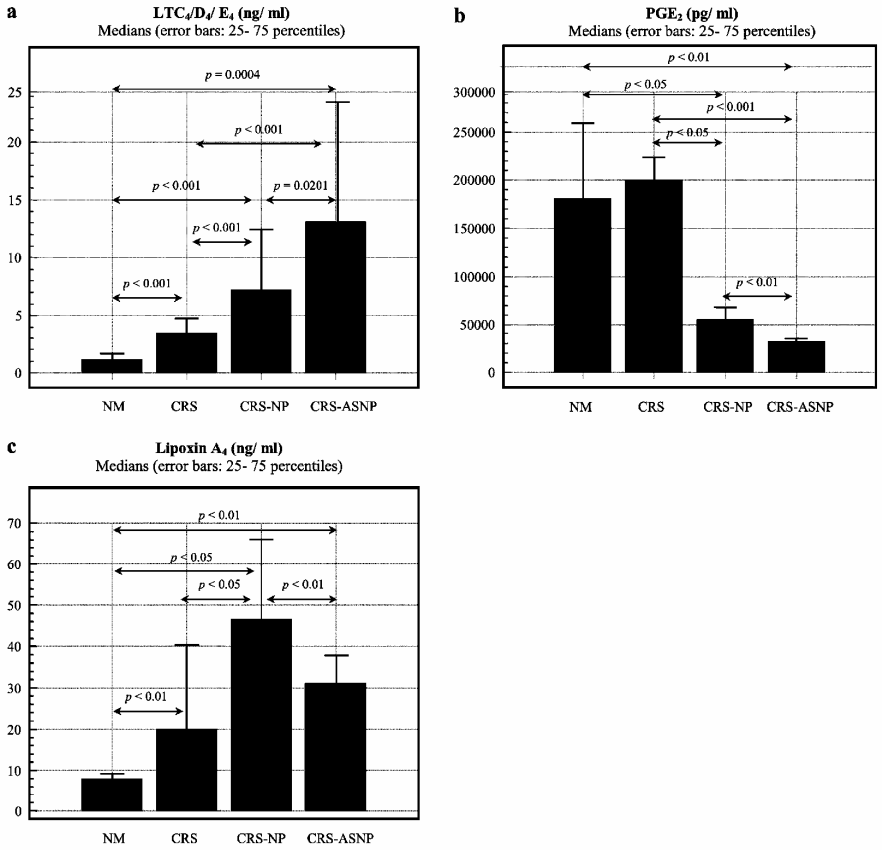

FIG 2. Concentrations of the eicosanoids cysLTC$_4$, cysLTD$_4$, and cysLTE$_4$ **(A)**; LXA$_4$ **(B)**; and PGE$_2$ **(C)** in sinonasal tissue of patients with CRS without nasal polyps (CRS group), patients with CRS with nasal polyps (CRS-NP group), patients with CRS with nasal polyps and aspirin sensitivity (CRS-ASNP group), and inferior turbinate tissue (normal mucosa) from healthy subjects (NM group). *P* values after the unpaired Wilcoxon test are shown.

pestle and mortar. Total RNA was isolated by using the Tri-reagent Kit according to the manufacturer's instructions (Sigma-Aldrich, St Louis, Mo). cDNA was synthesized from 2 μg of total RNA, as described previously.[29] Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad laboratories, Hercules, Calif) with specific primers (Table II). PCR reactions contained 20 ng of cDNA of unknown samples, 1× SYBR Green I Master mix (Bio-Rad Laboratories, Hercules, Calif), and 300 nM of primer pairs, and the PCR protocol consisted of 1 cycle at 95°C for 10 minutes, followed by 45 cycles at 95°C for 30 seconds and 60°C for 1 minute. β-Actin and hydroxymethyl-bilane synthase were used as internal control genes.[30] The relative number of molecules of each gene, expressed in relative expression units quantified per 20 ng of cDNA sample, was determined by using the ΔCT value method, as described previously.[31]

Eicosanoid production

CysLTC$_4$, cysLTD$_4$, cysLTE$_4$, PGE$_2$, and LXA$_4$ concentrations were measured by using ELISAs purchased from Oxford BioMedicals (Oxford). Sample extraction procedures for protein removal and eicosanoid stabilization were performed according to the provider's instructions. Briefly, nasal or sinus tissues were first homogenized in ethanol (5 mL/g) for LXA$_4$, LTC$_4$, LTD$_4$, and LTE$_4$ measurements and in 15% methanol/0.1 M sodium phosphate buffer, pH 7.5, for PGE$_2$ measurements and then centrifuged for 5 minutes at 3000 rpm at 4°C. Supernatants were diluted in water, pH 3.5, and processed by using the C18 Sep-Pak light column (Waters Corp) according to the manufacturer's instructions (Oxford BioMedical Research, Oxford, Miss). The detection ranges for all assays were between 0.02 and 10 ng/mL. The sensitivity value was 0.2 ng/mL for all assays, and the intra-assay and interassay coefficient of variation was less than 10%.

Eosinophilic inflammation markers

IL-5 concentrations in supernatants from nasal or sinus tissue homogenates were measured by means of ELISA (detection range, 7.8-500 pg/mL; sensitivity, <3 pg/mL) purchased from R&D Systems (Minneapolis, Minn). Tissue samples were homogenized in 0.9% NaCl solution (homogenizer B. Braun, Melsungen, Germany) at 1000 rpm for 5 minutes on ice and then centrifuged at 3000 rpm at 4°C for 10 minutes to collect supernatants. Quantification

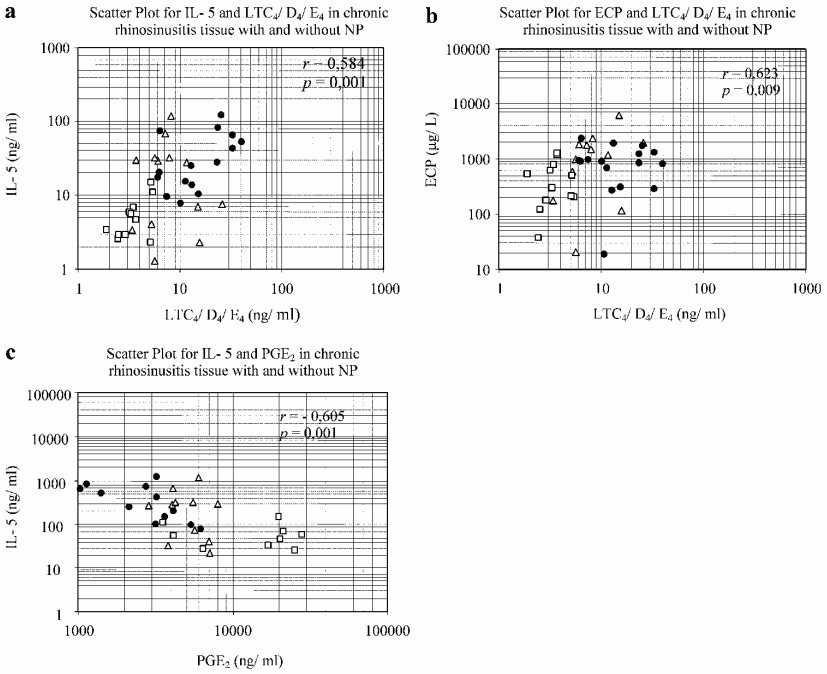

FIG 4. Logarithmic scatterplot after rank correlation analysis showing the direct correlation between LTC_4, LTD_4, and LTE_4 and IL-5 **(A)**, ECP **(B)**, and the inverse correlation between PGE_2 and IL-5 **(C)** in sinonasal tissue from the following: *open squares*, patients with CRS without nasal polyps (CRS group); *open triangles*, patients with CRS with nasal polyps (CRS-NP group); *filled circles*, patients with CRS with nasal polyps and aspirin sensitivity (CRS-ASNP group).

disease groups compared with in the control subjects (NM group; $P < .05$) and in the CRS-NP and CRS-ASNP groups compared with in the CRS group ($P < .05$); however, no statistically significant difference was observed between the aspirin-nonsensitive (CRS-NP) and aspirin-sensitive nasal polyp (CRS-ASNP) groups ($P > .1$). The Spearman rank correlation analysis showed that LTC_4, LTD_4, and LTE_4 concentrations significantly correlated with IL-5 ($r = 0.584$, $P = .001$) and ECP ($r = 0.423$, $P = .009$) levels in all patient groups. Furthermore, IL-5, but not ECP, inversely correlated to PGE_2 in all patient groups ($r = -0.6053$, $P = .001$), as shown in Fig 4.

DISCUSSION

The development of aspirin sensitivity remains unclear and has been related to several processes, including changes in AA metabolism, allergy, viral infection, and severity of inflammation.[32,33] We here show that COX-2 mRNA and PGE_2 concentrations were significantly decreased in nasal polyp tissue, especially in patients with aspirin sensitivity (CRS-ASNP group). In contrast, the lowest levels of LTC_4S, 5-LO mRNA, LTC_4, LTD_4, and LTE_4 were observed in control subjects, and the highest levels were found in CRS-ASNP tissues. 15-LO and LXA_4 levels were increased in all CRS groups compared with in the control subjects but were significantly downregulated in the CRS-ASNP group when compared with the CRS-NP group. Finally, IL-5 and ECP levels were increased in both nasal polyp tissues compared with in the NM and CRS groups and significantly correlated with LTC_4, LTD_4, and LTE_4 but inversely correlated with PGE_2 concentrations. These results demonstrate that changes in the AA pathway in sinonasal tissue occur in patients with different conditions of CRS, even in the absence of a clinical manifestation of aspirin sensitivity, and lead us to speculate that severe inflammation might form a pivotal condition for its manifestation.

It has been shown that leukotriene production is increased in nasal polyp tissue, eosinophils, and urine from patients with sensitivity to aspirin (references 11, 13, and 17). In our study mRNA levels of 5-LO and LTC_4S mRNA were not only significantly increased in the aspirin-sensitive patients but also in the tolerant CRS-NP group compared with the CRS group. The same pattern was observed for LTC_4, LTD_4, and LTE_4 production, which was also related to the eosinophilic inflammation markers ECP and IL-5. These results are in line with those of previous studies showing an upregulation of cysLTs in the nasal polyp tissue of patients without aspirin sensitivity and a relation to eosinophil

numbers.[34] According to these results, we propose that changes in these eicosanoids might not only represent a pathophysiologic hallmark of aspirin sensitivity but also a phenomenon linked to inflammatory reactions.

Lipoxins are receiving increased attention in the field of inflammation research because the role of these molecules in airway diseases becomes more than controversial. Our results confirm that nasal polyp tissue can produce lipoxins. We also report for the first time that the levels of these eicosanoids were significantly higher in all patients with CRS compared with in control subjects but decreased in the aspirin-sensitive patients compared with in the aspirin-tolerant subjects. It is known that lipoxin biosynthesis occurs mainly during cell-cell interactions between infiltrating leukocytes and tissue-resident cells, such as cytokine-primed endothelial or epithelial cells.[35] In addition, the severity of asthma has been associated with an increase and activation of 15-LO enzymes, collagen deposition, and eosinophil accumulation.[36] We have observed an increased eosinophilic infiltration in the epithelium of CRS-NP tissues (data not shown), which could partially account for the high levels of lipoxins in these patients. In addition, this compound has been reported to increase chloride secretion in human bronchial epithelial cells,[37] which might affect water retention and contribute to polyp formation. However, this does not explain the reduced capacity to produce lipoxins observed in tissue from patients of the CRS-ASNP group harboring abundant numbers of eosinophils. This phenomenon probably is due to intrinsic changes in the lipoxin biosynthetic pathway and appears to be the only specific finding in aspirin sensitivity in our study.

Furthermore, according to the literature, downregulation of PGE_2 can be considered as a hallmark for patients with rhinosinusitis with aspirin sensitivity.[38] However, previous studies performed in nasal polyp tissue have suggested that abnormalities in the cyclooxygenase pathway are not exclusively present in aspirin-sensitive patients but are also present in nasal polyps in the absence of this syndrome.[39] These findings are supported by our study showing that local tissue downregulation of PGE_2 and COX-2 was specifically observed in the nasal polyp groups and especially in the subgroup with aspirin-sensitive subjects but might not represent a unique characteristic of aspirin sensitivity.

In this study we demonstrate that levels of cysLTs increase with the severity of disease per patient group and are related to eosinophilic inflammation. In contrast, downregulation of the cyclooxygenase pathway seems to play a crucial role in the pathophysiology of nasal polyposis and might not directly be linked to the manifestation of hypersensitivity to aspirin. Finally, increase of lipoxin production in all CRS groups compared with that in control subjects and its downregulation in the aspirin-sensitive group points to the role of this metabolite in the development of the disease.

In conclusion, we show that changes in AA metabolism might occur in CRS without aspirin sensitivity, and they might not be specific for the clinical manifestation of aspirin syndrome but represent a secondary phenomenon linked to a certain degree of inflammation in the airway mucosa. However, because all patients with CRS had bronchial asthma, the influence of lower airway disease or systemic disease on local inflammation cannot be excluded. The question of whether these changes can precede the manifestation of this syndrome awaits a long- term follow-up study in patients with CRS and nasal polyposis in whom aspirin sensitivity has not yet developed.

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Expression of Eicosanoid Receptors Subtypes and Eosinophilic Inflammation: Implication on Chronic Rhinosinusitis

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ABSTRACT

Background: Eicosanoid receptors are G-protein-coupled receptors playing an important immunomodulatory role in airway diseases; however, there is little information on the expression of these receptors and their link with eosinophilic information in paranasal sinus diseases. We aimed with this study to investigate the tissue expression of leukotrienes and prostaglandin E_2 receptors in chronic rhinosinusitis patients with and without nasal polyposis and the link of this regulation with the inflammatory process in these subjects. **Methods:** Samples were prepared from nasal tissue of patients with chronic rhinosinusitis without nasal polyps (CRS, n=11), with nasal polyps (CRS-NP, n=13) and 6 healthy subjects (Controls). mRNA expression of $CysLT_1$, $CysLT_2$, BLT_1, BLT_2, E- prostanoid receptors (EP_1, EP_2, EP_3, EP_4) and IL-5R was determined by real- time PCR. Concentrations of PGE_2, $LTC_4/D_4/E_4$, LTB_4 and IL-5R (were determined by ELISA and of ECP by ImmunoCap. Protein expression and tissue localization of eicosanoid receptors and activated eosinophils were evaluated by immunohistochemistry. **Results:** $CysLT_1$ mRNA expression was significantly increased in CRS-NP compared to CRS and controls, and CRS compared to controls, whereas $CysLT_2$ mRNA was enhanced in both CRS groups without differences between them. In addition, levels of $CysLT_1$ and $CysLT_2$ receptors correlated to the number of activated eosinophils, IL-5R protein, ECP and $LTC_4/D_4/E_4$ concentrations in the disease groups. PGE_2 protein concentrations and prostanoid receptors EP_1 and EP_3 were down-regulated in the CRS-NP tissue vs. CRS and controls, whereas EP_2 and EP_4 expression was enhanced in CRS and CRS-NP patients vs. controls. No differences in BLT receptors or LTB_4 were observed between patients and controls. **Conclusions:** Contrasting to LTB_4, we here show that protein and receptor expression for cys-leukotrienes and prostaglandin E_2 is differentially regulated in CRS-NP, showing a parallel regulation pattern between eicosanoids and their receptors. Within the group of EP receptors, we found a differential shift of EP_1 and EP_3 molecules vs. EP_2 and EP_4, which may be important in the mechanisms operating in chronic rhinosinusitis/ nasal polyposis.

ABBREVIATIONS:

AA:	arachidonic acid
AAM:	arachidonic acid metabolism
BLT_1:	leukotriene B_4 receptor 1
BLT_2:	leukotriene B_4 receptor 2
CRS:	chronic rhinosinusitis
CRS-NP:	chronic rhinosinusitis- nasal polyp
CysLTs:	cysteinyl leukotrienes
$CysLT_1$:	cysteinyl leukotriene receptor 1
$CysLT_2$:	cysteinyl leukotriene receptor 2
EG_2:	eosinophil granulocyte
EP_1:	prostanoid receptor 1
EP_2:	prostanoid receptor 2
EP_3:	prostanoid receptor 3
EP_4:	prostanoid receptor 4
Sol- IL5Rα:	soluble interleukin –5 receptor alpha
$LTC_4/ D_4/ E_4$:	leukotriene $C_4/ D_4/ E_4$
LTB_4:	leukotriene B_4
PGE_2:	Prostaglandin E_2

Key words: prostanoid E receptors, nasal polyposis, chronic rhinosinusitis, leukotriene receptors, eosinophilic inflammation, eicosanoid metabolism.

INTRODUCTION

The role of eicosanoids in the pathophysiology of chronic inflammatory airway diseases especially asthma have been well documented. However, the key steps in the regulation leading to the production of these molecules remain unclear. Eicosanoid signalling operates through lipid G- protein- coupled receptors (GPCRs) (1). According to the International Union of Pharmacology (IUPHAR), eicosanoid receptors are classified in four main groups: the BLT receptors, with biological activities related to LTB_4, the cysteinyl leukotrienes (CysLTs) receptors related family, the lipoxin (ALX) receptors and the prostanoid receptors class (1).

Cysteinyl leukotrienes play a disease-regulating role in rhinosinusitis and asthma, particularly in the aspirin-sensitive syndrome. These eicosanoids mediate their biologic activities through interactions with the $CysLT_1$ and $CysLT_2$ receptors, which play an important role in inducing bronchospasm, endothelial cell adherence, myofibroblast proliferation, bronchoconstrictions, vascular hyper-permeability, mucus secretion and chemokine production (2). Immunohistochemical studies have revealed that the $CysLT_1$ receptor is expressed on eosinophils, mast cells, macrophages, neutrophils and vascular endothelial cells in the nasal mucosa (3). $CysLT_1$ receptor has been found up- regulated in inflammatory cells from aspirin-sensitive patients compared to non-aspirin-sensitive patients and its down- regulation was associated to the aspirin sensitization performed in these patients (4). Expression of CysLTs receptors has been also demonstrated in key allergic and inflammatory cells from the upper airway of patients with active seasonal allergic rhinitis (5). Cysteinyl leukotrienes may also regulate vascular permeability in the airways, leading to airway edema. *In vitro* studies have shown that administration of CysLTs receptor antagonists markedly reduced plasma extravasation and vascular endothelial growth factor levels in allergen-induced asthma, indicating the role of CysLTs receptors in the modulation of vascular permeability (6). Genetic studies have also suggested the $CysLT_2$ receptor to be one of the genes contributing to susceptibility to asthma, based on a polymorphism associated to the development of this disease in a Japanese population (7). In addition, a recent study performed in patients with chronic rhinosinusitis/ nasal polyps showed that $CysLT_1$ expression predominates on inflammatory leukocytes in patients with aspirin-sensitive rhinosinusitis, but the effects of cysteinyl leukotrienes on glands and epithelium may be mediated predominantly through $CysLT_2$ (8).

Together with CysLTs, leukotrienes B_4 receptors (BLTs) have been reported as potential molecules in the regulation of airway inflammation. However, in contrast to CysLTs, controversial results about LTB_4 and BLT receptors have been reported in several studies. The human high-affinity LTB_4 receptor BLT_1 has been found in myeloid leukocytes, including neutrophils, macrophages, and eosinophils, but also T cells (9). In vitro studies have demonstrated that LTB_4 can induce T helper type 1 and 2 cell chemotaxis and firm

adhesion to endothelial cells and early $CD4^+$ and $CD8^+$ T cell recruitment into the airway in an asthma model (10). Furthermore, BLT receptor- LTB_4 mediated action was found to contribute to neutrophil influx into the airways in patients suffering from chronic obstructive pulmonary disease, suggesting its role in disease progression (11). However, in upper airway diseases, the levels of LTB_4 and BLT receptors have been found increased in nasal polyp patients with allergy in comparison to non- allergic subjects (12) but no differences were observed between aspirin intolerant compared to tolerant subjects (13). Until now, no clear mechanism regulating the synthesis of these molecules in airway has been demonstrated.

Although a large body of studies has been focused in the expression leukotrienes and leukotrienes receptors in different airway diseases; a few experiments have been conducted related to the mechanisms regulating prostanoid and lipoxin receptor signalling. The prostanoid receptor subfamily comprises eight members: the prostaglandin D (DP) receptor, the prostaglandin E_2 receptors (EP_1, EP_2, EP_3 and EP_4), the prostaglandin F receptor (FP), the prostaglandin I receptor (IP), the tromboxane A receptor (TP), and a ninth prostaglandin receptor identified recently, the chemoattractant receptor homologous molecule expressed on Th_2 cells (CRTH2) (14). The roles of prostaglandin receptors in physiology and immune system are determined by multiple factors including cellular context, receptor expression profile, ligand affinity, and differential regulation of signal transduction pathways (14). In airways, effect of PGE_2 in bronchodilation and airway relaxation has been found to be mediated by EP_2 receptor (15, 16). In addition, a study performed in wild-type and EP receptor-deficient mice demonstrated that interaction of PGE_2 with EP_1 and EP_3 receptors induce airway constriction through activation of neural pathways, whereas activation of EP_2 on airway smooth muscle induced bronchodilation. These results suggest that modulation of airway tone by PGE_2 involves multiple receptors mechanisms and different cell populations (17). Basal expression of EP_2 and EP_4 receptors has been observed to be increased on bronchial inflammatory cells from asthmatic patients and may be altered *in vitro* on eosinophils in response to inflammatory stimuli, suggesting the immunomodulatory role of these receptors in asthma, (18). Furthermore, Burgess and col. have also shown a high expression of EP_2 and EP_3 receptors on smooth muscle cells from asthmatic patients compared to non-asthmatics. Interestingly, cells from asthmatic subjects had increased sensitivity to proliferation inhibition by EP_2- but not by EP_3 specific agonists, suggesting the role of EP_2 in airway cell proliferation process (19). Furthermore, recent studies have shown that EP_3 knockout mice developed a more severe allergic inflammation than wild-type mice or mice deficient in other prostaglandin E receptor subtypes. Of interest, a EP_3- selective agonist suppressed this reaction, suggesting this receptor as an important molecule in the modulation of allergic reactions (20). The aim of this study was to compare the tissue expression of CysLTs and PEG_2 receptors in chronic rhinosinusitis with and without nasal polyps, and to study their relation with eosinophilic inflammation in these patients.

MATERIALS AND METHODS

Patients and clinical diagnosis

Samples from ethmoidal and maxillary sinuses were collected during functional endoscopic sinus surgery (FESS) procedures in the Department of Othorhinolaryngology at the Ghent University Hospital. Nasal tissues were obtained from 13 patients with chronic rhinosinusitis and nasal polyposis (CRS-NP) (10 males, 3 females, age range: 30- 54 years) and 11 subjects with chronic rhinosinusitis without nasal polyposis CRS (8 males, 3 females, age range: 21- 53 years) who were scheduled for sinus surgery in the department of Othorhinolaryngology of the Ghent University Hospital. As control group (Controls), we included 6 subjects, who underwent septal surgery and removal of parts of the inferior turbinate due to anatomical variations, without any acute or chronic clinical, endoscopic or imaging signs of sinusitis or polyposis. The mean age of this group was 36.6 years (4 males, 2 females, age range: 21- 53 years) and none of the subjects had a history of atopic, sinus or lower airway disease.

Diagnosis of CRS was based on the presence of typical symptoms (headache, nasal obstruction and discoloured nasal drainage) longer than 12 weeks and a positive CT- Scan showing swelling of the ethmoidal and maxillary mucosa and bilateral obstruction of the osteomeatal complex but without polyp formation, visible during nasal endoscopy or during surgery. Nasal polyposis was diagnosed based on symptoms history (nasal congestion, lost of smell, changes in sense of taste and persistent postnasal drainage), clinical examination, nasal endoscopy and paranasal sinuses CT- Scan, defined as presence of visible bilateral polyps growing from the middle meatus into the nasal cavities, and affecting the ethmoidal sinuses. In the CRS-NP group, 4 patients had asthma and in the CRS group, there was one patient with allergic rhinitis and one with asthma, none of them did manifest any aspirin clinical manifestation of intolerance to aspirin or NSAIDs. Diagnosis of asthma was based on clinical history, typical symptoms and lung (pulmonary) function tests (Spirometry and Peak Expiratory Flow), following the Global initiative for asthma (GINA) guidelines. The study was approved by the ethical committee of the Ghent University Hospital, and all patients gave informed consent before their participation. The use of any oral medication with possible impact on measurements of enzymes or mediators, including systemic glucocorticoids and anti-leukotrienes, was stopped in all subjects 4 weeks before surgery. The use of topical glucocorticoids was interrupted 2 weeks before surgery.

Real time PCR for eicosanoid receptors

Quantitative real time PCR was used to determine the mRNA levels of enzymes and receptors involved in the AA cascade. Nasal tissue (30 mg) was homogenized in Tri- reagent buffer (Sigma-Aldrich, MO, USA), 1

ml per 50-100 mg of tissue, in a chilled pestle mortar. Total RNA from homogenates was isolated using Tri-reagent Kit following the manufacturer's instructions (Sigma-Aldrich, MO, USA). cDNA was synthesized from 2 µg of total RNA using Oligo(dT)$_{12-18}$, random hexamers and the Superscript RNase H$^-$ Reverse Transcriptase (Life Technologies, CA, USA), as described previously (21). Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad laboratories, CA, USA) using specific primers (Table 1) designed with the Primer3 software (22). PCR reactions contained 20 ng cDNA (total RNA equivalent) of unknown samples, 1x SYBR Green I Master mix (Bio-Rad laboratories, CA, USA) and 300 nM of primer pairs in a final volume of 25 µl. PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 45 cycles at 95°C for 30 seconds and at 60°C for 1 minute. The expression of two housekeeping genes Beta actin (ACTB) and Hydroxymethyl-bilane synthase (HMBS) was used to normalize for transcription and amplification variations among samples and control specimens. The relative number of molecules of each gene, expressed in relative expression units quantified per 20 ng of cDNA sample, was determined by the *ΔCT* value method as described previously (23).

Table 1. Primer sequences used for real time PCR amplification

Eicosanoid receptors	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon size	Genbank Accession number
EP_1	GATGGTGGGCCAGCTTGTC	GCCACCAACACCAGCATTG	73 bp	NM_000955
EP_2	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA	73 bp	NM_000956
EP_3	AAGGCCACGGCATCTCAGT	TGATCCCCATAAGCTGAATGG	76 bp	NM_000957
EP_4	ACGCCGCCTACTCCTACATG	AGAGGACGGTGGCGAGAAT	63 bp	NM_000958
BLT_1	CCTGAAAAGGTGCAGAAGC	AAAAAGGGAGCAGTGAGCAA	93 bp	NM_000752
BLT_2	CTTCTCATCGGGCATCACAG	ATCCTTCTGGGCCTACAGGT	88 bp	NM_019839
$CysLT_1$	TCCTTAGAATGCAGAAGTCCGTG	AAATATAGGAGAGGGTCAAAGCAA	80 bp	NM_006639
$CysLT_2$	GCTGATCATTCGGGTTCTGT	GGTGATGATGATGGTGGTCA	91 bp	NM_020377

Eicosanoid levels

Concentration of cysteinyl leukotrienes $C_4/D_4/E_4$ ($LTC_4/D_4/E_4$), prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) were measured by Enzyme Linked Immunoassays (ELISAs) purchased from Oxford BioMedicals (Oxford, USA). Sample extraction procedures for protein removal and eicosanoid stabilization were performed according to the provider's instructions. Briefly, nasal or sinus tissues were first homogenized in ethanol (5ml/g) for LTB_4 and $LTC_4/D_4/E_4$, and in 15% methanol/ 0.1M sodium phosphate buffer, pH 7.5 for

PGE_2 measurements and then centrifuged for 5 minutes at 3,000 rpm at 4°C. Supernatants were diluted in water, pH 3.5 and following manufacturer's instructions (Oxford BioMedicals, Oxford, USA). The detection ranges for all assays were between 0.02- 10 ng/ml. The sensitivity was of 0.2 ng/ml for all assays and the intra- and inter- assay coefficient of variation less than 10%.

Eosinophil inflammatory markers

Soluble IL-5α receptor isoform was quantified using a real time PCR, as described previously (21). Briefly, a standard curve was constructed from a plasmid containing the cDNA sequence for this receptor isoform. A fragment of this plasmid was amplified with specific primers, purified and subsequently quantified and used in equimolar 10-fold dilutions to generate standard curve. Real time amplifications were performed in a 25 µl volume reaction containing 1X SYBR Green I Master mix (Bio- Rad laboratories, CA, USA), 300 nM of primer pairs and a set of primers specific for this hIL-5 Rα isoform (21). PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 64°C for 1 minute. Each sample was tested in duplicate. The quantity of each amplicon was calculated from the values of the standard curve and normalized by the quantities obtained for beta actin (ACTB) and hydroxymethyl-bilane synthase (HMBS).

Soluble IL-5α receptor protein concentrations were measured by a research ELISA as described previously (24) with a sensitivity of 8 pg/ ml. and an intra- and inter- assay coefficient of variation less than 10%. Quantification of Eosinophil Cationic Protein (ECP) was carried out, on supernatants obtained after nasal tissue homogenization, by the UniCAP system (Pharmacia & Upjohn, Sweden), with a detection limit of < 0.5 µg/L and a coefficient of variation less than 10%.

Number of activated eosinophils was determined by staining the eosinophil granulocyte (EG_2) and semiquantitative scoring of positively stained cells on the different tissues. For that, frozen tissue sections were fixed in acetone for 10 minutes, washed in TBS buffer and incubated with (1:1000) mouse anti- human ECP/EPX monoclonal antibody (Pharmacia & Upjohn Uppsala, Sweden) for 1 hour. Then, the slides were incubated with (1:50) rabbit anti-mouse IgG for 10 minutes and developed with (1:100) alkaline phosphatase anti-alkaline phosphatase (Dako, Glostrup, Denmark) for 10 minutes at room temperature. Signal detection was performed using the New Fuchsin Substrate System, following the manufacture's instructions (Dako, Glostrup, Denmark). Semiquantitative scoring was performed by a pathologist, who was blinded for the clinical data, on a four-point scale adapted from an already validated system of semiquantitative evaluation. Zero represented the lowest and three the highest score. The analysis included all areas of the biopsies and a global score was given for each parameter.

Immunohistochemical staining for prostanoid and leukotriene receptors

Frozen tissue sections were fixed in acetone for 10 minutes at room temperature and washed in 1x TBS buffer. Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxidase (VWR International, Pennsylvania, USA) in PBS containing 0,001 % NaN_3 for 20 minutes at room temperature. Sections were than washed for 10 minutes with 1x TBS and incubated with foetal bovine serum during 30 minutes. Sections were then incubated for 1 hour at room temperature with primary antibodies: (1:250) rabbit IgG polyclonal Antibodies for (1:250) EP_1 receptor, (1:250) EP_2 receptor, (1:200) EP_3 receptor, (1:250) EP_4 receptor, (1:50) $CysLT_1$ and (1:50) $CysLT_2$, purchased by Cayman Chemicals, MI, USA. Signal was detected with $LSAB^+$ kit (HRP Rabbit/Mouse/Goat) purchased from Dako, using the AEC^+ High Sensitivity Substrate Chromogen Kit (Dako, Glostrup, Denmark). Number of inmunoreactive cells was determined by semiquantitative scoring of positively stained cells on the different tissues as described above.

Statistical data analysis

All data was analyzed using the MedCalc software version 6.0 (Mariakerke, Belgium). Results are presented in Box- and- Whisker plots, where the central box represent the values from the lower and upper interquartile range, and the middle line the median. Data comparison within different patient groups was performed using the Kruskal-Wallis test (*H-test*). The Wilcoxon test (or *Mann-Whitney U test*) for unpaired samples was applied to evaluate the statistical differences between two patient groups. Spearman's rank correlation analysis was performed to determine statistical significance of differences between two parameters in a classification group. *P* values equal or less than 0.05 was regarded as significant.

RESULTS

mRNA expression of eicosanoid receptors by real time PCR

Expression of leukotrienes and prostanoid receptors analyzed by quantitative real time PCR showed an up-regulation of $CysLT_1$ and $CysLT_2$ receptors in CRS patients compared to controls, but only $CysLT_1$ was significantly higher in CRS-NP in comparison to CRS patients as showed in figure 1a. In contrast, the four EP receptors showed an up- regulation of EP_2 and EP_4 mRNA in both CRS groups when compared with normal subjects. In contrast, EP_1 and EP_3 expression was similar in controls and CRS patients but significantly down-regulated in the CRS- NP group (Figure 1b). Concentrations of BLT_1 and BLT_2 receptors were similar in the three groups of patients (data not shown). In addition, mRNA expression profile for the four EP and the two CysLTs receptors were analyzed in each individual group of patients as showed in figures 2 and 3. In the control group, the expression of EP receptors did not show any differences. However,

in the CRS and CRS-NP we observed a down- regulation of EP_1 and EP_3 receptors compared to EP_2 and EP_4 and this difference was more accentuated in the CRS- NP subjects. On the other hand $CysLT_1$ and $CysLT_2$ mRNA levels were similar in CRS and normal mucosa, however significantly higher concentrations of $CysLT_1$ were observed in the CRS-NP group.

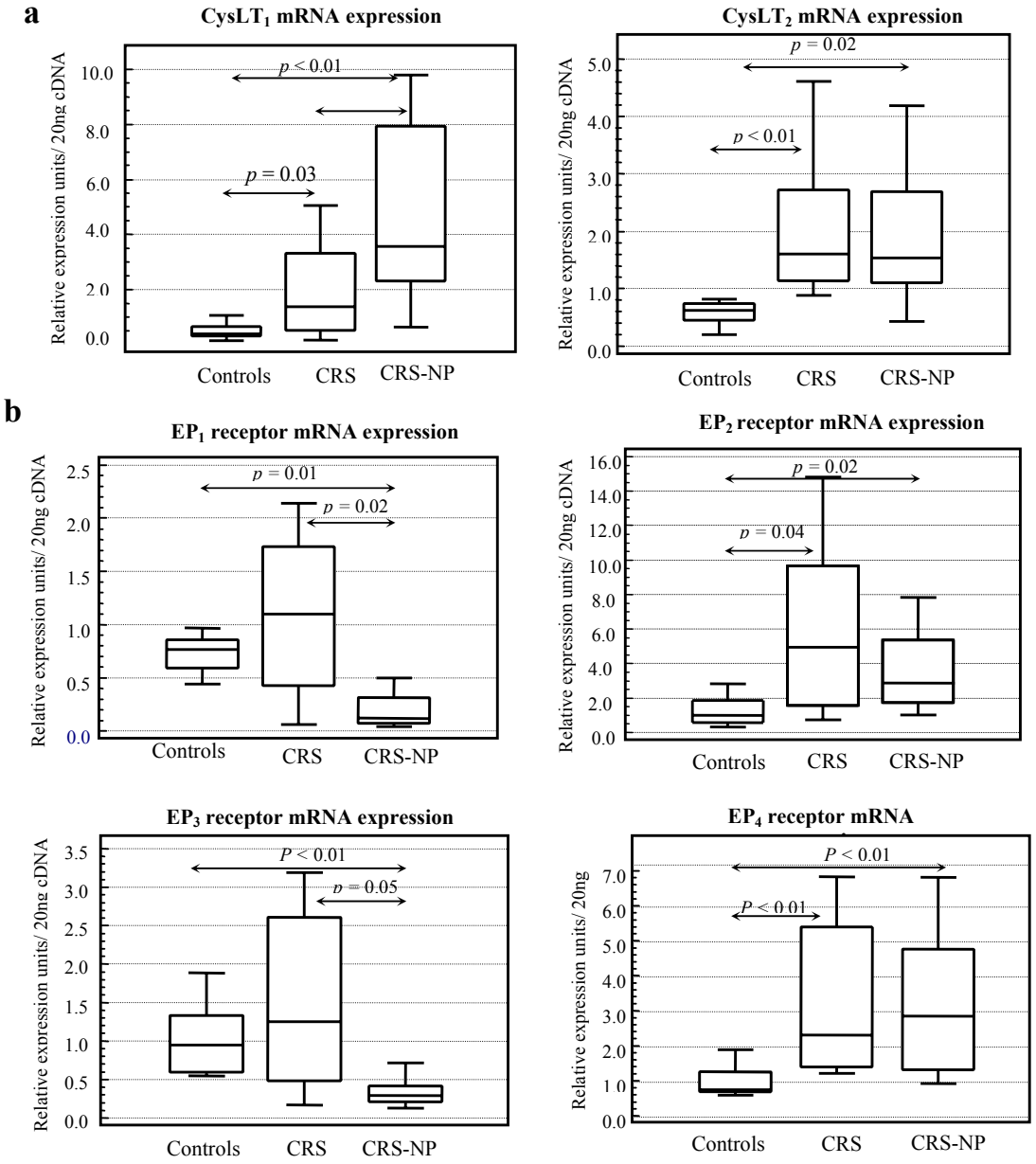

Figure 1. mRNA levels of eicosanoids receptors in nasal mucosa; **a)** CysLTs receptors, **b)** prostaglandin E receptors. *Controls:* healthy subjects, *CRS*: chronic rhinosinusitis, *CRS-NP:* chronic rhinosinusitis/ nasal polyps. *P: p* value after unpaired *Mann-Whitney U* test.

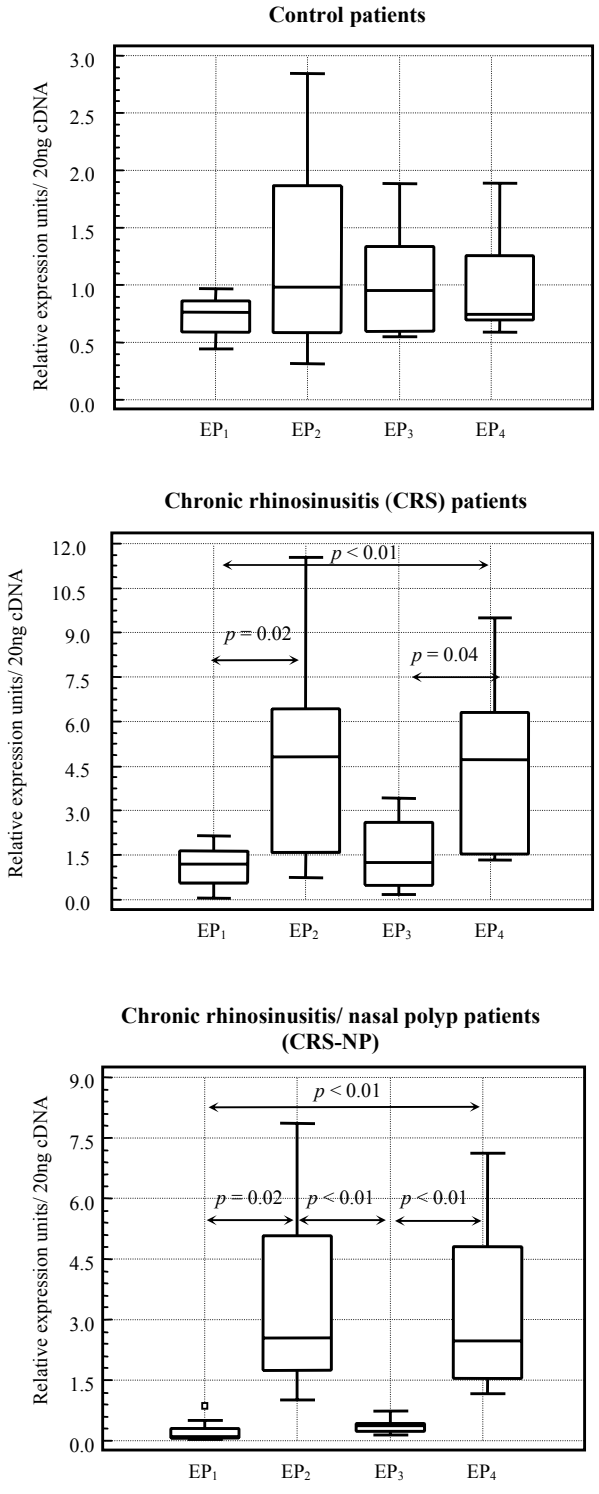

Figure 2. Balance of mRNA levels of prostanoid E receptors in nasal mucosa *P:* p value (unpaired *Mann- Whitney U* test).

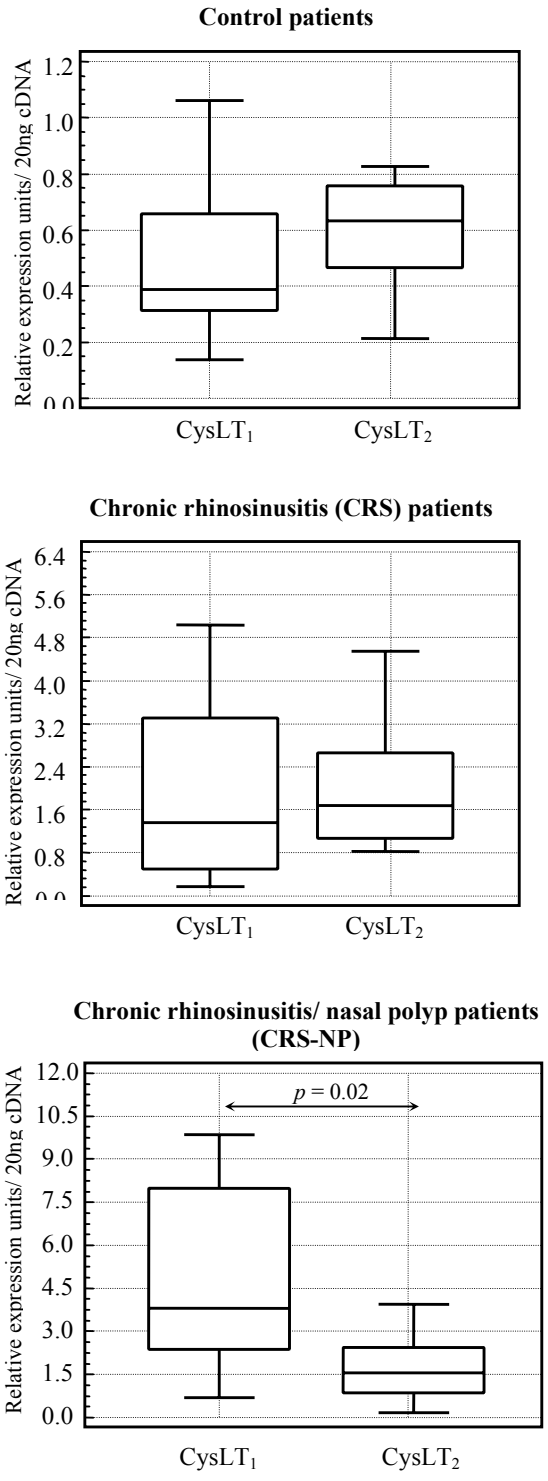

Figure 3. Balance of mRNA levels of CysLTs receptors in nasal mucosa. *P: p* value (unpaired *Mann- Whitney U* test).

Eicosanoid production and eosinophil inflammatory markers

Levels of eicosanoids were quantified by Enzyme immunoassays. While concentrations of LTC_4/ D_4/ E_4, were significantly higher in CRS-NP compared to CRS and controls, no differences were observed in LTB_4 levels between the groups. PGE_2 concentrations however, were similar in CRS and control ($p > 0.10$) but statistically lower in the nasal polyp tissue ($p = 0.001$) as showed in table 2. Real time PCR for sol- IL-5αR showed a significantly increase in CRS-NP subjects compared to CRS and in CRS compared to control subjects. Accordingly sol- IL-5αR protein was also statistically higher in the CRS- NP compared to CRS and in this group compared to control tissue. ECP was significantly increased in CRS compared to the control group ($p < 0.05$) and even more in CRS-NP ($p < 0.02$), as showed in table 2.

Immunohistochemistry results demonstrated a strong infiltration of inflammatory cells in the nasal polyp compared to the CRS and inferior turbinate tissues (data not shown) and the median score for EG_2 positive cells was significantly higher in NP tissue compared to the control and CRS tissues as summarized in table 2.

Table 2. Concentration of eicosanoids and eosinophilic inflammation markers in chronic rhinosinusitis patients. Results are expressed as median and interquartile ranges (IQR). *§:* $p < 0.05$ is due to differences between the three sample groups; *(¶):* $p < 0.05$ is due to differences in the levels between CRS-NP and controls and CRS patients. *N.S:* No statistical differences, *p* value > 0.05. *CRS:* Chronic rhinosinusitis, *CRS-NP*: chronic rhinosinusitis/nasal polyp, *Controls*: inferior turbinate tissues from healthy subjects.

	Controls	**CRS**	**CRS-NP**	***Kruskal Wallis- test***
Eicosanoids				
LTC_4/ D_4/ E_4 (ng/ ml)	1.16 (IQR: 0.85- 1.68)	3.34 (IQR: 2.70- 5.35)	7.24 (IQR: 4.65- 12.40)	$P < 0.01$ §
LTB_4 (ng/ ml)	25.25 (IQR: 8.26- 63.91)	21.95 (IQR: 9.40- 31.90)	19.44 (IQR: 12.80- 29.71)	N.S
PGE_2 (ng/ ml)	180.63 (IQR: 101.44- 258.86)	199.83 (IQR: 59.10- 223.52)	55.00 (IQR: 40.59- 67.87)	$p = 0.02$ ¶
Eosinophilic inflammation markers				
ECP (μg/ L)	602.51 (IQR: 309.90- 894.30)	2090.00 (IQR: 1437.60- 5442.40)	11880.00 (IQR: 1862.70- 17920.74)	$p < 0.01$ §
IL-5Rα protein (ng/ ml)	20.62 (IQR: 15.77- 26.43)	50.95 (IQR: 28.62- 67.78)	175.24 (IQR: 37.11- 309.67)	$P < 0.05$ §
sol- IL-5Rα mRNA	14757.50 (IQR: 12493.97- 23015.35)	159065.30 (IQR: 45909.00- 185796.90)	458449.55 (IQR: 267447.00- 796387.30)	$p = 0.02$ §
EG_2 positive cells	1,00 (IQR: 1,00- 1,15)	1,05 (IQR: 1,00- 1,40)	2,10 (IQR: 1,90- 2,25)	$p < 0.01$ ¶

The Spearman's rank correlation analysis showed a strong correlation between both CysLTs receptors mRNA with IL-5R protein concentrations ($CysLT_1$: r = 0,574, p = 0.01; $CysLT_2$: r = 0,523; p < 0.05), ECP ($CysLT_1$: r = 0,544, p = 0.02; $CysLT_2$: r = 0,413; p = 0.03) and the total number of activated eosinophils ($CysLT_1$: r = 0,546, p = 0.02; $CysLT_2$: r = 0,614; p = 0.03). No correlations were found between the levels of these receptors and eosinophilic inflammation markers with exception of EP_1 that inversely correlated with the number of activated eosinophils (r = 0,636; p = 0.01).

Immunohistochemical staining for prostanoid and leukotriene receptors

Immunohistochemical staining for $CysLT_1$ and $CysLT_2$ receptors in the nasal tissue is represented in figure 3. Immunoreactivity of both CysLTs receptors was observed in inflammatory cells in the lamina propria in both CRS groups. In addition, these receptors were expressed in the sub-epithelial layer of the nasal mucosa and to a lesser extend in the epithelium. Prostanoid E receptors were mainly expressed in the epithelium and in mucosal glands. In inflammatory cells, immunoreactivity for EP_1 and EP_3 was higher compared to EP_2 and EP_4 as showed in figure 4.

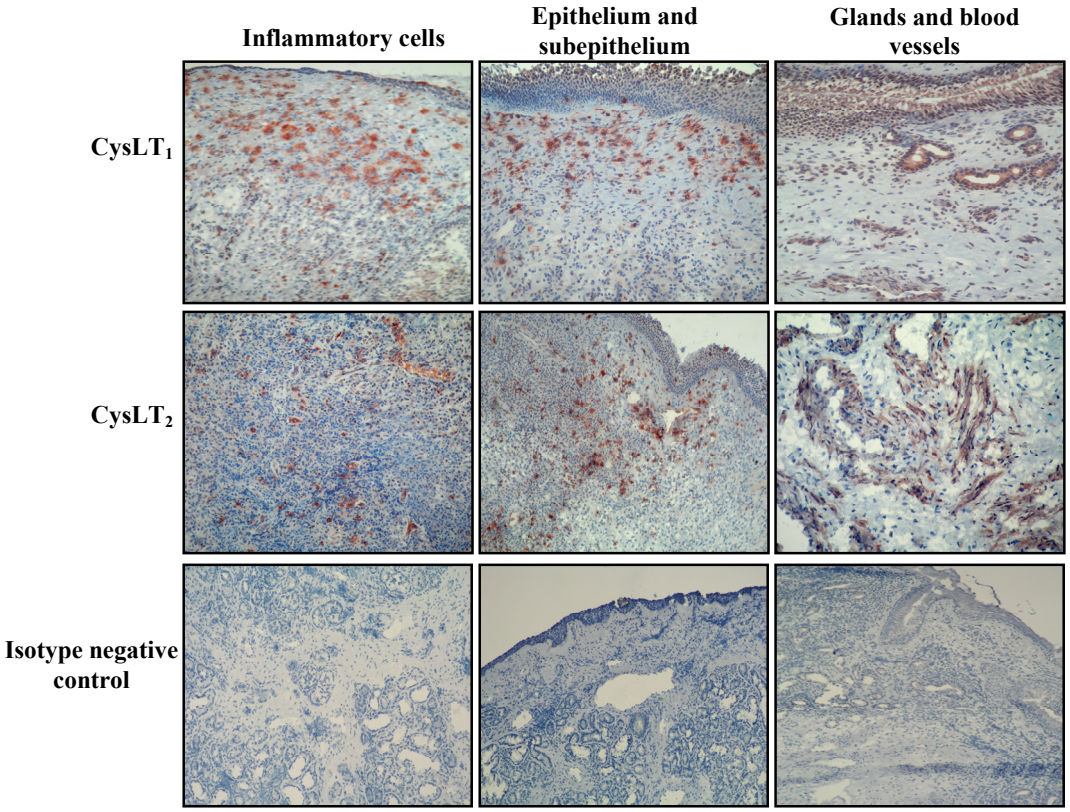

Figure 4. Representative photomicrography (original magnification 20X) of nasal polyp specimens immunostained for $CysLT_1$ and $CysLT_2$ receptors in inflammatory cells, epithelium, glands and blood vessels.

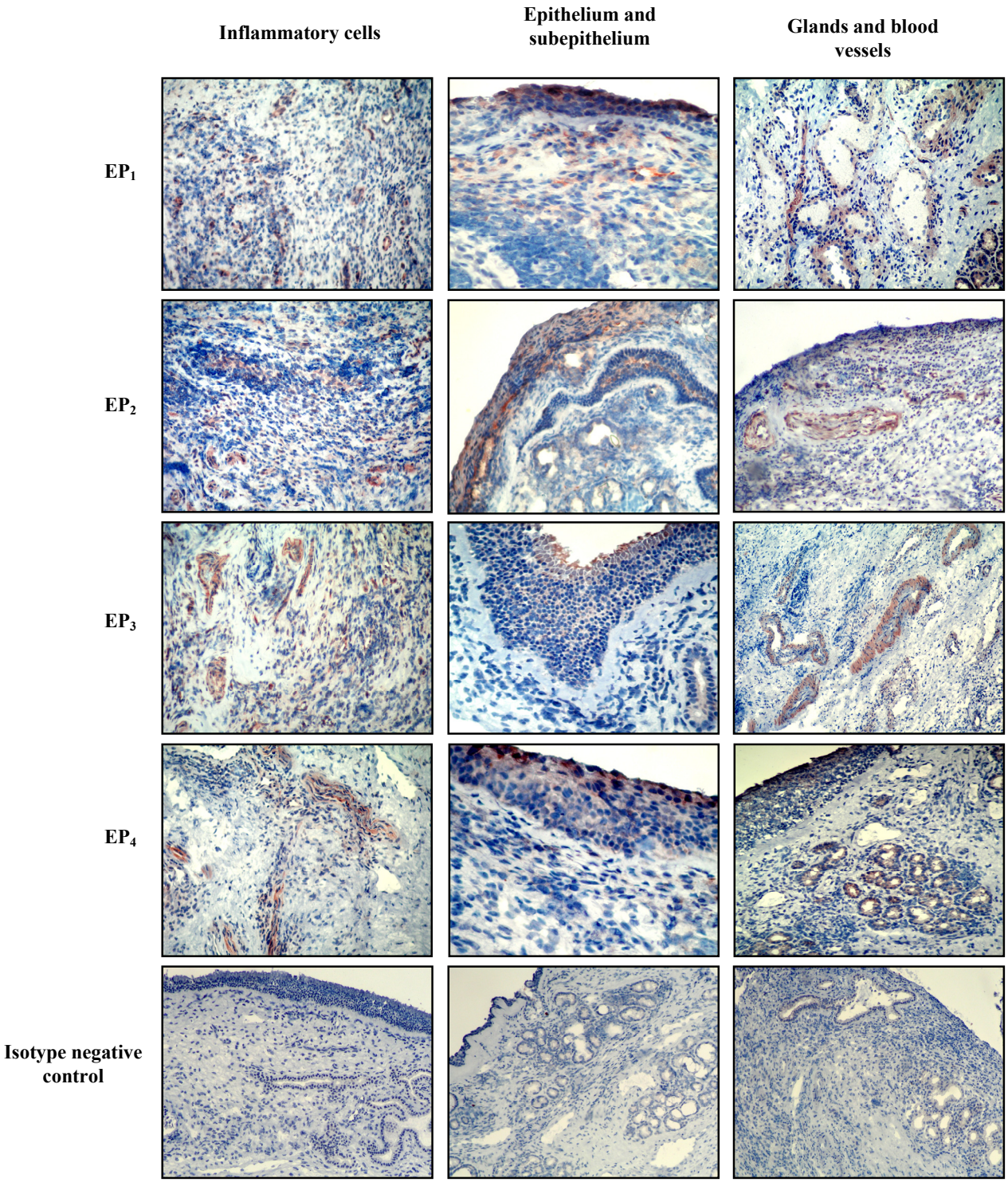

Figure 5. Representative photomicrography (original magnification 20X) of nasal polyp specimens immunostained for EP_1, EP_2, EP_3 and EP_4 receptors in inflammatory cells, epithelium, glands and blood vessels.

DISCUSSION

Several studies have suggested changes in the eicosanoid regulation patterns as one of the factors involved in the pathophysiology of chronic rhinosinusitis and nasal polyposis; however, the effect of eicosanoids in the tissue, greatly dependents of the differential expression of the distinct subtypes of their receptors.

In this study, we confirmed that $CysLT_1$ and $CysLT_2$ receptors are up- regulated in chronic rhinosinusitis and nasal polyp patients. Interestingly, the balance of these receptors was similar in healthy and chronic rhinosinusitis subjects, in contrast to the nasal polyp group where expression of $CysLT_1$ was significantly higher when compared to $CysLT_2$. Furthermore, we evaluate the link between these receptors and eosinophilic inflammation and we observed that both $CysLT_1$ and $CysLT_2$ correlated with markers of eosinophil activation like IL-5αR, ECP and the number of activated eosinophils. These results are in line with previous results showing that eosinophils are one of the most important sources of these receptors in inflamed upper airways (25, 26); and that $CysLT_1$ maybe involved in several stages of eosinophil differentiation, recruitment and maturation (27-29). In other hand, we did not found any changes in LTB_4 or BLTs receptors between controls and disease groups. These findings correspond with previous studies performed in aspirin intolerant nasal polyp patients (13) and with perennial allergic rhinitis (30). However are in contrast with other reports showing increased levels of this eicosanoid in allergic versus non- allergic nasal polyp patients (31). Accordingly, there are no clear evidences about the role of this molecule in chronic rhinosinusitis and nasal polyposis and following our results; we question its role in these diseases.

As well as leukotrienes, prostaglandins and especially PGE_2 play an important role in the regulation of the inflammatory process observed in chronic rhinosinusitis patients (28, 29). Little is known about the function and distribution of these receptors in airways and there are no studies reporting the action or regulation of these receptors in upper airway tissue

We show for the first time that mRNA profile of prostanoid E receptors differs between chronic rhinosinusitis with and without polyps, again being different from healthy controls. We also observed that EP_2 and EP_4 receptors are up- regulated in chronic rhinosinusitis and nasal polyp tissue compared to control subjects; however, EP_1 and EP_3 transcripts were statistically decreased only in the nasal polyp patients.

It has been reported, that action of PGE_2 via EP_1 and EP_3 receptors has been reported to be mediated by an increase of intracellular cAMP (33). In inflammatory cells, this phenomenon is associated with an inhibition of effector's cell functions such as activation, or response to certain stimulus (33). Accordingly, we can assume that down-regulation of these receptors may increase functionality and susceptibility of inflammatory

cells to inflammatory stimulus like IL-5 and LTs contributing to the chronic inflammation observed in these diseases. Controversially, interaction of PGE_2 with EP_1 and EP_3 variants is translated in an increase of intracellular calcium inducing immune cell activation (33). This last action may be of great importance in chronic rhinosinusitis/ nasal polyposis due to increase of intracellular calcium may induce the activation of cytosolic phospholipase A_2 leading to the production of leukotrienes. However, this contradicts our previous hypothesis that PGE_2/ EP_1/ EP_3 interaction may has anti- inflammatory action.

An *in vitro* study performed on inflammatory cells demonstrated that eosinophils express high levels of EP_2 and EP_4 mRNA in comparison with EP_1 and EP_3, which were almost not present in these cells (34). In addition, interaction of PGE_2 with these receptors may induce cellular cAMP production that can contribute to CysLTs production that has been reported to be mediated by EP_4 activation (34). Levels of EP_2 and EP_4 receptors in the chronic rhinosinusitis and chronic rhinosinusitis/ nasal polyp groups did not correlate with eosinophil number or activation but it was increased in the disease groups compared to controls. In addition, CysLTs synthesis was also enhanced in the chronic rhinosinusitis patients compared to chronic rhinosinusitis/ nasal polyps and healthy subjects. These results may suggest the existence of important sources other than eosinophils expressing these receptors and contributing to inflammation in these patients. In addition, the lack of correlation and the similar mRNA levels of these receptors between the disease groups may be because regulation and functionality of these receptors greatly depend of post-transcriptional regulation mechanisms. Here we were not able to analyze the protein expression of these molecules to confirm our PCR results.

Finally, it has also been suggested that deficiency of PGE_2 production may up-regulate the expression of EP_2 and EP_4 receptors (34). We have previously reported that concentrations of PGE_2 are decreased in chronic rhinosinusitis/ nasal polyp compared to chronic rhinosinusitis patients (25) that could explain the high levels of the receptors observed in the patients. However, of interest, expression of these receptors is similar between the two chronic rhinosinusitis groups, suggesting again the role of post- transcriptional events in the functionality of these receptors.

Summarizing, we here describe that mRNA pattern of prostanoid E receptors is different in chronic rhinosinusitis from chronic rhinosinusitis/ nasal polyp patients. In the last group a down- regulation of EP_1 and EP_3 receptor was observed suggesting that these molecules may have some implications in polyp development. This is a descriptive preliminary study which opens the door to more specific experiments including protein regulation and functional studies that will reveal more information about the role of these receptors in chronic rhinosinusitis diseases.

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CHAPTER 6

IMPLICATION OF STAPHYLOCOCCAL SUPERANTIGENS ON EOSINOPHILIC INFLAMMATION

Introduction

Infection with *staphylococcus aureus* is a common feature in patients suffering from chronic rhinosinusitis and nasal polyposis. The implication of the enterotoxins (SEs) released from this bacterium in the pathogenesis of these diseases has been extensively suggested. In this study, we wanted to investigate whether the immune response triggered against *S. aureus* enterotoxins could be associated with clinically documented aspirin intolerance and how these two criteria are linked to eosinophilic inflammation. We here demonstrated that patients with aspirin intolerance showed an exacerbated eosinophilic inflammation compared to tolerant and healthy subjects and an up-regulated immune response against SEs in terms of specific IgE antibodies to these enterotoxins. There was not direct association between this response and the manifestation of aspirin intolerance to aspirin; suggesting that eosinophilic inflammation and response to SEs both contribute to the pathophysiology of this syndrome in an independent manner.

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Aspirin Sensitivity and IgE Antibodies to *Staphylococcus aureus* Enterotoxins in Nasal Polyposis: Studies on the Relationship

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Key Words

Aspirin sensitivity · Nasal polyposis · *Staphylococcus aureus* enterotoxins · ECP · IgE · Interleukin-5

Abstract

Background: Nasal polyposis is a multifactorial disease characterized by a chronic eosinophilic inflammation of the sinus mucosa, often associated with asthma and aspirin sensitivity. We have recently shown that the presence of IgE antibodies to *Staphylococcus aureus* enterotoxins (SAEs) was related to the severity of eosinophilic inflammation in nasal polyp tissue. In this study, we therefore aimed to determine, whether aspirin sensitivity was related to an immune response to SAEs, and how both criteria would be related to eosinophilic inflammation. ***Methods:*** 40 subjects with nasal polyposis (NP) were classified as aspirin-sensitive (n = 13, ASNP) or aspirin-tolerant (n =27, ATNP) based on a bronchial aspirin challenge test. Homogenates prepared from nasal polyp tissue and inferior nasal turbinates from healthy subjects (n =12) were analyzed for concentrations of IL-5 by enzyme immunoassay and for ECP, total and IgE to a mix of SAEs (A, C, TSST-1) using the ImmunoCAP system. ***Results:*** Concentrations of IL-5, ECP, total IgE, and IgE to an SAE mix were significantly increased in ASNP compared with ATNP patients and controls. In addition, a subgroup analysis showed an increase in eosinophilic markers in ATNP-SAE(+) compared to ATNP-SAE(–). This relationship, however, was not found in ASNP-SAE(+) and ASNP-SAE(–) subjects, indicating that SAE immune response is overlapped or not relevant in this condition. ***Conclusions:*** Aspirin sensitivity was associated with increased concentrations of eosinophil-related mediators, as well as IgE antibodies to SAEs in nasal polyp tissue. However, a direct impact of *S. aureus* could not be established. It seems that aspirin sensitivity and immune reactions to SAEs are independently related to eosinophilic inflammation.

Introduction

Nasal polyposis (NP) is a multifactorial disease characterized by chronic eosinophilic inflammation of the upper airway mucosa, i.e. the nose and sinuses [1]. This pathology is often associated with asthma and aspirin sensitivity and both T helper 1 (Th1) or 2 (Th2) type cytokines are upregulated in tissue independent of the atopic status [2]. Sensitivity of the airways to acetylsalicylic acid (aspirin) is characterized by polypous rhinosinusitis, severe, often steroid-dependent asthma and adverse airway reactions upon ingestion of aspirin or other nonsteroidal anti-inflammato-

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ry drugs (NSAIDs) [3]. The disease pattern in aspirin-sensitive patients is characterized by chronic inflammation of the upper and lower airways, often accompanied by an increase in blood and tissue eosinophils and elevated numbers of T lymphocytes, macrophages and mast cells in bronchial mucosa, nasal secretions and serum [4, 5]. Of interest, patients with aspirin-sensitive NP (ASNP) frequently show more severe disease and local eosinophilic inflammation compared with nonsensitive or aspirin-tolerant (ATNP) NP subjects, as well as an increased rate of recurrence after treatment including surgery [6, 7].

In addition, abnormalities in arachidonic acid metabolism have been found in ASNP patients characterized by a significant difference in cyclooxygenase/lipoxygenase product ratios compared with normal nasal mucosa [8]. Also, a low production of PGE_2, which downregulates the cyclooxygenase-2, and over-expression of LTC_4 synthase resulting in increased release of cys-LTs [9, 10] have been demonstrated in these patients.

Bacterial infection may also be implicated in chronic eosinophilic inflammatory disorders [11]. Superantigens, especially *Staphylococcus aureus (S. aureus)* enterotoxins (SAEs) have been demonstrated to potentially influence the pathogenesis of chronic skin and respiratory diseases, such as dermatitis, perennial allergic rhinitis and NP [12–14].

Clinical and experimental studies indicate that nasal polyp formation and growth are initiated and perpetuated by an integrated process involving the mucosal epithelium, extracellular matrix, and inflammatory cells, which in turn may be triggered by both infectious and noninfectious inflammation [15]. In a recent study, we discussed the possible role of SAEs in NP. We demonstrated that the presence of IgE antibodies to SAE A/SAE B correlated to the severity of eosinophilic inflammation in terms of eosinophilic cationic protein (ECP), interleukin-5 (IL-5) and eotaxin protein levels [16]. In this study, we therefore aimed to determine whether clinically documented aspirin sensitivity was related to an immune response to SAEs, and how both criteria would be related to eosinophilic inflammation.

Material and Methods

Patients

For this study, polyp tissue was obtained from 40 patients with bilateral nasal polyps, who were scheduled for sinus surgery. The diagnosis of NP was based on a typical history and symptoms of NP such as nasal congestion, rhinorrhea and loss of smell, and confirmed by nasal endoscopy prior to surgery. The diagnosis of aspirin sensitivity was made on the basis of a positive bronchial aspisol provocation test. In the NP group, 13 individuals showed sensitivity to aspirin and 27 were not sensitive to this drug (table 1). All of the aspirin-sensitive subjects also suffered from asthma, and had a history of at least one bronchial reaction to nonsteroidal anti-inflammatory drugs (NSAIDs).

Table 1. Characteristics of the patient groups used in the study

	Control	ATNP	ASNP
Subjects	12	27	13
Age, years	39 ± 13.4	57 ± 15	52 ± 13.7
Male/female	6/6	17/10	4/9
Inhaled steroids	0	100	100
IgE-SAE (+)	0	7	7
IgE-SAE (–)	12	20	6
Allergic patients	0	3	10
Asthmatic patients	0	6	13

A skin prick test for a panel of inhalant allergens including house dust mites, grass and tree pollen, cat dander and feathers was performed. 13 out of 40 NP patients had at least one positive test, with 10 of these patients belonging to the aspirin-sensitive NP group (ASNP) (table 1). As control group, we included 12 subjects, who underwent septal surgery and removal of parts of the inferior turbinate due to anatomical variations. None of these subjects had a history of atopic disease, NP, asthma or adverse reactions to NSAIDs. The ethics committee of Medical University of Lodz approved the study and all patients gave informed consent before their participation. The use of any medication related to the disease, including glucocorticoids and anti-leukotrienes was stopped in all subjects 4 weeks before surgery.

Asthma and aspirin sensitivity highly coincided in our patient population, and it was impossible to differentiate the two clinical parameters. It should be noted that, as we selected the patients on the basis of aspirin sensitivity, aspirin-sensitive asthmatics are overrepresented in comparison to aspirin-tolerant subjects, and no conclusions can be deduced for asthmatics in general.

Aspirin Challenge Protocol

Lysine-acetylsalicylic acid (lysine-ASA; Aspisol, Horby Bayer AG, Germany) was administered with a dosimeter-controlled jet nebulizer (Spira Electro 2, Respiratory Care Center, Hameelinna, Finland). The lysine-ASA challenge test was only performed if the patient's FEV_1 was ≥ 60% of predicted on the day of challenge. Three fresh stock solutions of 2 *M*, 1 *M* solution and 0.1 *M* of lysine-ASA were prepared and used within 4 h of preparation. The solution in the nebulizer chamber was kept at 12 °C between administrations of doses, but brought to room temperature before each administration. The nebulizer delivered an aerosol with 80% of particles being less than 5.8 μm, and a median mass diameter of 4.1 μm. The use of 3 different concentrations of lysine-ASA solution and varying the number of tidal breaths (1–23) resulted in stepwise increments in the dose of inhaled lysine-ASA. Baseline FEV_1 was measured as the best of three efforts before (pre-diluent baseline) and 20 min after the inhalation of 7 breaths of 0.9% sodium chloride (post-diluent baseline). If the post-diluent FEV_1 was <90% of the pre-diluent FEV_1, the lysine-ASA challenge was not performed due to instability of the bronchial tree. The post-diluent baseline was used to calculate the percent drop

Table 2. Concentrations of inflammatory markers and IgE in NP, ASNP, ATNP and control groups (median + interquartile ranges)

	IL-5, pg/ml	ECP, µg/l	IgE to SAEs, kU/l	Total IgE, kU/l
NP	108.14 (22–231)	3.0 (1.5–8.8)	1.9 (2–7)	164.1 (50–502)
ASNP	274.6 (127–667)	8.7 (3.0–14.1)	10.2 (2–20)	472.7 (296–706)
ATNP	57 (22–127)	2.4 (1.2–6.6)	1.93 (1.9–3)	82.1 (35–266)
Controls	21.4 (21.3–21.5)	51.4 (17–1.3)	0.5 (0.5–0.5)	14.9 (0.5–32)

in FEV_1 caused by lysine-ASA, which was inhaled in half-log incremental doses every 30 min. FEV_1 was obtained at 10, 20 and 30 min after the last inhalation of each dose. The lysine challenge was completed when FEV_1 decreased by ≥20%, compared to the post-diluent baseline or the maximum dose of ASA had been reached (540 µmol cumulated dose). The patient was observed for at least 1 h after the termination of the provocation, and if necessary, 2 puffs of salbutamol were administered.

Tissue Collection and Processing

Tissue samples were collected during sinus or septal surgery and immediately frozen in liquid nitrogen and stored at –70°C. 100 mg of tissue was homogenized in 1 ml of 0.9% NaCl solution (mechanical homogenizer B. Braun, Melsungen, Germany) at 1,000 rpm for 5 min on ice [17, 18]. After homogenization, the suspensions were centrifuged at 3,000 rpm, 4°C for 10 min to collect supernatants for ELISA. All prepared samples were stored at –70°C until analyzed.

Quantification of IL-5 and ECP in Tissue Homogenates

IL-5 was measured by an Enzyme Immunoassay (EIA) purchased from R&D Systems (Minneapolis, USA). The assay has a detection range between 7.8 and 500 pg/ml and a sensitivity of <3 pg/ml. Amounts of ECP and total IgE in supernatants obtained after homogenization of nasal polyp tissue were quantified by a UniCAP system (Pharmacia & Upjohn, Sweden), with a detection limit of <0.5 µg/l for ECP and 0.35 kU/l for total IgE measurements.

Quantification of Total IgE and Specific IgE in Tissue Homogenates

To screen for IgE antibodies to SAEs in nasal polyp samples, we established a mix of three SAEs (SAE A, SAEC and TSST-1) coupled to a solid-phase (ImmunoCAP- system, Pharmacia Sweden, cut-off 0.35 kU_A/l). No nonspecific IgE reactivity to the ImmunoCAP SAE mix was found for non-antibody active IgE (E myeloma) at concentrations up to 1,000 kU/l. Samples positive for IgE antibody to the SAE mix tested negative to a control ImmunoCAP without allergen.

Statistical Analysis

Data are expressed as median and interquartile ranges. The Mann-Whitney U test was applied using MedCalc version 6.0 to evaluate the statistical differences between groups. The Kruskal-Wallis test (H test) was applied to compare the data within the different patient subgroups. To evaluate statistical significance of differences between two parameters, Spearman's rank correlation analysis was applied. The χ^2 test was used to test the statistical significance of differences between different classification groups. A p value of 0.05 or less was considered as statistically significant.

Results

Eosinophil-Related Mediators and IgE in NP versus Controls

A significant increase in IL-5 concentrations ($p < 0.01$) was observed in samples from supernatants in NP patients compared to controls. IL-5 strongly correlated with ECP ($r = 0.70$, $p < 0.01$), which was also significantly upregulated ($p < 0.01$) in the NP group. A significantly higher concentration of total IgE and IgE antibodies to SAEs in nasal polyp tissue was observed compared to healthy subjects ($p < 0.01$). In addition, levels of IgE to SAEs correlated with the IL-5 ($r = 0.37$, $p = 0.02$) and ECP ($r = 0.49$, $p = 0.002$) levels in the NP group. Data are summarized in table 2.

Eosinophil-Related Mediators and IgE in ASNP and ATNP Patients

NP patients were further analyzed according to two classification groups, with or without aspirin sensitivity (ASNP and ATNP, respectively). Results are summarized in figure 1 and table 2. Quantities of IL-5 and ECP were upregulated in ASNP and differed significantly from ATNP and control subjects. Concentrations of total IgE and IgE antibodies to a mix of SAEs (SAE A, SAE C, TSST-1) showed significantly higher levels in ASNP patients compared to ATNP and control groups as well.

Eosinophil-Related Mediators and IgE in ASNP (ATNP) – IgE to SAE Subgroups

A post hoc analysis, considering aspirin sensitivity as the main classification criterion, was performed in order to investigate whether the immune response to SAEs (expressed in levels of IgE antibodies to SAEs) was related to the inflammatory reactions and changes in arachidonic acid metabolites in NP patients. ASNP and ATNP patients were each divided in two subgroups: SAE(+) and SAE(–).

Patients with levels of IgE antibodies to SAEs above 0.35 kU_A/l were included in the group we assume had pre-

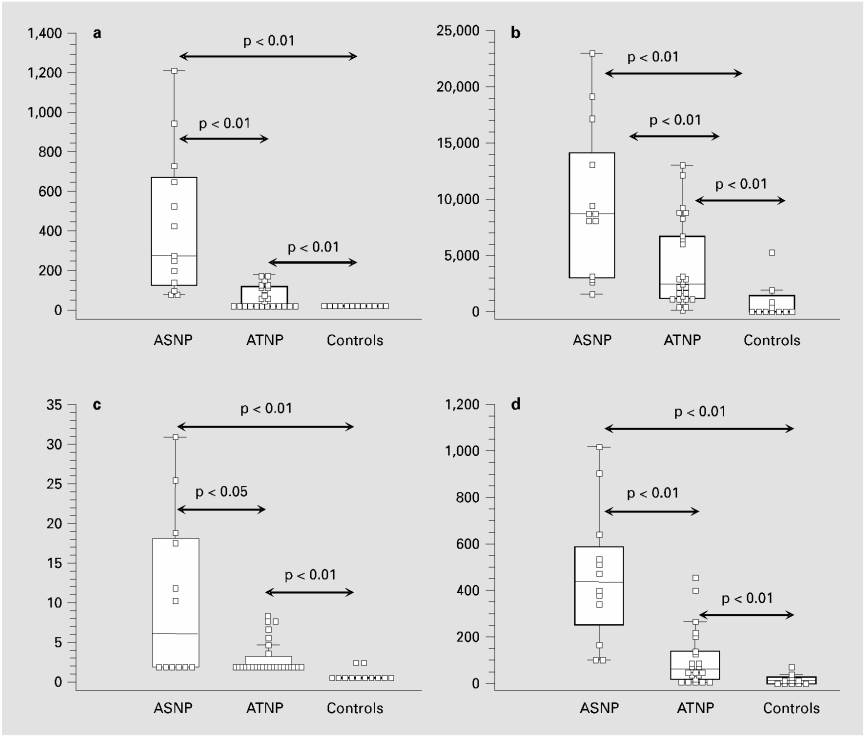

Fig. 1. Concentrations of IL-5 (pg/ml) (**a**), ECP (μg/l) (**b**), specific IgE antibodies (kU_A/l) (**c**) and total IgE antibodies (kU_A/l) (**d**) to SAEs, in nasal polyp tissue from ASNP (n = 13), ATNP (n = 27) and in nasal turbinate tissue from the control group (n = 12). The box and whiskers plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding outside and far out values. Statistical analyses were performed by using the Mann-Whitney U test.

vious or current exposure to SAEs [SAE(+)]. Subjects containing no IgE antibodies or concentrations of less than 0.35 kU_A/l, were considered as SAE negative [(SAE(–)]. Out of 13 patients with ASNP, 7 were SAE(+) (54%) in contrast to 7 out of 27 in the ATNP group (26%) and none out of 12 subjects in the control group (0%, p = 0.003).

Data were analyzed by the Kruskal-Wallis test and results are summarized in table 3 and figure 2. Concentrations of inflammatory markers (IL-5 and ECP) did not differ between ASNP-SAE(+) and SAE(–) groups, but were upregulated with respect to the control group. Interestingly, the pattern for ATNP groups was different, as IL-5 and ECP levels were significantly increased in the SAE(+) compared to SAE(–) patients and controls.

Discussion

In this study, we have demonstrated that concentrations of eosinophil-related markers such as IL-5 and ECP, as well as total IgE and IgE antibodies to SAEs in polyp tissue were significantly increased in ASNP compared with ATNP patients and controls. A post hoc analysis did not reveal any direct link between SAEs and inflammatory reactions in ASNP. However, in ATNP, the immune response to these enterotoxins was linked to the severity of eosinophilic inflammation.

We have previously demonstrated that IgE antibodies to SAEs in NP patients were related to the severity of eosinophilic inflammation, and possibly to the clinical

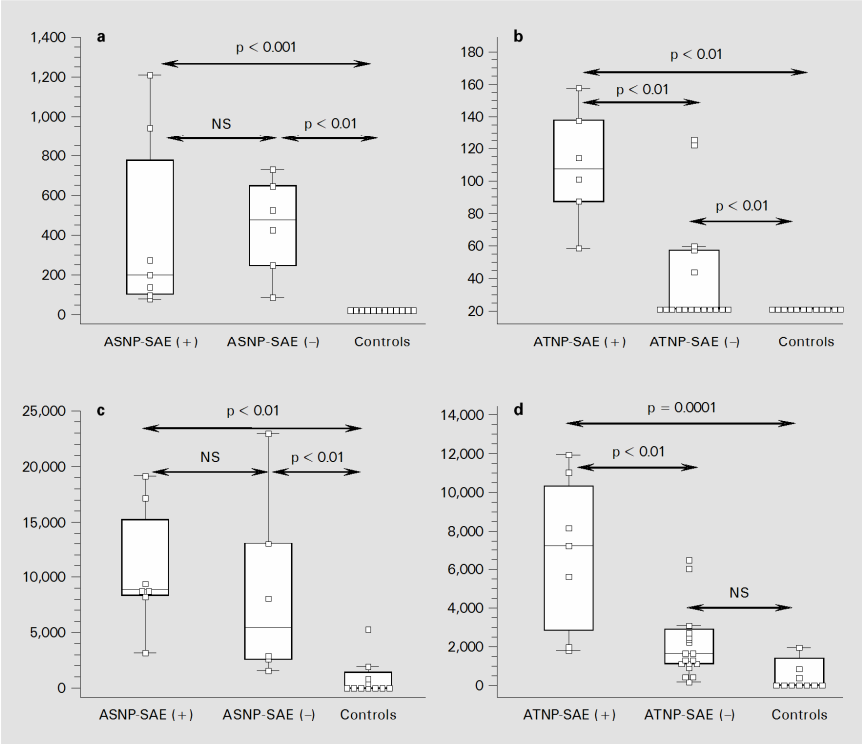

Fig. 2. Concentrations of IL-5 (pg/ml) (**a, b**) and ECP (μg/l) (**c, d**) in nasal polyp tissue homogenates and nasal turbinates from ASNP–SAE(+), ASNP–SAE(–), control subjects and ATNP-SAE(+), ATNP-SAE(–) and control subjects. The box-and-whiskers plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding outside and far out values. Statistical analyses were performed using the Mann-Whitney U test.

Table 3. Concentrations of inflammatory markers and IgE in ASNP, ATNP-SAEs subgroups (median + interquartile ranges)

	IL-5, pg/ml	ECP, μg/l	IgE to SAE, kU_A/l	Total IgE, kU/l
ASNP-SAE (+)	198.8 (106–775)	8.8 (8.3–152.2)	18.8 (13–29)	472.7 (383–838)
ASNP-SAE (–)	475.2 (250–647)	5.4 (2.6–13.0)	1.9 (1.9–1.9)	426.5 (107–533)
ATNP-SAE (+)	128.2 (105–167)	8.3 (3.9–11.4)	6.6 (4.9–7.9)	787.6 (201–2,270)
ATNP-SAE (–)	21.6 (21–57)	1.6 (1.1–2.9)	1.9 (1.9–1.9)	50.8 (15–105)

manifestation of asthma [16]. Fleischer [19] has shown that staphylococcal enterotoxins can act as superantigens by stimulating accessory or target T cells by cross-linking the variable part of the beta chain of the T cell receptor with MHC class II molecules. This leads to a multiclonal stimulation of up to 20–25% of the naive T-cell population in a nonspecific way, and also induces a multiclonal IgE synthesis and severe eosinophilic inflammation [16]. In addition, SAEs has been demonstrated to inhibit eosinophil apoptosis and modulate cell surface markers on these cells [20] and to induce the production of Th2-type cytokines such as IL-5 [13].

In the present study, we were able to confirm former results [17] showing significant upregulation of IL-5 protein in samples from NP tissue homogenates versus controls, which showed a firm correlation to ECP in NP samples. Also confirming earlier results [16], concentrations of total and IgE antibodies to SAE mix (A, C and TSST-1) were significantly higher in NP tissue homogenate compared to control nasal mucosa, and IgE antibodies to SAEs correlated with the concentrations of ECP and IL-5 in the NP group. We now show that concentrations of IL-5 and ECP were especially increased in ASNP subjects compared with controls and/or ATNP patients, in line with earlier work [7]. We furthermore show that concentrations of total IgE antibodies and of IgE antibodies to SAEs were significantly increased in ASNP subjects compared to controls and ATNP patients. 54% of ASNP patients demonstrated IgE antibody production against SAEs, compared to 26% in the ATNP and none in the control group, which reflects a significant difference between these groups. Finally, data from a subgroup analysis of ASNP and ATNP for the criterium 'IgE antibodies to SAEs' showed that severity of inflammation is related to the presence of IgE to SAE in the group of ATNP patients only. The increase in the levels of inflammatory mediators in the ATNP-SAE(+) compared to SAE(–) but not in ASNP-SAE(+) compared to SAE(–) patients reveals that immune response to SAEs represents a disease modifier unrelated to aspirin sensitivity.

Aspirin sensitivity was associated with increased concentrations of eosinophil-related mediators, as well as IgE antibodies to SAEs in NP tissue. However, a direct impact of *S. aureus* on NP could not be found. It seems that aspirin sensitivity and immune reactions to SAEs are independently related to eosinophilic inflammation.

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CHAPTER 7

ROLE OF STAPHYLOCOCCAL SUPERANTIGENS IN THE REGULATION OF EICOSANOID PRODUCTION IN NASAL TISSUE AND STRUCTURAL CELLS

Introduction

Several authors have suggested the implication of bacterial superantigens in the regulation of arachidonic acid cascade. These enterotoxins have been shown to induce leukotrienes and affect several immune-modulatory cell functions. In the previous chapters we suggested that the presence of a *S. aureus* induced immune response may increase eosinophilic inflammation in nasal polyp patients, and showed that IgE-antibodies to SEs was significantly increased in aspirin- intolerant subjects. We also showed that the up-regulation of the arachidonic acid cascade runs parallel with the severity of inflammation. Based on these findings, we here aimed to study the tissue regulation of eicosanoid production in nasal polyp patients and its possible relation to an immune response to *S. aureus* enterotoxins. In this study, we found that leukotriene and lipoxin pathway is up- regulated in nasal polyp patients with an immune response to SEs and this seems to be correlated to the inflammatory reaction probably derived by these enterotoxins.

Furthermore, it has been demonstrated that SEs influence the regulation of the airway remodeling process in both upper and lower airway diseases. That is why we aimed to investigate the capacity of SEs to regulate the production of COX- 2 and PGE_2, in fibroblasts isolated from nasal tissue. Furthermore, we evaluated the capacity of these molecules to influence basic cell functions like cell growth and migration. This preliminary work showed for the first time that SEB down- regulate COX- 2 expression, endogenous PGE_2 release and migration capacity but blocks cell growing. Of interest, it seems that this enterotoxin may influence nasal fibroblast metabolism by interacting with the MHC-II (classical binding) and also by an alternative mechanism independent of these molecules. This study open a new door to the implication of superantigens in the regulation of the remodelling process in upper inflammatory airway diseases.

Eicosanoid Metabolism and Eosinophilic Inflammation in Nasal Polyp Patients with Immune Response to *Staphylococcus aureus* Enterotoxins

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ABSTRACT

Background: *Staphylococcus aureus* derived enterotoxins (SEs) have been implicated in the pathogenesis of airway inflammatory diseases, esp. nasal polyposis. However, the exact role of these molecules in the regulation of eicosanoid synthesis in this pathology remains unexplored. **Objective:** We studied the possible impact of SE-induced immune responses on the eicosanoid production in nasal polyp (NP) patients. **Methods:** Tissue sample homogenates from NP patients, with (NP-SEs(+)) and without detectable IgE-antibodies to SEs (NP-SEs(-)) (ImmunoCap system), were assayed for IL-5, MPO, $LTC_4/D_4/E_4$, LTB_4, LXA_4, total IgE and ECP. **Results:** Inflammatory makers, eicosanoids and total IgE were significantly increased in NP-SEs(+) compared to NP-SEs(-) tissues, with the exception of MPO, which was similar in both groups. Eicosanoid concentrations correlated to IL-5 and ECP, however, only cys-leukotriene levels correlated with IgE-antibodies to SEs, independently of allergy and asthma. **Conclusions:** Eicosanoid synthesis is up- regulated in polyp tissue of patients with immune response to SEs and seems to be related to the inflammatory reaction induced by these enterotoxins.

ABBREVIATIONS

ECP :	eosinophil cationic protein
IgE-abs to SEs :	IgE antibodies to *S. aureus* enterotoxins
IL-5:	interleukin- 5
LTB_4:	leukotriene B_4
LTC_4/ D_4/ E_4:	leukotriene C_4/ D_4/ E_4
LXA_4:	lipoxin A_4
MPO:	myeloperoxidase
NP:	nasal polyps/ nasal polyposis
NP-SEs(-):	nasal polyp patients without IgE to SEs
NP-SEs(+):	nasal polyp patients with IgE to SEs
SAgs:	superantigens
SEs:	*S. aureus* enterotoxins

Key words:

Nasal polyposis, *S. aureus* enterotoxins, eosinophilic inflammation, leukotrienes, lipoxin A_4, IgE

INTRODUCTION

In the normal population, 30% of individuals are long-term carriers of *Staphylococcus aureus* (1). This Gram- positive bacterium can often be found in the normal microflora of the human skin, but also in the upper respiratory and intestinal tracts (2), and constitutes an important cause of community- and hospital-acquired infections (3). *S. aureus* pathogenicity is generally related to the production of coagulase enzymes; however, these organisms are also able to secrete a variety of cellular antigens and toxins named enterotoxins, which have potent superantigenic properties (4). *S. aureus* enterotoxins (SEs) may influence the activity of both immuno- modulatory and pro-inflammatory effector cell types, and therefore may have a potentially important role in the pathogenesis of chronic inflammatory disease, including eosinophil-related airway diseases (5). It has previously been shown that local application of superantigens (SAgs) to the airways in mice develops a unique inflammatory immune response, characterized by T cells and eosinophils influx, increased levels of IL-4 and marked macrophage activation resembling intrinsic asthma (5). In addition, in humans, recent studies performed in nasal polyp patients and asthma have demonstrated that an increased colonization and/or a local immune response to enterotoxins, including the formation of polyclonal IgE-antibodies, is related to disease severity and eosinophilic inflammation (6- 9).

The implication of bacterial superantigens in the regulation of inflammatory pathways such as the arachidonic acid cascade has also been suggested. Stimulation of basophiles from patients with atopic eczema with staphylococcal enterotoxins has been shown to induce the release of histamine and leukotrienes, indicating a role for these toxins as possible allergens in this group of patients (10). Additionally, incubation of permeabilized human polymorphonuclear granulocytes with staphylococcal toxic shock syndrome toxin 1 and streptococcal erythrogenic toxin A resulted in the up-regulation of signal transduction pathways (release of LTB_4) in these cells, affecting immune-modulatory functions (11). Finally, studies done in plasma samples from mice challenged with SEB showed that incubation with this enterotoxin resulted in an increase of 5-hydroxyeicosatetraenoic acid and LTD_4 production, suggesting that these metabolites may be important in SEB- induced illness and shock (12).

Previous research from our group suggested that the presence of a *S. aureus* induced immune response may increase eosinophilic inflammation in nasal polyp patients, and the finding of IgE-antibodies to SEs was significantly increased in aspirin-sensitive subjects (7, 8). This report has consequently been confirmed by Suh et al (9). We also showed that the up-regulation of the arachidonic acid cascade runs parallel to with the severity of inflammation and disease (13). Based on these findings, we here aimed to study the tissue regulation of eicosanoid production in nasal polyp patients and its possible relation to an immune response to *S. aureus* enterotoxins.

MATERIALS AND METHODS

Patients

Study subjects were selected based on a documented medical history, visible polyps upon endoscopy, and a positive CT- Scan. Samples from ethmoidal and maxillary sinuses were collected during routine functional endoscopic sinus surgery (FESS) at the Department of Othorhinolaryngology in the Ghent University Hospital. The patients were later classified into 2 groups: subjects with nasal polyps with IgE to SEs (NP-SEs(+), n= 10) and nasal polyp without IgE to SEs (NP-SEs(-), n= 10). Patients undergoing septoplasty or conchotomy due to anatomical variations were considered as controls (n= 10). All patients gave informed consent before their participation and the study was approved by the ethical committee of the Ghent University Hospital.

Table 1. Characteristics of patients groups

	Controls	**NP- SEs (-)**	**NP- SEs (+)**
Number of patients	10	10	10
Sex (female/ male)	2/ 8	1/ 9	2/ 8
Age, years (mean + standard error)	36 (± 4.3 years)	40 (± 4.1 years)	51.7 (± 2.5 years)
Number of patients with asthma	0	3	6
Number of patients with aspirin sensitivity	0	2	4
Number of patients positive for specific IgE to SEs	0	0	10
Number with positive skin prick test	0	3	5

Clinical diagnosis of nasal polyposis

Clinical diagnosis of nasal polyposis was performed following the guidelines of the American Academy of Otolaryngology, Head and Neck Surgery. It was based on a documented medical history, typical symptoms and the presence of endoscopically visible bilateral polyps growing from the middle nasal meatus into the nasal cavities, and affecting ethmoidal and maxillary sinuses according to a computed tomography (CT)-Scan of the paranasal sinuses. Diagnosis of asthma was based on the clinical history, and pathological lung function tests, following the Global initiative for asthma (GINA) guidelines. Atopy was evaluated on the basis of the results of a skin prick test performed for a panel of allergens including: house dust mites, grass and tree pollen, cat dander and moulds. The use of any medication related to the disease, including systemic glucocorticoids, antibiotics and anti-leukotrienes, was stopped in all subjects 4 weeks before surgery. The use of topical glucocorticoids was interrupted 2 weeks before surgery. Patient's characteristics are summarized in table 1.

Sample preparation

Tissue specimens were collected from the inferior turbinate for control patients, and from the paranasal sinuses from polyp patients during routine septal and sinus surgery and snap frozen until use. All tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added per every 0.1 g tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun, Melsungen, Germany) at 1000 rpm during 5 min on ice. After homogenization, the suspensions were centrifuged at 1500 rpm for 10 minutes at 4 °C, and the supernatants separated and stored at -80°C until analysis. Supernatants were collected and stored at -80°C until use. For measuring eicosanoids levels, specific sample extraction procedures were applied as described bellow.

Quantification of Total and Specific IgE

Screening for total IgE and IgE antibodies was performed by using the UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden). For IgE to SEs measurements, we used a mix of three SEs (SE A, SE C and TSST-1) coupled to a solid phase, using a cut- off of 0.35 kU_A/L. Non- specific binding of IgE was evaluated using an ImmunoCAP coated with glycine- human serum albumin.

Levels of Eicosanoids

Concentration of cysteinyl leukotrienes (LTC_4/ D_4/ E_4), LTB_4 and LXA_4 were measured by Enzyme Linked Immunoassays (ELISAs) purchased from Oxford BioMedicals (Oxford, USA). Sample extraction procedures for protein removal and eicosanoid stabilization were performed according to the provider's instructions. Briefly, nasal tissues were first homogenized in ethanol (5 ml/g) and then centrifuged for 5 minutes at 3,000 rpm at 4°C. Supernatants were diluted in water, pH 3.5 and processed by using C_{18} Sep-Pak light column (Waters Corporation, USA) following manufacturer's directions (Oxford BioMedicals, Oxford, USA). The assays have a detection range between 0.04 and 10 ng/ ml and a sensitivity of about 0.2 ng/ ml. The intra- and inter- assay coefficient of variation was less than 10% for all assays.

Markers for inflammation

IL-5 and MPO concentrations were measured tissue homogenates by ELISAs purchased by R&D Systems (Minneapolis, USA) and OxisResearch (Portland, OR, USA), respectively. The detection range was 7.8- 500 pg/ ml and the sensitivity < 3 pg/ ml for IL-5, and 1.6 - 100 ng/ ml and 1.5 ng/ ml for MPO, respectively. The intra- and inter- assay coefficient of variation was less than 10% for all ELISAs. Quantification of Eosinophil Cationic Protein (ECP) was carried out on supernatants obtained after homogenization of NP tissues by the UniCAP system (Pharmacia & Upjohn, Sweden), with a detection limit of < 0.2 µg/L.

Statistical data analysis

All data were analyzed using the MedCalc software version 7.6 (Mariakerke, Belgium). Results are presented in Box-and-Whisker plots or Bars. In the Box and Whisker plots, the central box represents the values from the lower to upper quartile (25 to 75 percentile or interquartile range (IQR)), the middle line represents the median and line extends from the minimum to the maximum value, excluding "outside" and "far out" values. In the bars chart, the height represents the median and the errors bars represents the values from the lower to upper quartile (25 to 75 percentile or interquartile range (IQR). Data comparison within different patient subgroups was performed using the Kruskal-Wallis test (*H-test*). The Wilcoxon test (or *Mann-Whitney U test*) for unpaired samples was applied to evaluate the statistical differences between patient groups. Spearman's rank correlation and multiple regression analyses were performed to determine statistical significance of differences between two parameters in a classification group. *P* values equal or less than 0.05 were regarded as significant.

RESULTS

Levels of total and specific IgE

Nasal polyp patients were divided in two groups according to the presence of IgE-antibodies to SEs. Patients with IgE antibodies to SEs above 0.35 kU_A/L were considered to be previously or currently exposed to SEs. *Subjects containing no IgE antibodies or concentrations of less than 0.35 kU_A/L were considered as SE negative.* Levels of total IgE were significantly higher in the NP- SEs(+) patients compared to the NP-SEs(-) and control subjects as shown in table 2.

Concentrations of eicosanoids and inflammatory markers

Concentrations of eicosanoids are summarized in figure 1. Levels of LTB_4, $LTC_4/D_4/E_4$ and LXA_4 were significantly higher in NP-SEs(+) compared to NP-SEs(-), $p < 0.05$ and control groups. The inflammatory markers ECP and IL-5 were also significantly increased ($p < 0.05$) in NP-SEs(+) compared to NP-SEs(-) and healthy subjects, however, MPO concentrations were comparable in both disease groups, and significantly higher than in control patients (table 2).

DISCUSSION

Bacterial colonization have been suggested as possible triggers of the inflammatory events in the development of nasal polyposis (2, 6- 9). It has been demonstrated that *S. aureus* enterotoxins are capable of

reacting with T- lymphocytes, and that these lymphocytes are stimulated to produce Th_2 cytokines, with the consecutive increase of eosinophil survival and IgE synthesis (14). The possible influence of SEs on eicosanoid release has been described in atopic eczema (10). The purpose of this study was to investigate the possible impact of an immune response to *S. aureus* enterotoxins on the regulation of the eicosanoid pathway, which is clearly implicated in the pathophysiology of upper airway diseases, and the development of inflammation (13). We here show that eicosanoid production in terms of $LTC_4/D_4/E_4$, LTB_4 and LXA_4 is up- regulated in nasal polyp tissue of patients with IgE-antibodies to *S. aureus* enterotoxins, and the levels of these mediators correlated to eosinophilic inflammation markers and specific IgE to SEs.

Table 2. Median and interquartile ranges (IQR) for inflammatory markers in tissue nasal polyp samples with and without immune response to *S. aureus* enterotoxins. (*): $p < 0.05$ is due to differences between the three sample groups; (**): $p < 0.05$ is due to differences in the levels between control and NP patients. (***): $p < 0.05$ is due to differences between the control and NP-SEs(-) compared to NP-SEs(+) groups.

	Controls	**NP- SEs (-)**	**NP- SEs (+)**	**Kruskal- Wallis test**
Eosinophil cationic protein (µg/ L)	602.5 (IQR: 309.9- 894.3)	9806.9 (IQR: 1686.5 - 17673.8)	25583.0 (IQR: 17226.0 - 29870.3)	$p < 0.01$ (*)
Interleukin- 5 (pg/ ml)	20.9 (IQR: 16.9- 25.0)	81.5 (IQR: 38.9- 291.9)	327.9 (IQR: 106.2- 385.5)	$p < 0.01$ (*)
Myeloperoxidase (ng/ ml)	4882.8 (IQR: 3007.1- 7015.0)	8013.90(IQR: 4912.1- 11476.0)	9705.2 (IQR: 7426.1- 17427.1)	$p = 0.01$ (**)
Total IgE (kU/ L)	1.9 (IQR: 1.9- 1.9)	323.9 (IQR: 67.2- 387.5)	1564.0 (IQR: 739.1- 2039.7)	$p < 0.01$ (*)
IgE to SEs (kU_A/ L)	1.9 (IQR: 1.9- 1.9)	1.9 (IQR: 1.9- 1.9)	8.6 (IQR: 6.3- 17.0)	$P < 0.01$ (***)

Eicosanoids are important mediators of inflammatory reactions in sinonasal diseases. These mediators are released locally from most inflammatory cells present in, or recruited to the airways, by a variety of biological signals (15, 16). In this study, leukotrienes $LTC_4/D_4/E_4$ were significantly higher in the nasal polyp tissue from NP-SEs(+) patients and correlated with levels of IgE-abs to SEs and markers of eosinophil activation (ECP) and survival (IL-5). So far, there is no evidence that SEs can directly influence the leukotriene regulatory pathway; however, it is known that these enterotoxins can induce the synthesis of IL-5 and modulate eosinophil functions (17), and IL-5 can activate cytosolic phospholipase A_2, resulting in the translocation and activation of 5- lipoxygenase enzyme, and hence in the induction of cysteinyl leukotriene synthesis (18). LTB_4 is known to be synthesized by activated neutrophils, monocytes and alveolar macrophages with neutrophils representing the main source in inflammation. In this study, increased concentrations of LTB_4 did not correlate with myeloperoxidase (marker for neutrophil activation), which levels were similar in both NP groups, suggesting that neutrophils might not be the main source of this eicosanoid. *S. aureus* superantigens have been suggested to induce neutrophil migration and activation (19)

and LTB_4 has been demonstrated to be a potent neutrophil, but a weak eosinophil chemoattractant, which partially seem to contradict our results. However, other studies have also shown that the responsiveness of eosinophils to this eicosanoid may be increased in the presence of IL-5 (20) which is clearly increased in the NP-SEs(+) patients.

Table 3. Spearman's correlation analysis between arachidonic acid metabolites, inflammatory markers and specific IgE to SEs in nasal polyp groups. (*): *p* values less than 0.05; *r*: correlation coefficient.

	LTB_4 (ng/ml)	**$LTC_4/D_4/E_4$ (ng/ml)**	**LXA_4 (ng/ ml)**
IgE to SEs (kU_A/L)	$r = 0.63$, $p = 0.02$ (*)	$r = 0.60$, $p = 0.01$ (*)	$r = 0.26$, $p = 0.27$
total IgE (KU/L)	$r = 0.39$, $p = 0.12$	$r = 0.61$, $p = 0.01$ (*)	$r = 0.37$, $p = 0.11$
Interleukin-5 (pg/ ml)	$r = 0.72$, $p = 0.01$ (*)	$r = 0.59$, $p = 0.01$ (*)	$r = 0.60$, $p = 0.01$ (*)
Eosinophil cationic protein (μg/ L)	$r = 0.62$, $p = 0.01$ (*)	$r = 0.58$, $p = 0.02$ (*)	$r = 0.77$, $p < 0.01$ (*)
Myeloperoxidase (ng/ ml)	$r = 0.06$, $p = 0.79$	$r = 0.14$, $p = 0.56$	$r = -0.19$, $p = 0.44$

Additionally, activated mast cells can also considerably regulate leukotriene synthesis via IgE and cytokine mediated mechanisms (21). Although we did not measure any marker of mast cell degranulation in this study, previous research from our group showed no differences in the levels of tryptase and histamine in nasal polyp tissue from patients with and without specific IgE to SEs (6) which suggests that leukotriene production may not mainly derive from these cells.

Furthermore, contribution of the 15-lipoxygenase- derived eicosanoids such as lipoxins is receiving increased attention in the field of inflammatory diseases. These compounds are synthesized during cell-cell interactions between infiltrating leukocytes and tissue-resident cells, such as cytokine-primed endothelial or epithelial cells (16). Of interest, although lipoxins have been defined as anti- inflammatory lipid mediators, severity of asthma has been associated with an increase and activation of 15-LOX enzymes, collagen deposition and eosinophil accumulation (22), and lipoxins have been reported to increase chloride secretion in human bronchial epithelial cells (23). In this study, tissue levels of LXA_4 were increased in NP-SEs(+) compared to NP-SEs(-) and correlated with ECP and IL-5, but not with IgE-abs to SEs in the NP groups. Accordingly, we can suggest that the eosinophilic infiltration observed in NP-SEs(-) and even more in the NP-SEs(+) group, may crucially account for the high levels of lipoxins observed. The role of this eicosanoid in the pathogenesis of nasal polyposis remains unclear.

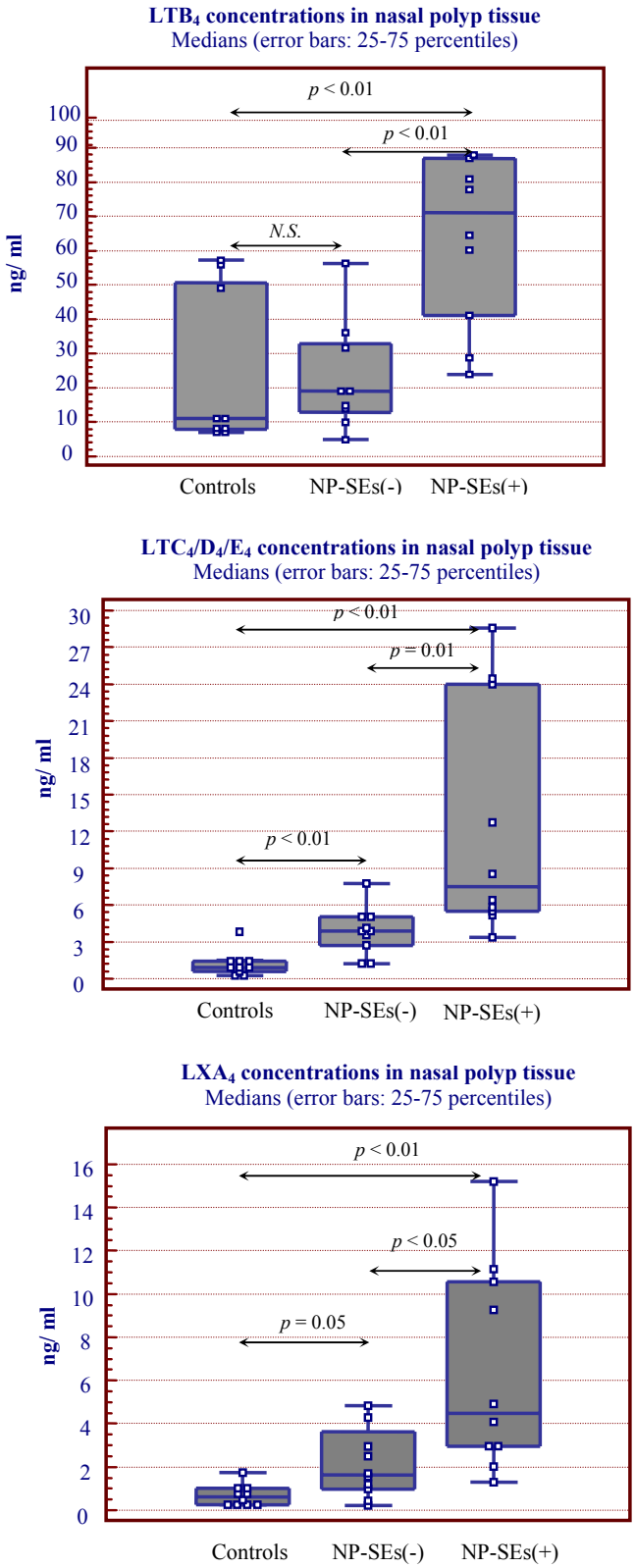

Figure 1. Concentrations of eicosanoids: LTB_4, cysteinyl leukotrienes ($LTC_4/D_4/E_4$) and lipoxin A_4, in nasal polyp tissue from patients with (NP-SEs(+)) and without (NP-SEs(-)) immune response to *S. aureus* enterotoxins. *P*: *p* value after Mann-Whitney test for independent samples.

Finally, the possible impact of allergy and asthma on eicosanoid levels was studied. This issue was difficult to address due to the limited number of patients per group. We did not observe any differences in eicosanoid levels between NP-SEs(+) and NP-SEs(-), asthmatics and non- asthmatics, or allergic and non- allergic patients, with the exception of LXA_4, which was increased in asthmatics. In addition, no correlation was found between local mucosal levels of IgE to SEs and skin prick positivity, confirming previous results indicating that the local immune response to SEs may be unrelated to allergy (6, 7).

In conclusion, we show in this study that tissue leukotriene and lipoxin synthesis is significantly up-regulated in nasal polyp patients with an IgE involving immune response to *S. aureus* enterotoxins. In addition, this increase of eicosanoids seems to be correlated to the inflammatory reaction derived by SEs immune response and seems to be unrelated to asthma and allergy conditions. This work is a preliminary evidence of the possible impact of *S. aureus* infection in the regulation of inflammatory mechanisms like eicosanoid pathway, playing an important role in the pathophysiology of upper airway diseases.

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Staphylococcus aureus Enterotoxin B Regulates Prostaglandin E_2 Synthesis, Growth and Migration in Nasal Tissue Fibroblasts

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ABSTRACT

Background: Superantigens have been identified to interact with T-cells, granulocytes and structural cells to orchestrate inflammatory processes, and are likely to amplify airway diseases such as nasal polyposis and asthma. With the strength of inflammation, the release of anti-inflammatory prostanoids is reduced. So far, a direct mechanism by which enterotoxins could modify the prostanoid metabolism and related functions of the cells is unknown. **Objective:** We studied the possible role of *S. aureus* enterotoxin B (SEB) on COX-2 expression, PGE_2 release, and basic cell functions like growth and migration in fibroblasts isolated from nasal tissue. **Methods:** Fibroblasts were isolated from inferior turbinate tissue and cultured in the presence of different concentrations of SEB. Pre-incubation with IFN-γ was performed to induce the expression of MHC-II receptors, facilitating the interaction with SEB. PGE_2 production was assayed by Enzyme Linked Immunoassays. Expression of COX-2 and HLA-DR genes was assayed by real-time PCR. Migration and growth tests were performed, and the localization of SEB within the cells was assayed by confocal microscopy. **Results:** Nasal fibroblasts express high baseline levels of PGE_2. Stimulation with SEB failed to induce any change in COX-2 expression or PGE_2 release. However, after pre- incubation with IFN-γ, the enterotoxin significantly down-regulated PGE_2 synthesis and COX-2 mRNA expression. In addition, the migration capacity of the fibroblasts was increased after SEB stimulation with, but not without IFN-γ pre-stimulation. Cellular growth was blocked by the enterotoxin, and this effect was inhibited by IFN-γ. **Conclusion:** We here show that SEB down-regulates COX-2 mRNA expression, PGE_2 release, and growth, and increases cell migration in nasal fibroblasts. SEB partially acts via the HLA complex, but also directly translocates into the cells.

ABBREVIATIONS:

COX-2:	cyclooxygenase- 2
HLA-DR:	major histocompatibility complex, class II, DR alpha
IFN-γ:	interferon gamma
MHC-II:	major histocompatibility complex, class II
PGE_2 :	prostaglandin E_2
SEB :	*staphylococcus aureus* enterotoxin B
SEs:	*staphylococcus aureus* enterotoxins
SAgs :	superantigens

Keywords

Nasal fibroblasts, cell growth, cell migration, *staphylococcus aureus* enterotoxin B, PGE_2, COX-2, interferon gamma.

INTRODUCTION

Fibroblasts are important sentinel cells of the immune system playing a critical role in chronic inflammation and tissue repair (1). However, the implication of these cells in the regulation of immune responses has been considered during a long time as relatively unimportant. Recently, several studies have demonstrated that fibroblast can interact with CD40 receptors. This interaction induce the activation of the transcription factor NF-κB and stimulate fibroblasts to synthesize high levels of cytokines (e.g. IL-6, IL-8), adhesion molecules (e.g. ICAM-1, VCAM-1) and cyclooxygenase metabolites (2, 3). In the airways, fibroblasts play a crucial role in the remodelling process where they are the primary cellular source of collagens I and III as well as of growth factors (4). Additionally, prostanoids, metabolites of arachidonic acid have been suggested to be crucial molecules in the control of remodelling process (5- 6).

Moreover, recent studies have suggested the possible implication of bacterial superantigens (SAgs) in the pathogenesis of inflammatory airway diseases (7- 8). *In vitro* experiments, have demonstrated that staphylococcal enterotoxins (SEs) can selectively induces the production of interstitial collagenase over the expression of the tissue inhibitor TIMP in fibroblasts; and that phenomenon maybe mediated in part by PGE_2 and cyclooxygenase production (9). In addition, staphylococcal enterotoxin A (SEA) can selectively induce the secretion of stromelysin (MMP-3) from synovial fibroblasts, suggesting the role of bacterial SAgs in the regulation of articular destruction observed in inflammatory joint disease (10). More recently, in a study performed in bovine mononuclear cells, SEA and SEB induced PGE_2 production, which had a modulating role on the proliferative response of bovine $CD4^+$ and $CD8^+$ T cells (11).

Role of superantigens in the pathogenesis of upper airway diseases has become an important issue in the last years (6, 7). In a previous study, we showed that immune response to SEs was linked to an exacerbation of eosinophilic inflammation and up- regulation of leukotrienes and lipoxins within the nasal polyp tissue (19). Based on those findings, we aimed with this study to investigate the capacity of *S. aureus* enterotoxin B to regulate the production of PGE_2 release, growth and migration processes in nasal tissue fibroblasts.

MATERIALS AND METHODS

Samples

Fibroblasts were isolated from inferior turbinate tissue obtained from patients (n= 5) undergoing septoplasty or rhinoseptoplasty due to anatomical variations, and not suffering from any sinus disease. Surgery was performed in the the Department of Othorinolaryngology at the Ghent University Hospital. All patients gave informed consent before their participation and the study was approved by the ethical committee of the Ghent University Hospital.

Reagents

Dulbecco's Phosphate Buffered Saline (PBS), Penicillin-Streptomycin (5000 IU/ml and 5000 µg/ml), Tryptan Blue (0.4 % solution in phosphate buffered saline) were obtained from Invitrogen, Merelbeke, Belgium. Minimum essential medium (MEM), Opti-MEM I Reduced Serum Medium (Optimem I), L-glutamine (200 mM), Trypsin- EDTA (1X) and Foetal Bovine Serum (FBS), qualified, origin USA) were purchased from GIBCO BRL (Life Technologies, Grand Island, NY. 5% Ultroser G were obtained from Biospera (Cergy- Saint- Christophe, France). Recombinant human IFN-γ was from R&D Systems, Minneapolis, USA and *S. aureus* enterotoxin B (SEB) were obtained from Sigma- Chemicals, MO, USA. SYBR Green I Master mix, Aurum total RNA and the Script cDNA synthesis Kits were obtained from Bio-Rad laboratories (CA, USA).

Fibroblast isolation from nasal tissue

Nasal tissues obtained during surgical operations were rinsed several times with Opti-MEMI suplmented with 5 % FBS, 5 % Ultroser G, 2 mM glutamine, 50 IU/ ml Penicillin- 50 µg/ ml Streptomycin and cut into small pieces (approximately 1 mm^2). Diced specimens were then plated (density of 9 pieces/ 6 well tissue culture dishes) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C until a monolayer of fibroblast-like cells was observed to be confluent. Then, the explanted tissues were removed, cells were trypsinized, and replated into 250 cm^2 tissue culture falcons at a final volume of 5 ml. The medium was changed every 3 days for 2 to 3 weeks until 90% confluence was obtained. Subsequently, the cells were split and passaged. The cells were characterized with antibodies against vimentine, cytokeratin and alpha- smooth muscle actin using the Peroxidase- anti- peroxidase technique. Stimulated cell cultures were centrifuged at 4°C and the supernatants were collected and stored at -20°C until use. Subsequently, cells were trypsinized, washed, resuspended in Opti-MEM I, and then centrifuged. After the supernatants were removed one part of the cells were resuspended in lysis buffer for posterior RNA extraction and another part was stored at -80°C.

Cell stimulation

Cells were stimulated with 0.01, 0.1 and 1 µg/ ml of SEB or 10 ng/ ml of IL-1β for 24 hours at 37°C and 5% CO_2. To induce the MHC-II receptors cells were pre- treated with IFN-γ (10ng/ ml) during 24 hours at 37°C and 5% CO_2 and then were stimulated with 0.01, 0.1, 1 and 1 µg/ ml of SEB during 24 hours at 37°C and 5% CO_2. We used a negative control per sample in each experiment that consisted in cells incubated only with culture medium (MEM).

Enzymatic immunoassays

Concentration of PGE_2 were measured by Enzyme Linked Immunoassays (ELISA's) according to the manufacturer's instructions (Oxford BioMedicals, Oxford, USA) with an intra- and inter- assay coefficient of variation for all ELISAs was less than 10%.

Quantitative real- time PCR

Cell cultures were centrifuged and pellets containing 1x106 cells were disrupted and homogenized in Lysis solution and total RNA was extracted using the Aurum total RNA Mini Kit. cDNA was synthesized from 1µg of total RNA by using the Script cDNA Kit following the manufacturer's instructions. Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System with the following primers set: Fw- (HLA-DR1α): 5'- CATCAAGGGAGTGCGCA-3' and Rev (HLA-DR1α): 5'-CTCCATGTGCCTT-ACAGAGGC-3' (product size: 64 bp, Genbank accession number: NM_019111) and Fw (COX-2): 5'-GCTGGAACATGGAATTACCCA-3' and Rev (COX-2): CTTTCTGTACT-GCGGGTGGAA (product size: 88 bp and Genbank accession number: XM_051899). PCR reactions contained 20 ng of cDNA from cultured cells, 1x SYBR Green I Master mix and 300 nM of each primer. PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 45 cycles at 95°C for 30 seconds and at 62°C for 1 minute. Ubiquitin C (UBC) and Hypoxanthine phosphoribosyl-transferase 1 (HPRT1) were used as internal control genes for normalization. Primer sequences for these genes were obtained from the Real Time PCR Primer and Probe Database from the Dept. of Medical Genetics from the University of Ghent (medgen.ugent.be/rtprimerdb).The relative number of molecules of each gene was expressed in relative expression units quantified per 20 ng of cDNA sample and was determined by the *ΔCT* value method.

Growth curve assay

Confluent cells in a 25 cm^2 falcon were trypsinized with preheated trypsin- EDTA and resuspended in 5 ml of Opti-MEM I. Living cells were counted using a Neubauer counting chamber and seeded (10.000 cells/well) in a 24-well plate and incubated with Opti- MEM I, IFN-γ (10 ng/ml) or SEB (0.1 µg/ ml) at 37°C, 5 % CO_2 for 24, 48 and 72 hours. Per each time point, cells were again trypsinized with pre- heated trypsin-EDTA and resuspended in 1 ml Opti- MEM I and number of living cells (per each time point) were quantified with Tryptane blue using the Neubauer counting chamber.

Cell migration and wound healing assays

Fibroblasts were seeded in a 35 mm diameter 6 well plate and incubated at $1x10^5$ cells/ well in serum free medium MEM and allowed to grow until confluency during 7 days. Then, the medium was replaced by 1x

Dulbecco's Phosphate Buffered Saline and cells were incubated at 37°C, 5 % CO2 for 5 minutes. The monolayer was wounded by pressing a sterile razor blade down onto the well making a sharp visible demarcation at the wound edge. The blade was then gently moved to one side to remove a part of the cell-monolayer. Two separated 15- 20 mm wounds were made in the same well. The wounded layers were washed two times with Opti-MEM to remove the cell debris and incubated with Opti-MEM I and IFN-γ (10 ng/ml) or SEB (0.1 µg/ ml) at 37°C, 5 % CO_2. Migrating cells were counted after 24 and 48 hours using a counting- eyepiece with grid graticule (10 mm x 10 mm). The first line of the grids falls together with the sharp beginning demarcation of the wound. Ten different places of each wound were counted.

Statistical Analysis

All data was analyzed using the MedCalc software version 6.0 (Mariakerke, Belgium), and presented as median and interquartile range (IQR). Data comparison within different patient subgroups was performed using the Kruskal-Wallis test (*H-test*). The Wilcoxon test for unpaired samples (or Mann-Whitney U test) was applied to evaluate the statistical differences between patient groups. Spearman's rank correlation analysis was used to determine statistical significance of differences between two parameters in a classification group. *P* values equal or less than 0.05 were regarded as significant.

Cell localization of SEB

Cell localization of SEB was assayed by immuno-fluorescence confocal microscopy. Cryostat (Shandon) sections from nasal tissues (7 to 8 µm thickness) were prepared and collected on poly-D-lysine-coated glass slides, air dried for 24 hours at room temperature and stored at –20°C. Tissue sections were fixed in acetone for 10 minutes at 4°C and blocked using 0.4% fish skin gelatin/ 1X PBS for 2 hours at room temperature. The sections were then incubated with (1: 500) polyclonal antibody anti- SEB (Sigma, MI, USA) for 1 hour and with the secondary antibody (1:2000) anti- rabbit Alexa Fluor 526 (Molecular Probes, Leiden, The Netherlands) for 1 hour at room temperature; both primary and secondary antibodies were diluted in 0.4% fish skin gelatin/ 1X PBS. The sections were then counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Leiden, The Netherlands) for 5 minutes and mounted in Vectashield mounting medium (Vector Laboratories, CA, USA). The localization of the protein was evaluated using a Bio- Rad Radiance 2100 Laser Scanning Confocal Microscope System with the 4- line Argon primary laser, Green HeNe laser and the Blue Laser Diode. Analysis was performed using Adobe Photoshop (version 7.0) and Confocal Assistant (version 4.02, Todd Clark Brelje) software programs.

RESULTS

Effect of SEB on PGE_2 production

Nasal tissue fibroblasts cultured in MEM in absence of stimulus produced significant baseline concentrations of mRNA of COX-2 and PGE_2. Stimulation with SEB without pre- incubation with IFN-γ did not induce any change on PGE_2 production or COX-2 mRNA expression. Only IL-1β increased significantly the release of these molecules (data nor shown). Induction of the expression of MHC-II molecules in fibroblasts after stimulation with IFN-γ was verified by real-time PCR, which showed an expression of the HLA-DR1α genes in all IFN- γ pre- stimulated compared to non-stimulated cells (Figure 1).

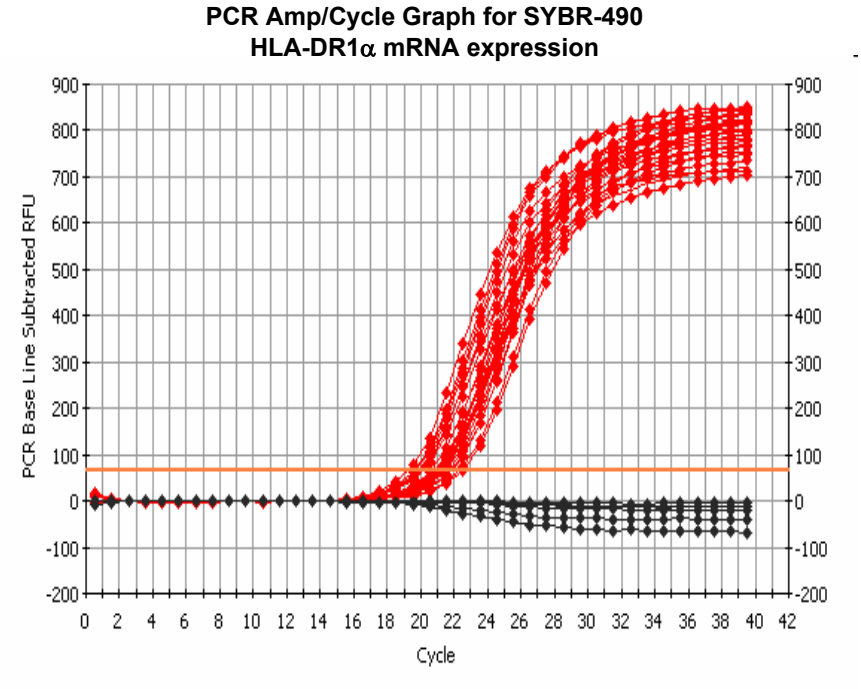

Figure 1. Curve after real- time PCR for HLA-DRα gene in nasal tissue fibroblasts showing amplification of the gene after pre- incubation with IFN-γ (red curves) and no amplification in cells without pre- incubation with the cytokine (black curves). After Mann-Whitney test for independent samples: *: p <0.05 in comparisson to MEM, *§: $p < 0.05$ in comparisson to MEM and IFN-γ.

Pre- incubation with IFN-γ lead to a significant decrease of endogenous PGE_2 and COX-2 mRNA (Figure 2) compared to non- stimulated cells. Interestingly, this down- regulation was acentuated after incubation with SEB at 0.1 and 1 μg/ml, but in a dose independent manner (Figure 2). In addition, incubation with IL-1β significantly increased the expression COX-2 and PGE_2 release.

Furthermore, expression of the transcripts expressing the genes encoding for prostanoids E recptors (EP_1, EP_2, EP_3 and EP_4) was analyzed. Real time PCR results showed no changes in expression of EP_1 and EP_3 receptors (Figure 3) but a SEB dose dependent down- regulation of EP_2 receptor.

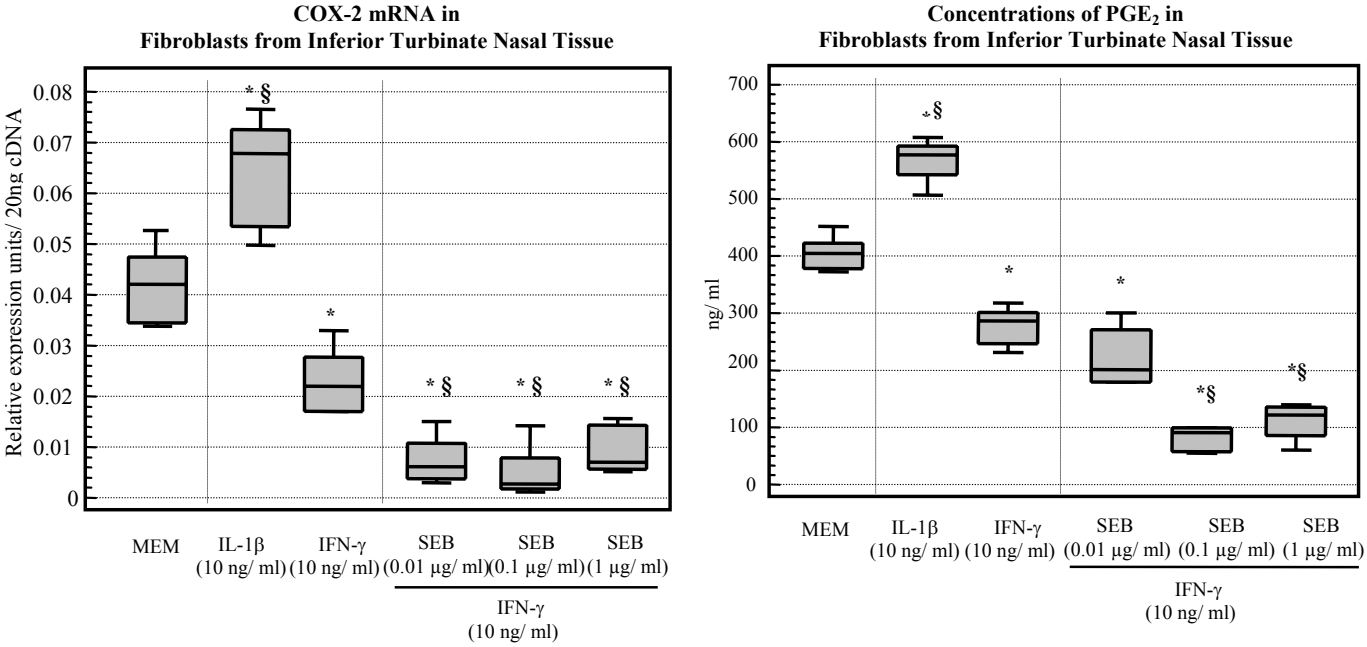

Figure 2. mRNA levels of COX-2 and concentrations of PGE_2 in nasal tissue fibroblasts after stimulation with IL-1β, IFN-γ, and SEB (0.01, 0.1 and 1 µg/ ml). (*): statistical differences compared with IFN-γ and SEB stimulated cells; (***§**): statistical difference compared with non- stimulated cells (MEM); *p* value < 0.05 after *Mann-Whitney test* for independent samples.

Effect of SEB on growth and migration

Results of growing test were expressed as number of living cells after 24, 48 and 72 hours. Number of cells per each stimulus was compared to cells cultured only in MEM, which were considered as controls for normal growing. Cells in MEM showed a significant increase in proliferation after 24, 48 and 72 hours. The same pattern was observed in cells stimulated with IFN-γ and IL-1β (Figure 4). When cells were incubated with SEB however, no differences were observed between the number of cells after 24, 48 and 72 hours. The number of living cells after 24 and 48 hours was similar in cultures containing IFN-γ and stimulated with SEB, but significantly increased after 72 hours. Cell number however, was similar for all cultures after 24 and 48 hours only at 72 hours a stattistical difference was only observed between plates containing SEB when compared **w**ith the other cultures.

Furthermore, migration test results show the number of cells reaching a line (L2) situated at 240 µm from the culture wound after 24 (L2D1) and 48 hours (L2D2) of incubation with SEB, IFN-γ, IL-1β or MEM (Figure 4).

This test showed that pre- incubation with IFN-γ did not change migration capacity of the cells. However, SEB in absence of the cytokine significantly induced cell migration (Figure 5). In addition, stimulation with SEB after pre- incubation with IFN-γ attenuated the effect of SEB. IL-1β as expected increased migration in fibroblasts and this effect was statistical significant compared to the one obtained with SEB.

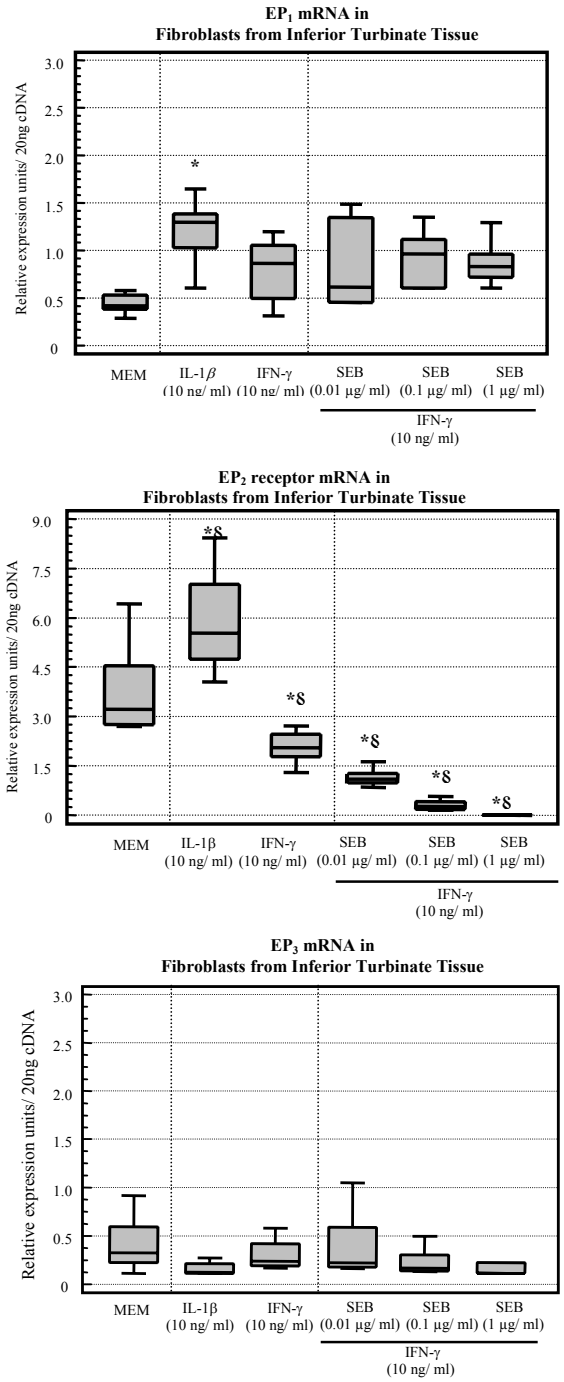

Figure 3. mRNA levels of E prostanoid receptors (EP_1, EP_2 and EP_3) in nasal tissue fibroblasts after stimulation with IL-1β, IFN-γ, and SEB (0.01, 0.1 and 1 µg/ ml). (*): statistical differences compared with IFN-γ and SEB stimulated cells; (*§): statistical difference compared with non- stimulated cells (MEM); *p* value < 0.05 after *Mann-Whitney test* for independent samples.

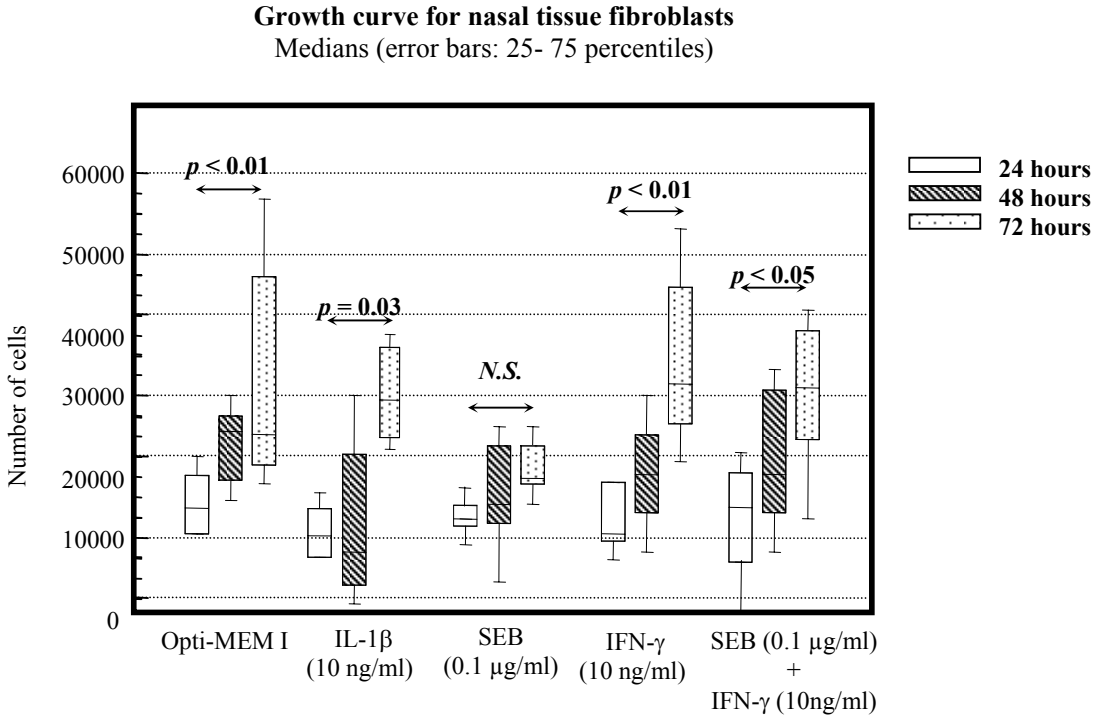

Figure 4. Growth curve for nasal tissue fibroblasts after incubation with SEB, IFN- γ and IL-1β. *P*: *p* value after Mann-Whitney test for paired samples (comparing samples within a group):

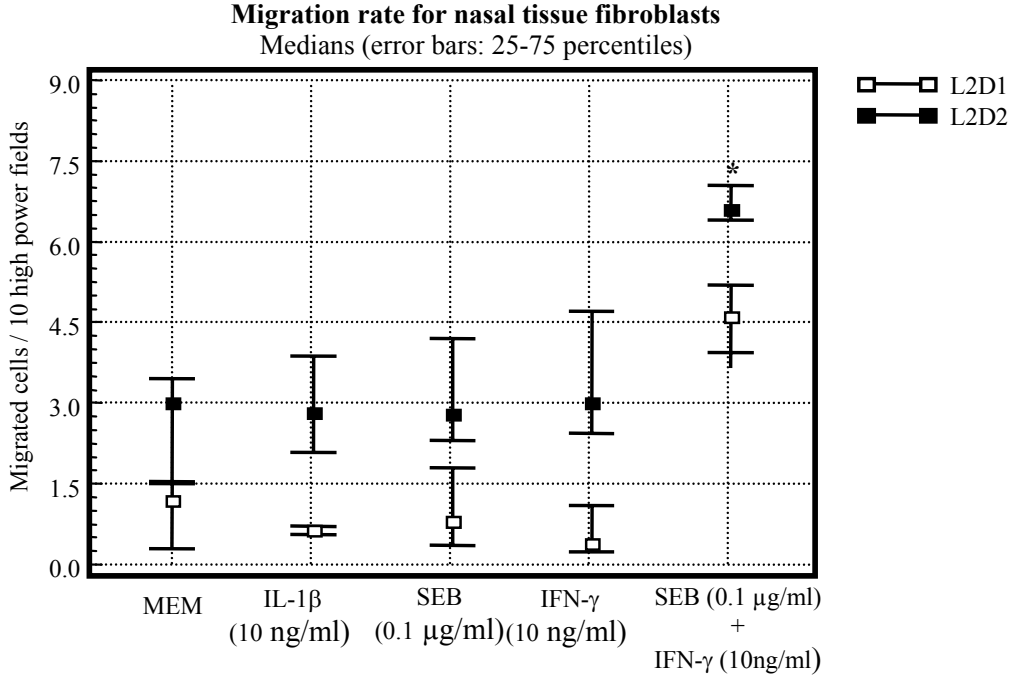

Figure 5. Migration test for nasal tissue fibroblasts after stimulation with SEB, IFN-γ and IL-1β. (*): statistical difference compared to non- stimulated (MEM), IFN-γ, SEB and IL-1β stimulated cells; *p* value < 0.05 after *Mann-Whitney* test for independent samples. Graph shows the number of cells that migrating 240 µm from the wound site after 24 hours (L2D1) and 48 hours (L2D2).

Cell localization of SEB

Cellular localization of SEB was assayed by confocal microscopy. Confocal images of nasal fibroblasts after incubation with 0.1 µg/ ml of SEB showed that the enterotoxin was localized in the cytoplasm but also in some regions of the nucleous of the cell. Of interest the same effect was observed after pre- incubation with IFN-γ followed by stimulation with 0.1 µg/ ml of SEB. However, in this case the enterotoxin was more localized in the periphery of cell cytoplasm and around the nucleous. Also in some cells nuclear localization was oberved.

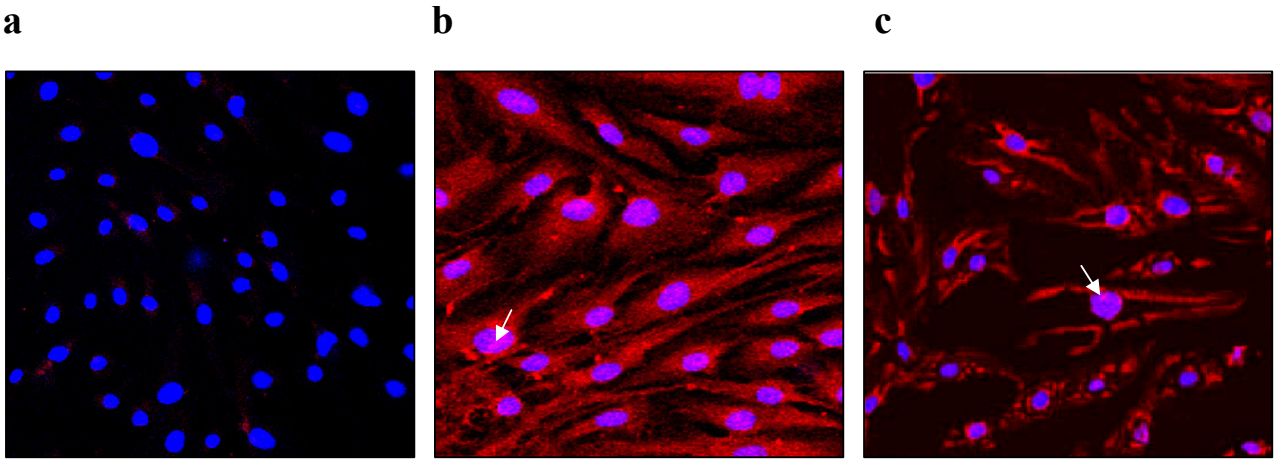

Figure 5. Confocal microscopy image (magnification 40X) performed in nasal tissue fibroblasts (a) non-stimulated, (b) stimulated with SEB (0.1 µg/ ml) during 24 hours, and (c) stimulated with SEB (0.1 µg/ ml) during 24 hours after pre-incubation with IFN-γ. The figure shows that SEB is internalized in the nasal fibroblasts and can be localized in the cytolpams and in the nucleous (white arrow) of the cell.

DISCUSSION

Fibroblasts have been demonstrated to be key cells in the remodelling process in airway diseases. These cells are ubiquitous and provide mechanical strength to tissues by providing a supporting framework of extracellular matrix. It has recently postulated that fibroblasts from different anatomic regions display characteristic phenotypes that are remarkably stable even after prolonged culture *in vitro* (12). In this study we confirm that nasal tissue fibroblasts can spontaneously express the COX-2 enzyme and release PGE_2. In addition, we found that SEB down- regulated the expression of these molecules, stops the growth and increased the migration capacity of these cells.

PGE_2 is the major prostanoid product of fibroblasts in upper and lower airways (13) and is a potent inhibitor of important functions in these cells including cell proliferation and chemotaxis, collagen synthesis, and growth factors expression (14, 15). The role of PGE_2 in airways has been mainly related to anti-inflammatory actions, where this prostanoid blocks collagen synthesis and attenuates inflammatory cell

activation (18). However, Liu et al., (17) have reported a rich vascularity around COX-2 mRNA positive fibroblasts in nasal polyp tissue; and suggested that inducible COX-2 and probably PGE_2 may contribute to nasal polyp development by promoting vasodilatation (17). In contrast, deficient production of this eicosanoid has been observed in fibroblasts from asthmatic patients and especially in the ones having aspirin intolerance (16). And this study is in line with the one reporting a PGE_2 down- regulation in homogenates from nasal polyp and aspirin intolerant patients (20). Although the contribution of this prostaglandin in airways diseases remains unclear, we here suggest that staphylococcal enterotoxins may contribute to the pro- inflammatory reactions observed in upper airway diseases by down- regulating PGE_2 release.

In this study, SEB failed to induce changes in the cyclooxygenase pathway in cells that were not pre-incubated with IFN-γ. IFN-γ is the only cytokine able to induce both mRNA and protein expression of MHC-II molecules via the HLA-DR gene. In cultures containing IFN-γ, the cytokine significantly reduced the mRNA expression of COX-2 and the release of PGE_2. Interestingly, SEB acentuated the effect of this cytokine but in a partial dose dependent manner. These results suggest that SEB may influence this pathway by acting via the classical binding to MHC-II. IFN-γ is released in response to infection by bacteria or parasites. The implication of this cytokine in upper airway diseases like chronic rhinosinusitis and nasal polyposis is not yet clear. In several studies, IFN-γ has been observed to be highly expressed in nasal polyp tissue and has been shown to induce expression of COX-2 in nasal polyp explants (21). Here we hypothesize that IFN-γ may provide the binding site allowing the SEB enter the cell and regulate PGE_2 synthesis. In addition, the existence of a possible alternative superantigen binding molecule (p85) in monkey renal fibroblasts was reported in 1995 by Rogers and col. (22). The binding of SEB to this site had an affinity equivalent to SEB binding by MHC-II, but at a site which does not permit recognition by the TCR. Although, no further studies have been done regarding this molecule we cannot exclude the presence of this site allowing the entrance of the enterotoxin to the cell.

Finally, accumulation of fibroblasts is an important event in tissue response to injury and can occur via chemotactic recruitment accompanied by local proliferation of these cells. The second aim of this study was to investigate if SEB could affect the growing and migration capacity of nasal tissue fibroblasts. Of interest, this molecule blocked the growing process of these cells after 72 hours and in absence of IFN-γ. This effect seems to be independent of IFN-γ and is in contradiction with the findings of COX-2 and PGE_2 in these cells. Increase of PGE_2 has been reported to inhibit cell proliferation. According to our data, it seems that SEB may regulate fibroblast's growing by a PGE_2 independent mechanism. In addition, this stop in growing may be due to a toxic effect from the enterotoxin. The changes in this process were induced after 48 and 72 hours that may suggest a long period of incubation. However, this was partiallly confirmed with the confocal staining showing that SEB is internalized by the cell in absence of IFN-γ; maybe by altering cell membrane permeability?. Accordingly, although some asumptions can be made suggesting an alternative binding of the

enterotoxin to fibroblasts, studies evaluating different time points of incubation will give a better vision of the interaction and toxicity of these molecules.

Finally, cell migration is one of the steps involved in wound healing process in the airways and constitute a subject of interest since patients with nasal polyp and chronic rhinosinusitis patients often suffer of fibrosis and healing problems after surgery. Cell migration of nasal fibroblasts was significantly enhanced after stimulation with SEB only in cultures pre- incubated with IFN-γ. Surprisingly, it seems that there is a synergistic effect between thes two molecules due to no changes compared to non-stimulated cells were observed in cells incubated with the cytokine or SEB separately. Of interest, the number of cells that migrated but not the number that proliferated increased with the time. This suggests that SEB may has a primary effect on the rate of migration in these cells. The rate of migration of a cell depends on several interacting factors, including the ability of the cell to polymerize cytoskeletal elements resulting in protrusion of cytoplasmic processes at the cell edge, to adhere to subjacent matrix at the leading edge, and detach from substrate at the trailing edge (23). Influence of any of these processes by the enterotoxin could result in an increased rate of nasal fibroblasts migration by the enterotoxin.

In summary, the current study demonstrates for the first time that SEB decreases COX-2 expression, PGE_2 release and stops growing but increase migration processes in human nasal tissue fibroblasts. The mechanism behind this action was not the objective of this work however, it seems that there are mechanisms other than the conventional binding via MHC-II that allows the enterotoxin enter the cell and influence its metabolism. This study has to be completed with experiments using PGE_2, and MHC-II inhibitors that can add more information to the regulatory mechanisms acting in these cells. This work add a new clue to the implication of superantigens in the development of upper airway diseases.

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DISCUSSION AND CONCLUSIONS

Chronic sinus diseases constitute one of the most common respiratory tract conditions in humans with a prevalence of 4- 15% in the general population and representing a considerable socioeconomic problem. Chronic rhinosinusitis has been understood as one disease that can be linked to different clinical appearances like nasal polyps. However, recent studies based on clinical parameters, release of cytokines, and regulation of inflammatory pathways lead to the thinking of considering it as a heterogeneous group of diseases with different underlying etiologies and pathomechanisms. During the last decade, extensive studies have been performed on the field of eicosanoid metabolism and its regulation in chronic rhinosinusitis. These studies have shown the importance of this pathway in the balance of pro- inflammatory mechanisms operating in this disease. More recently, the idea of infection as an important disease modifier has been suggested. Both viral and bacterial infections have clear implication on the clinical picture of chronic rhinosinusitis. In this work, we aimed to find a link between eosinophilic inflammation, imbalance of eicosanoid metabolism and the possible role of *S. aureus* enterotoxins in the regulation of these processes.

Quantitative real time PCR for gene expression analysis in chronic Rhinosinusitis/ Nasal polyposis

Chronic rhinosinusitis with bilateral nasal polyps are characterized by abundant eosinophilic infiltration activation, and high amounts of IL-5 and eotaxin. IL-5 is one of the main cytokines detected at mRNA as well as at protein level in nasal polyp tissue (Bachert et al., 1997; Hamilos et al., 1998). The biological signal of this cytokine is mediated through its receptor consisting of a specific IL-5-binding α-chain, and a signal-transducing common β-chain (Tavernier et al., 1992).

Function and expression of this receptor can be regulated through splicing events generating two different transcripts: one encoding a membrane-anchored protein through alternative splicing, and a second one encoding a soluble form of this receptor, by normal splicing events (Tavernier et al., 1992). Adequate tools to investigate the regulation of the hIL-5Rα expression *in vivo* are mandatory to understand the pathomechanisms involved as well as to design future therapeutic approaches for eosinophil inflammatory diseases. However, no sensitive technique was available for the quantification of the small differences at the transcription level of this receptor.

Real time PCR is increasingly used to quantify physiological changes in gene expression due to its large dynamic range of quantification and its high sensitivity. With the methodology established, we could quantify the hIL-5Rα splice isoforms in human peripheral blood and eosinophilic chronic rhinosinusitis tissue with an efficiency ranged between 95% and 97% for both splice variants. Specific amplification and coefficients of variation less than 2% for C_T values and 25% for calculated quantities demonstrated the accuracy of the technique. We also showed an up-regulation of both isoforms in nasal tissue and blood from eosinophilic chronic rhinosinusitis patients compared to healthy subjects. Additionally, a significant over-expression of the soluble isoform in peripheral blood from chronic rhinosinusitis patients compared to

control subjects was also found, which confirmed previous studies reporting an up- regulation of this isoform at protein level in subjects with nasal polyposis (Gevaert et al., 2003). Finally, the development of this assay will greatly help in the study of the regulatory mechanisms of this receptor in other eosinophil- related diseases such as asthma, atopic dermatitis, and hypereosinophilic syndrome, and hence will contribute to the development of future therapeutic strategies for eosinophilic inflammatory diseases.

Internal control genes and RNA quality in expression studies in chronic rhinosinusitis/ nasal polyposis

To ensure the accuracy and reliability of gene expression studies it is necessary to guarantee the quality of the starting material, and the selection of a good reference gene to normalize for differences in RNA quantity and cDNA synthesis efficiency. RNA degradation constitutes a major problem for gene quantification analysis specially when working with biological samples resulting in misinterpretations of the results. However, although this problem has been extensively discussed, control of RNA integrity is often not performed due to the limited availability of samples or the lack of the adequate methodology. In addition, stability of reference gene expression is an issue that has been only addressed in a few studies and none of them in tissues from upper airways.

Reference genes are expressed constitutively in every cell. However, it has been demonstrated that their expression can vary according to cellular proliferation, cellular composition, and by individual genetic background (Glare et al., 2002). Our results show for the first time that the stability of reference genes is different in chronic rhinosinusitis and nasal polyp mucosa (as can be expected because of the differences in cellular origin). Additionally, gene-specific variation was always higher in degraded versus intact samples, with more pronounced differences in chronic rhinosinusitis compared with nasal polyp tissue. In non-degraded tissue samples from chronic rhinosinusitis without polyps, HMBS, SDHA, GAPD and ACTB were the most stable reference control genes, whereas in the chronic rhinosinusitis/ nasal polyp tissue: ACTB, TBP, SDHA and HPRT1 showed the highest stability. Furthermore, although not included in the article, analysis of the most stable references genes for normal nasal mucosa (inferior turbinate tissue) was also performed. Of interest, these samples suffered a much less degradation compared to specimens obtained from nasal polyp and chronic rhinosinusitis tissue and showed indeed a different stability pattern for internal control genes compared to the other tissues studied. In this case, HMBS, TBP, ACTB and RPL3A were the most stable genes. Furthermore, genNorm analysis performed for the three different sample groups showed that HMBS, SDHA, GAPD and ACTB are the most suitable genes for normalization. Among the most used reference genes used in gene expression analyses in chronic rhinosinusitis and nasal polyps we found B2M, GAPD, ACTB and HPRT1 (Song et al. 2003; Rhyoo et al., 1999 and Mullol et al., 2002). From these genes, only GAPD and ACTB were found to show a high stability. However, it is important to remark that as stated before, stability of these genes may vary according to the sample treatment and the type of sample, so we cannot fully exclude these genes as stable genes in the samples analysed.

According to these results, we can conclude that HMBS, GAPD, ACTB, and SDHA are the most suitable genes for normalization in gene expression analysis performed in samples from chronic rhinosinusitis with and without polyps and inferior turbinate tissue. Additionally, we strongly recommend performing RNA integrity analysis for each sample before carrying out gene expression studies.

Regulation of eicosanoid metabolism and eosinophilic inflammation

The arachidonic acid (eicosanoid) cascade is one of the most important pathways regulating the balance of pro- and anti- inflammatory reactions in the human body. As we described before this cascade contains two major different pathways that lead to the formation of prostaglandins, leukotrienes and lipoxins (Funk et al., 2001; McMahon et al., 1995). As we already known, changes in these pathways have been described in chronic rhinosinusitis patients with antrochoanal polyps (Jang et al. 2000), allergic rhinitis and cystic fibrosis (Wurm et al., 2001). More importantly, altered balance of these eicosanoids has been thought to be the main cause of the occurrence of the aspirin intolerance syndrome. In the second part of this study we analyzed the local tissue eicosanoid regulation in terms of leukotrienes, prostaglandins and lipoxins production and their receptors in three subgroups of patients with chronic rhinosinusitis and we evaluated its possible correlation with the severity of eosinophilic inflammation and the clinical manifestation of aspirin intolerance. The results discussed in this section are summarized in figures 7, 8 and 9.

CysLTs and LTRs have been described as potent inducers of airway smooth muscle contraction, vascular vasodilatation and vascular permeability, which are pivotal processes for the recruitment of leukocytes to the site of inflammation (Holgate et al., 2003). Concentrations of these eicosanoids as measured by means of ELISA, were significantly increased in chronic rhinosinusitis/ nasal polyp versus chronic rhinosinusitis and control subjects. This increase was in parallel with the up- regulation of the mRNA transcripts and protein of 5-LOX and LTC_4S enzymes. Additionally, $CysLT_1$ receptor mRNA was also increased in chronic rhinosinusitis/ nasal polyp versus chronic rhinosinusitis and control subjects. Interestingly $CysLT_2$ transcripts were similar in both chronic rhinosinusitis groups and higher than control subjects. In the chronic rhinosinusitis groups we found a direct link between lipoxygenase pathway and the severity of eosinophilic inflammation. These patients showed a high infiltration of activated eosinophils in the epithelium and tissue lamina propria (data not shown); and the number of these cells together with eosinophil mediators ECP, IL-5 and IL-5Rα directly correlated with the levels of CysLTs. Furtherly, we analysed the activation of this pathway in aspirin intolerant patients which showed a significant enhance in the tissue expression of CysLTs and their enzymes: LTC_4S and 5-LOX.

Eosinophils are one of the principal cell types recruited to and activated at sites of inflammation, and are capable of elaborating high amounts of lipid mediators, including leukotrienes. The enzymatic pathways by which eosinophils synthesize CysLTs have been established (Bandeira- Melo et al., 2003), but the

mechanisms that physiologically regulate their biosynthesis remain to be elucidated. Potentially ‘‘activated’’ eosinophils may elaborate greater quantities of LTC_4. Some known priming stimuli like eotaxin, PAF and IL-5 may participate in the recruitment and activation of these cells to sites of inflammation and induce CysLTs production by inducing the formation of lipid bodies in these cells (Bandeira- Melo et al., 2003). In addition, IL- 5 has been shown to induce translocation of 5- LOX to the nucleus in normal human blood eosinophils resulting in the activation of the enzyme and enhancing the capability of cysLTs synthesis in these cells (Cowburn et al., 1999). Additionally, eosinophils also constitute one of the major sources for $CysLT_1$ in the nasal mucosa (Sousa et al., 2002), which suggests a possible autocrine regulation of the leukotrienes synthesis able to maintain the high levels of these eicosanoids in the chronic rhinosinusitis tissue. Alternatively, CysLTs are known to be chemotactic for eosinophils and to promote eosinophilic inflammation through their ability to promote adhesion molecule P-selectin and CCR3 expression and to inhibit eosinophil apoptosis (Zhu et al., 2001). Accordingly, we speculate that the enhanced presence of CysLTs in chronic rhinosinusitis tissues might be in part a result of an increased infiltration and activation of eosinophils. Consequently this leukotriene up- regulation acts as a paracrine and autocrine factor for survival and chemotaxis of these cells (Figure 7).

Together with CysLTs, LTB_4 and LTB_4 receptors (BLTs) were assayed. We found that protein levels of LTB_4 and mRNA for BLT_1 and BLT_2 did not differ between the disease groups and normal nasal mucosa. Leukotriene B_4 is thought to be an important mediator of inflammation as it is a potent activator and chemotactic agent for human neutrophils (Beeh et al., 2003). Of interest, controversial results regarding the regulation of this eicosanoid in upper airways diseases have been reported, difficulting the understanding of its role in these pathologies. Elevated concentrations of LTB_4 have been found in nasal polyp patients with allergy in comparison to non-allergic subjects (Pinto et al., 1997) and in aspirin sensitive patients compared to healthy subjects, but without differences with the tolerant subjects (Sousa et al., 2002). Production of LTB_4 depends of the action of LTA_4 hydrolase enzyme on its metabolite LTA_4. However, LTA_4 is a ubiquitous molecule that serves also as substrate for LTC_4S, 12-LOX and 15-LOX. We found that levels of this eicosanoid were similar in all our patient groups and we hypothesize that it may be due to intrinsic regulatory mechanisms operating in chronic rhinosinusitis that lead to a switch to the CysLTs pathway instead to the LTB_4 formation. Furthermore, it has been suggested that in some diseases the differences in eicosanoid levels between group of patients is due to the differences in number of the cells producing them an not due to a differential cellular synthesis. LTB_4 is mainly produced by neutrophils which were present in the same extend in our patients groups. In this study, we did not investigate why the levels of LTB_4 and BLTs are equal in all groups; this issue requires more investigation to be elucidated.

Cyclooxygenases (COX-1 and COX- 2) catalyze the initial step in the formation of biologically important prostanoids, such as prostaglandins and tromboxanes. As leukotrienes, prostaglandins play a central role in ing, wound healing and immune responses.

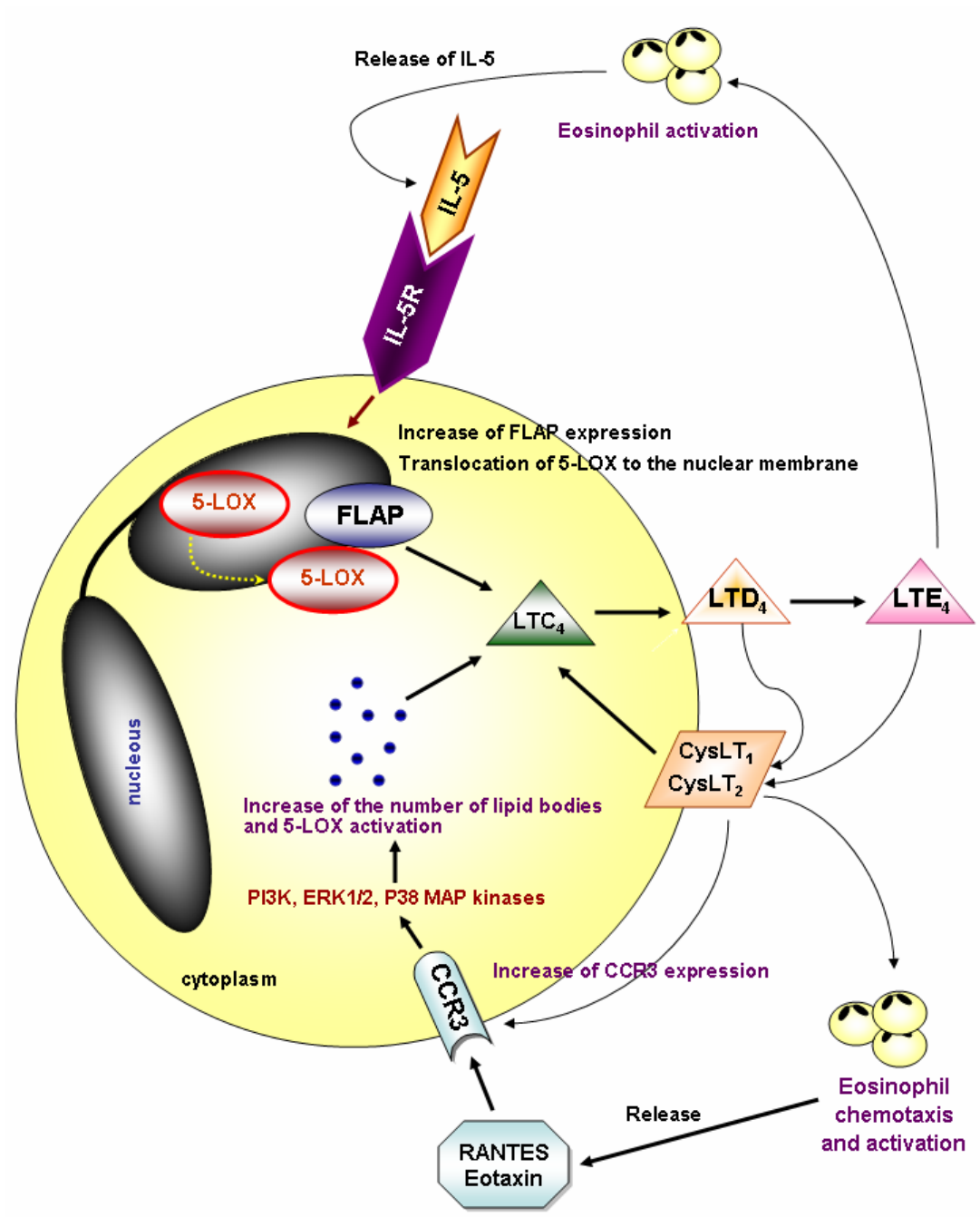

Figure 7. Autocrine and paracrine effects of leukotrienes on eosinophil activation and recruitment.

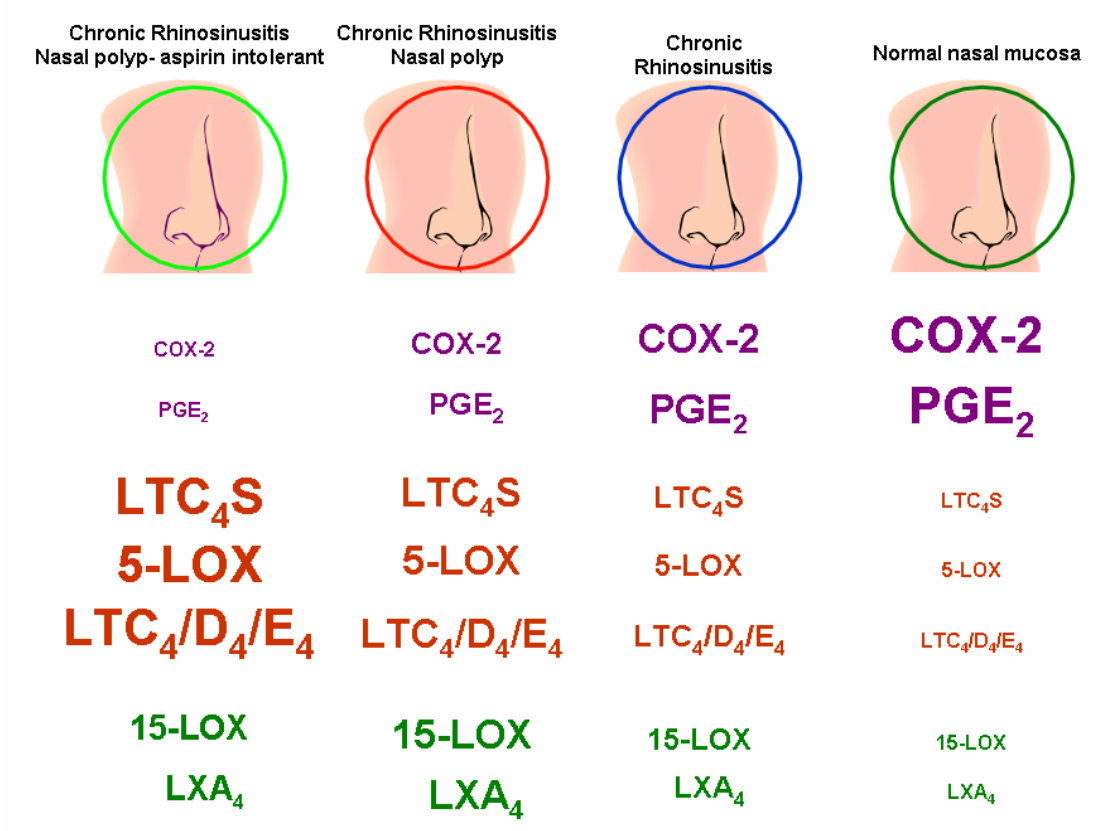

Figure 8. Profile of the arachidonic acid metabolism in patients with chronic rhinosinusitis, chronic rhinosinusitis/ nasal polyp and chronic rhinosinusitis/ nasal polyp- aspirin intolerance.

The role of these eicosanoids in airway diseases have been mainly described in rhinosinusitis- aspirin intolerant patients. It has been demonstrated that in this group of patients, blockage of COX-2 reduce asthmatics symptoms and CysLTs release contrary to COX-1 inhibition that precipitates asthmatic attacks (Szczeklik et al., 2001). Additionally, it has been found that COX-2 expression is down- regulated in nasal mucosa of aspirin-intolerant patients (Picado et al. 1999; Szczeklik et al., 1999) and this is associated with a decrease and activation of the NF-κβ transcription factor, which modulates activation of several genes involved in inflammation including that of COX-2 (Picado et al. 1999).

We first showed that COX-2 mRNA and PGE_2 concentrations were significantly decreased in chronic rhinosinusitis/ nasal polyp tissue and even more in patients with aspirin intolerance. Of interest, PGE_2 concentrations were also significantly decreased in chronic rhinosinusitis/ nasal polyp patients without aspirin intolerance. These findings are in line with the ones reported by Gosepath and col. (Gosepath et al., 2004), where COX-2 was found down- regulated in epithelial cells of nasal polyp tissue compared to normal nasal mucosa. Furthermore, we found an inverse correlation of PGE_2 and eosinophilic inflammation markers

such as ECP and IL-5 in the subgroups of chronic rhinosinusitis patients; which is in line with a study performed in sensitized mice showing an increase of eosinophil infiltration into the airway wall and eosinophil number in bronchoalveolar lavage fluid in COX-2 deficient mice (Nakata et al., 2005). Taking all together, we can suggest that down- regulation of COX-2 and PGE_2 may play an important role in the maintenance of eosinophilic inflammation and hence in the high levels of CysLTs produced in chronic rhinosinusitis/ nasal polyp patients. However, the related pathomechanisms may not be exclusive for aspirin intolerance.

In addition, evaluation of the expression of EP receptors, showed an increase in EP_2 and EP_4 receptors mRNA in chronic rhinosinusitis compared to control subjects whereas EP_1 and EP_3 were dramatically decreased in the nasal polyp patients. Until now, this is the first study reporting the gene regulation of these receptors in upper airway tissue. It has been demonstrated that EP_2, EP4, and one isoform of the EP_3 receptor can increase intracellular cAMP, which may be associated with inhibition of inflammatory cell functions (Tilley et al., 2001). On the other hand EP_1, as well the EP_3 variant can also increase intracellular calcium levels inducing activation of immune cells (Tilley et al., 2001). Down- regulation of PGE_2 production may increase the expression of EP_2 and EP_4 receptors (Mita et al., 2002). Prolongation of eosinophil survival by PGE_2 seems to be mediated by EP_4 receptors (Peacock et al., 1999), which are highly expressed together with EP_2 in eosinophils and inflammatory cells from asthmatic patients leading to the production of pro-inflammatory cytokines (Ying et al., 2004). The high levels of EP_2 and EP_4 receptors in the chronic rhinosinusitis and nasal polyp groups, together with the increase in eosinophil inflammation compared to healthy subjects support these previous results. However, remains unclear why the expression of these receptors is similar between the two chronic rhinosinusitis groups when the degree of eosinophilic inflammation is clearly higher in the nasal polyp group. Additionally, action of PGE_2 on EP_1/EP_3 receptors showing induction of airway and pronounced allergic inflammation in asthmatics mice lacking EP_3 receptor has been reported (Tilley et al., 2003). These findings together with the down-regulation of EP_1 and EP_3 receptors and the low concentrations of PGE_2 observed in chronic rhinosinusitis and even more in nasal polyp patients suggest that PGE_2- EP_3 interaction may play an important role in regulating allergic and inflammatory mechanisms in upper airways inflammatory diseases.

Furthermore, eicosanoids produced by 12/ 15-LOX enzymes, the lipoxins, have been implicated in several airway inflammatory conditions (McMahon et al., 2004). However, knowledge on the expression and role of 15-LOX and lipoxins in upper airways diseases is quite limited and controversial. In general, lipoxins (LXA_4 and LXB_4), are associated with anti- inflammatory effects (Kantarci et al., 2003). However, there are *in vitro* experiments showing that LXA_4 is able to induce a dose-dependent increase of the hyper- adhesiveness of human umbilical vein endothelial cells, leading to an increase in the binding of neutrophils to these cells (Bratt et al., 1995). In addition, it has been observed that patients with localized aggressive periodontitis produce more LXA_4 compared to healthy subjects, suggesting the possible link of this molecule with the

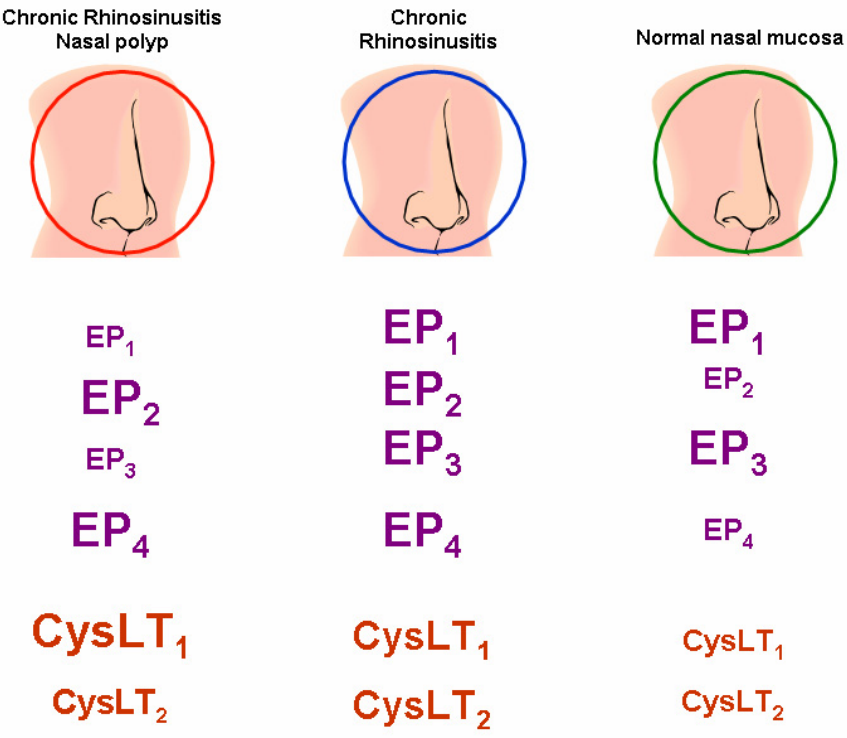

Figure 9. Profile eicosanoid receptors in patients with chronic rhinosinusitis and chronic rhinosinusitis/ nasal polyposis

inflammatory process in these patients (Kantarci et al., 2005). Additionally, certain 15- *HETE*s, intermediate metabolites of the lipoxygenase pathway, may have some pro-inflammatory action, specifically neutrophilic chemotaxis and severity of asthma has been associated with increased expression and activation of 15- LO enzymes, collagen deposition and eosinophil accumulation (Chu et al., 2002).

We here found an up-regulation of 15-LOX mRNA and LXA_4 levels in the chronic rhinosinusitis patients compared to control subjects and a down-regulation in aspirin intolerant patients compared to tolerants. In addition, 15-LOX enzyme was localized in the epithelium and sub- epithelium of chronic rhinosinusitis and even more in nasal polyp tissues (data not published) confirming its expression in the nasal tissue. Chopped human nasal polyps have been shown to have a high capacity of producing LXA_4 in presence of polymorphonuclear granulocytes (Edenius et al., 1990), which was confirmed in this study. The high amount of activated eosinophils infiltrating the nasal mucosa together with the strong immunohistochemical signal for 15-LOX observed in the epithelium of chronic rhinosinusitis and even more in the nasal polyp tissues, suggests that the increased levels of LXA_4 may be related to a transcellular interaction between inflammatory (eosinophils) and tissue-resident cells, such as cytokine-primed endothelial or epithelial cells.

Lipoxin synthesis by eosinophils could be via transference of LTA_4 to epithelial cells bearing 15-LOX enzyme or directly synthesis after stimulation by cytokines (e.g. IL-5) or other inflammatory mediators. However, the mechanism controlling the release of these mediators remains to be elucidated.

Finally, contribution of these molecules in the pathogenesis of chronic rhinosinusitis/ nasal polyp remains unclear. *In vitro* studies performed in bronchial epithelial cells have shown that lipoxins can increase chloride secretion, which might affect water retention (Bonnans et al., 2003). Increased local release of inflammatory mediators causing sodium absorption and chloride permeability in epithelial from polyps leading to the recruitment of inflammatory cells have been also proposed as a phenomenon involved in the development of polyps (Bernstein et al., 1994). The role of these molecules and the mechanism operating in upper airway diseases will need further investigation. However, it seems that down-regulation of this molecule only in the aspirin-intolerant group maybe an important hallmark for this syndrome. However, what is contradictory and extremely interesting is why this reduced capacity of lipoxin production in patients with aspirin intolerance harboring abundant numbers of eosinophils and other inflammatory cells. Accordingly, it may be hypothesized that lipoxins are synthesized in chronic rhinosinusitis with and without nasal polyp as a natural response to balance the high levels of leukotrienes and to maintain tissue integrity and down- regulation in aspirin intolerant patients may be due to intrinsic changes (maybe in epithelial or endothelial cells as has been suggested) in this biosynthetic pathway unrelated to eosinophilic inflammation.

Impact of superantigens on eosinophilic inflammation

Eicosanoid regulation and eosinophilic inflammation, as we stated before, constitute important mechanisms in chronic rhinosinusitis and even more in nasal polyp and aspirin intolerance. Additionally pathogenesis of these diseases has been related to a number of factors such as fungi, allergy, bacterial infection, and most recently, superantigens. In 2001, work from our group showed an association between increased levels of specific IgE to *S. aureus* enterotoxins and eosinophilic inflammation in nasal polyps, pointing to the possible implication of bacterial superantigens in the pathophysiology of upper airway diseases (Bachert et al., 2001). Based on the previous findings we decided to evaluate the implication of *S. aureus* immune response on eosinophilic inflammation in patients with chronic rhinosinusitis/ nasal polyp and the manifestation of aspirin intolerance. Furthermore, we explorated the regulation of eicosanoid metabolism in terms of leukotrienes and lipoxins production in patients with IgE to staphylococcal enterotoxins and the correlation with eosinophil activation markers. Finally, we explorated the possible role of these enterotoxins in cellular process and eicosanoid release in strucutral cells.

In our first study we found that concentrations of eosinophil-related markers such as IL-5 and ECP, as well as total IgE and IgE antibodies to SEs were significantly increased in nasal polyp patients compared to healthy subjects. In the nasal polyp tissue these eosinophilic markers strongly correlated with IgE antibodies

to SEs confirming our previous findings. Additionally, these mediators were also statistically higher in aspirin intolerant when compared to their tolerant counterparts. In these patient groups IgE to SEs was observed in 54% aspirin intolerant subjects and only 26% in the tolerant ones and these figures were un-related to the presence of allergy. Of interest, direct correlation between eosinophilic activation markers and IgE to SEs levels was only observed between patients with and without SEs immune response within the aspirin tolerant but not in the aspirin intolerant group. These results are partially supported by Suh et al., (Suh et al., 2004) who found that levels of ECP, total IgE, and specific IgE to SEs were higher in nasal polyp homogenates from aspirin intolerant compared tolerant subjects. However, on the contrary to our data these authors found a significant correlation between IgE to SEs and eosinophil activation markers in the aspirin intolerance patients.

SEs act as superantigens by stimulating accessory or target T-cells by cross-linking the variable part of the β chain of the T-cell receptor but also can bind with MHC class II molecules in antigen presenting cells. Binding to T- cells, leads to a stimulation (20- 25%) of the naive T-cell population inducing multiclonal IgE synthesis and the release of Th_1 and Th_2 cytokines (e.g. IL-1, IL-3, IL-4, IL-5, IL-8) that may regulate eosinophil activation and apoptosis processes. Moreover, enterotoxins may also modulate eosinophil surface expression of CD9, CD11a, CD16, CD40, CD44, or CD63 receptors and hence modify important eosinophil functions (Wedi et al., 2002). It is not clear yet what is the real contribution of these enterotoxins in the pathogenesis of chronic rhinosinusitis/ nasal polyposis and aspirin intolerance syndrome. It is important to emphasize that chronic inflammation is the major problem in chronic rhinosinusitis and bacterial superantigens may act as disease modifier by continuously stimulate lymphocytes to produce the cytokines necessary for the maintenance of this inflammation. The link between eosinophilic inflammation and immune response to SEs may be due to the release of certain molecules like IL-5, GM-CSF and RANTES, which greatly contribute, to eosinophil chemotaxis, activation and survival**.** Taking all together, we can hypothesize that staphylococcal superantigens may be a modulating factor of the local eosinophilic inflammation in chronic rhinosinusitis/ nasal polyp tissue. However, the contribution of this enterotoxin's immune response to the clinical manifestation of aspirin intolerance remains unclear. Our data points that SEs may be a modifier more than a causative factor being dependent on a former damage of the nasal mucosa by unrelated mechanisms as suggested before (Bachert et al., 2001).

Studies on the relationship between superantigens and eicosanoid metabolism

Once infection occurs, a cascade of inflammatory events act as an amplified loop of immune reactions until the infection is contained. These early actions are later replaced by mechanisms that are more specific and eventually become redundant. Thus, it is important for the inflammatory response to be limited and resolved. Many molecules like endogenous lipid-derived mediators (eicosanoids) play a crucial regulatory role in the maintenance or resolution stages of inflammatory response controlling its magnitude and duration.

Based in the later findings, we decided to investigate the role of these SAgs on eicosanoid release in chronic rhinosinusitis/ nasal polyp tissue and its relation with inflammatory process present in these patients. This study showed that eicosanoid production in terms of $LTC_4/D_4/E_4$, LTB_4 and LXA_4 is up- regulated in nasal polyp tissue of patients with IgE-antibodies to *S. aureus* enterotoxins. Additionally, the levels of these mediators correlated to eosinophilic inflammation markers and specific IgE to SEs and were unrelated to asthma and allergy, which are clinical factors related to exacerbated eicosanoid release.

So far, there is no evidence that SEs can directly influence the leukotriene regulatory pathway; however these enterotoxins can induce the synthesis of IL-5 which activates cytosolic phospholipase A_2, resulting in the translocation and activation of 5-LOX enzyme, and hence in the induction of cysteinyl leukotriene synthesis (Cowburn et al., 1999). On the other hand, LTD_4 which is secreted extracellularly may induce adhesion and behave as a potent chemoattractant for eosinophils which are recruited to the site of inflammation and then activated (Spada et al., 1994). Moreover, eosinophils may act as antigen presenting cells expressing the MHC-II surface molecules when activated with GM-CSF (Mawhorter et al., 1994), which can be expressed in high concentrations in the nasal polyp, resulting on cell activation and release of eosinophil granulocytes and pro- inflammatory mediators including leukotrienes.

Additionally, it is important to emphasize that although eosinophils constitute an important cell type in eicosanoid release in upper airway diseases, activated macrophages may also greatly contribute to this pathway (Wehner et al., 2001). The role of these cells during mucosal inflammation involves events like pathogen clearance, antigen presentation, lymphocyte activation and its ability to produce cytokines and lipid mediators such as leukotrienes. Parallel studies from our group have shown an infiltration of activated macrophages in the nasal polyp tissue with *S. aureus* immune response. These cells expressing phagocytotic receptors such as mannose receptor have the ability of recognize microorganisms and subsequently activate secretion of inflammatory products that may include lipoxygenase derivate molecules.

Another interesting observation of our study was that number of activated neutrophils did not differ between patients with and without SEs immune response. Activated neutrophils are important sources of LTB_4 and LXA_4 which concentrations were up- regulated in patients with IgE antibodies to SEs. *S. aureus* superantigens have been suggested to induce neutrophil migration and activation with subsequent release of LTB_4. In addition, LXA_4 have been shown to be secreted after bacterial infection and act as endogenous leukotriene antagonists and inhibitors of bacterial induced peripheral mononuclear cells recruitment in inflammation. The lack of correlation between LTB_4 and activated neutrophils points to the existence of another source for the production of this eicosanoid such as eosinophils and macrophages, also bearing the enzymes necessary for its synthesis. On the other hand, the high levels of LXA_4 may account not only from eosinophils but also from transcellular events between these cells containing LTA_4 and endothelial cells synthesizing 15-LOX. LXA_4 inhibits LTB_4-induced neutrophil chemotaxis and migration of these cells across the endothelium, which can in part explain the similar numbers of neutrophils in the nasal tissues.

Accordingly, endothelial cells can express high levels of LXA_4 receptor and may act as paracrine and autocrine factors regulating the expression of this eicosanoid in cell epithelium and circulating eosinophils and macrophages. However, the role of these molecules in the nasal polyp tissue of SEs positive patients is still a question mark; are they synthesized as natural counter- regulatory molecules or have some pro-inflammatory actions as has been suggested in periodontal diseases (Kantarci et al., 2005).

Importantly, we cannot exclude that contribution of allergy and asthma conditions on eicosanoid levels and this issue was addressed in this study. Allergen inhalation in asthma induces the activation of mast cells and macrophages. Antigen processing and presentation by macrophages to undifferentiated T- helper cells may induce differentiation to the Th_2 phenotype. This process results in the release of IL-4 and IL-5, causing IgE synthesis and eosinophil infiltration, respectively; which end in the release of pro- inflammatory mediators, including leukotrienes. Furthermore, activation of human lung mast cells with IgE has been shown to increase cytosolic free Ca^{2+}. This results in the activation of Ca^{2+} dependent phospholipase A_2 and the production of LTC_4 provoking inflammatory cell influx into the airways and bronchial smooth muscle contraction (Cruse et al., 2005). Of interest, we did not observe any differences in leukotriene levels between SEs positive or negative subjects with and without asthma or allergic and non- allergic. These findings suggest that regulation of leukotriene production is a general phenomenon parallel to inflammation and may not be directly link to asthma or allergy conditions. However, surprisingly, LXA_4 was significantly increased in asthmatics patients versus non- asthmatics with the highest levels in patients with IgE antibodies to SEs. Lipoxins have been identified in the bronchoalveolar fluid of patients with lung disease and found in decreased concentrations in patients with severe compared to subjects with mild asthma (Levy et al., 2005). However, in contrast to these previous results and partially in line with ours, Chavis et al., (2000) found that levels of lipoxins are the same in PBM from untreated and steroid dependent asthmatic patients, and that they are not synthesized in control subjects. Accordingly, these authors indicate that lipoxins can be produced at inflammation sites by transcellular metabolism associated to the inflammatory process operating in these patients. This made us suggest that lipoxins could be of interest as a biomarker to evaluate severity and the pathomechanisms not only in upper airway but also in lower airway diseases such as asthma.

To confirm our findings in tissue, we decided to study the influence of SEs (SEB) in the eicosanoid pathway in nasal tissue fibroblasts due to their clear implication in the remodelling process in both upper and lower airways. Fibroblasts are the major source of extracellular connective tissue matrix, and the recruitment, accumulation, and stimulation of these cells are thought to play important roles in both normal healing and the development of fibrosis in airway remodelling. In the airways, PGE_2 is mainly produced by smooth muscle cells, fibroblasts, and epithelial cells. In this study we demonstrated that nasal fibroblasts express high constitutive levels of PGE_2 and COX-2 mRNA in culture.

Additionally, SEB failed to induce changes in COX-2 and PGE_2 production when cells were cultured in absence of IFN-γ. Of interest, a significantly decrease in the levels of endogenous PGE_2 and mRNA of COX-2 was observed after stimulation with SEB in IFN-γ pre-treated cells. It is important to remark that in this study; cells were pre- incubated with IFN-γ to induce the MHC-II molecules, (which are normally not expressed in fibroblasts) and mimic the classical binding of superantigens to the cell. However, we cannot exclude the action of this cytokine on these cells. IFN-γ is present in elevated levels in upper airway inflammatory diseases and may induce the activation of lymphocytes and eosinophils within the tissue (Dellacono et al., 1997). This cytokine has been demonstrated to attenuate IL-1β induced PGE_2 production in orbital fibroblasts (Han et al., 2005) and induce the synthesis and activation of $cPLA_2$ protein in a human bronchial epithelial cell line (Wu et al., 1994). In line with these findings, our results show that IFN-γ induces COX-2 mRNA expression and PGE_2 release in nasal fibroblasts. Additionally, staphylococcal enterotoxin A has been demonstrated to induce PGE_2 production, COX-2 and cytosolic phospholipase A_2 ($cPLA_2$) expression in IFN-γ treated fibroblast-like synoviocytes (Mehindate et al., 1995), however in nasal fibroblasts, SEB acted as an antagonist of this cytokine. Although there are no studies performed in nasal fibroblasts showing the effect of superantigens in PGE_2 release, parallel results from our group have shown the presence of *S. aureus* and enterotoxins in epithelium of nasal polyps. It is known that interaction between fibroblasts and epithelial cells play a crucial role in the balance of eicosanoid levels via transcellular mechanisms. We here suggest that presence of SEB in chronic rhinosinusitis/ nasal polyposis may contribute to the exacerbation of inflammation in this disease by down- regulating fibroblasts PGE_2 production.

Moreover, staphylococcal superantigens have been shown to induce leukocyte infiltration that is dependent on the expression of ICAM-1 and TNF-α in mouse air pouches (Tessier et al., 1998). Additionally, stimulation of human alveolar macrophages with enterotoxin A (SEA) induced the synthesis and secretion of IL-8, which is a major neutrophil chemotaxin (Miller et al., 1996). Of interest, enterotoxin B only induced migration in presence of IFN-γ and it seems that this cytokine somehow attenuate the effect of the enterotoxin. These controversial results can only suggest that SEB may act as a chemoattractant for fibroblasts however, this effect seems to be mediated by the binding of MHC-II. IFN-γ has been reported to increase the expression of both intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in human lung fibroblasts (Spoelstra et al. 1999), which are molecules regulating the adhesion and migration processes. Although it was not the objective of this work, it will be interesting to evaluate the expression of these molecules in our cell model to add more data about the possible mechanisms modulating the migration capacity of these cells. Finally, PGE_2 has been reported to be involved in the regulation of the migration capacity of airway fibroblasts and hence modulate the wound healing process in the airways. Specifically, PGE_2 is a potent inhibitor of fibroblast proliferation and collagen synthesis (Saltzman et al., 1982) and appears to inhibit fibroblast chemotaxis by modulating the rate of fibroblast migration (Kohyama et al., 2001.) We here showed that SEB blocks cell proliferation probably by entering

the cell by altering membrane cell permeabilization. However, its effect on rate migration was IFN-γ dependent but did not correlate with the release of PGE_2. This suggests that SEB may influence these processes by acting on different regulatory pathways as the ones involving this eicosanoid.

In conclusion, we suggest that staphylococcal superantigens may influence significantly the pathogenesis of upper airway inflammatory diseases by not only modulating eosinophil activation but also by influencing fibroblast metabolism. The molecular mechanisms behind the action of these compounds remain unclear and futher studies are mandatory. The understanding of the regulatory mechanisms of important pathways like eicosanoid synthesis and cell migration by enterotoxins will surely be a pivotal element in our understanding of the role played by *Staphylococcus aureus* superantigens in upper airway diseases.

Clinical relevance of the study

Conventional medical treatment of chronic rhinosinusitis and nasal polyps consists of systemic glucocorticoids (decreasing doses), eventually associated with antibiotics, and then long-term use of intranasal steroids. The clinical efficacy of glucocorticoids depends mainly on their ability to reduce airway eosinophil infiltration and the concomitant massive plasma exsudation. Both topical and systemic glucocorticoids may affect the eosinophil function by reducing eosinophil viability and activation. Furthermore, they have been shown to reduce the secretion and release of chemotactic cytokines by nasal mucosa and polyp epithelial cells. However, a long-term use of steroids can induce severe side events including osteoporosis, growth retardation in children and thinning skin. Thus, in patients where corticosteroid treatment is contraindicated or induces side-effects, sinus surgery remains the only alternative. However, after surgery, some patients have been reported to have recurrency of polyp formation. This rate of recurrence is not only depending of technical skill of the surgeon, but also seems to be dependent of the type of nasal polyposis. Patients suffering from aspirin intolerance syndrome are more prone to have recurrences after sinus surgery. Our studies clearly demonstrated that patients suffering from nasal polyps with aspirin intolerance present different pathogenesis pathways and, subsequently, may differ in their clinical course. These evidences fully justify the urgent need for new alternatives in the management of chronic rhinosinusitis/ nasal polyposis and constitute a priority issue for research works related to these pathologies. The use of several 5-lypoxygenase inhitors and leukotriene receptor antagonists in the daily management of asthma and aspirin intolerance illustrates this trend. However, their use in chronic rhinosinusitis and nasal polyps is up till now limited.

Our work will surely open new perspectives for alternative therapies in these diseases. These therapies may not only include corticosteroids and antibiotics but could also find a place for lipoxin analogs and lipoxygenases and leukotriene antagonists, which are known to have a modulating role in eosinophil and eicosanoid-derived inflammation.

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FUTURE PERSPECTIVES

Based on the results discussed previously we intend to extend this work in the following directions:

Regulation of the 15-LOX enzyme and its metabolites 15-HETE and LXA_4 in upper airway diseases

LXA_4 has been considered to play a crucial role in resolving inflammation and stopping recruitment signals for neutrophils in the airways; however, the role of these metabolites in the regulation of inflammatory mechanisms in the airways is not yet fully understood. 15-LOX, the enzyme responsible for the synthesis of lipoxins exist in two isoforms, the 15-LOXa and the 15-LOXb. 15-LOX was shown in this work to be up-regulated in the diseases groups compared to controls, but down- regulated in the aspirin intolerant patients. Recent studies have shown the existence of three additional splice variants for 15-LOXb gene, which maybe have different regulatory (pro-) inflammatory functions (Bhatia et al. 2003). Based on the later we will first develop a quantitative real time PCR protocol (in collaboration with Prof. Marek Kowalski from the Medical University of Lodz, Poland) together with a methodology for the detection of single nucleotides polymorphisms to study the expression of these splice variants in the nasal tissue. Furthermore, the cellular expression of these enzymes on inflammatory cells in the nasal tissue will be studied by double confocal microscopy staining. This study will be performed in chronic rhinosinusitis with and without polyps and in cystic fibrosis patients, which have been reported to have a metabolic defect in this pathway. Furthermore, special attention will be focused in aspirin intolerant patients.

Role of prostanoid receptors on inflammatory upper airway diseases

We have demonstrated that prostaglandin E receptors mRNA is differentially regulated in chronic rhinosinusitis and nasal polyps compared to healthy nasal tissue. To extend this study we will evaluate the protein expression of these receptors by using western- blotting and double staining to identify their cellular localization. Furthermore, after the cellular source is identified, *in vitro* functional studies will be performed in cells isolated from peripheral blood and tissue of patients with chronic rhinosinusitis/ nasal polyposis. In this experiment, cells will be stimulated with different cytokines and pro- inflammatory mediators in presence of specific receptor antagonists to study the release of inflammatory molecules and the regulation of other eicosanoids like CysLTs.

Mechanism of aspirin desensitization and the implication of AA cascade changes

Aspirin desensitization is one of the most effective methods for patients with aspirin induced asthma with symptoms of chronic rhinosinusitis and recurrent nasal polyps. The regulatory mechanisms and eicosanoid metabolism during aspirin desensitization, has been a subject of very few studies to date. The implication of aspirin desensitization on the regulation of arachidonic acid cascade will be assayed in a double- blind study including chronic rhinosinusitis patients with and without aspirin intolerance. Here we will evaluate the

regulation of key enzymes and bioactive metabolites from the cyclooxygenase and lipoxygenase pathways including PGs, cysLTs and lipoxins. In addition, mRNA of eicosanoid receptor will be evaluated together with the incidence of polymorphisms in COX- 2 and LTC_4S in these patients. This data will be furtherly related to clinical parameters on the lower and the upper airways. This study will be performed in collaboration with Prof. Andrew Szczeklik, from the Medical University of Krakow, Poland.

Superantigens, eosinophilic inflammation and arachidonic acid pathway in upper airway diseases

We have provide some evidences that SEs may be involved in the regulation of eicosanoid production in tissue and structural cells from patients with chronic rhinosinusitis/ nasal polyposis. Although the study of the splicit mechanisms was not the objective of this work, we have now a very wide view of the possible role that these enterotoxins may play in the pathogenesis of airway diseases. To further go on with this project we are currently studying the effect of SEB in the regulation of leukotriene and lipoxin pathways in peripheral blood from aspirin intolerant and tolerant patients (in collaboration with Prof. Marek Kowalski, Medical University of Lodz, Poland).

In addition, our pre-liminar study on nasal fibroblasts will be extended to define the mechanisms of SEB action on these cells. For that, we will first perform stimulation experiments at different time points to study the time dependent action of the enterotoxin. Subsequently we will perform inhibition studies with antibodies against MHC-II to verify the participation of this molecule in the binding of the enterotoxin to the cell. Parallel to this work we will evaluate the influence of the staphylococcal enterotoxin in the regulation of other important mediators involved in fibroblasts metabolism like fibroblasts growth factors, adhesion molecules (VCAM-I, ICAM-I) and cytokines. Finally, we will evaluate the implication of SEB on the NF-κβ pathway. The nuclear factor kappa beta (NF-κB) is a transcription factor involved in the regulation of crucial mechanisms related to cytokine and lipid mediators' release during inflammation. In this work, we will first study the mRNA expression of IκBα and the IKKβ molecules, which are involved in the regulation of COX-2. IκBα is the endogenous inhibitor of NF-κB and IKKβ molecule is involved in the phosphorilation of IκBα, and their balance is responsible for the activation and deactivation of the NF-κB activity. Furthermore, we will correlate the mRNA findings with activity assays and the release of PGE_2 by the stimulated cells.

CURRICULUM VITAE

Curriculum vitae

PERSONAL

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EDUCATION

University graduated degree

1988- 1993: Bachelor in Pharmaceutical Sciences, Havana University, Cuba

Post- graduated degrees

1996- 1998: Master in Biotechnology, Havana University, Cuba

1998- 2000: Master in Molecular Biology, Vrije Universiteit van Brussel, Belgium.

Working experience

1993- 1998: Senior researcher in the Quality Control Department of the Center for Genetic Engineering and Biotechnology, Havana City, Cuba.

2000- 2001: Pre- doctoral training in the Department of Othorhinolaryngology, Ghent University Hospital, Belgium.

2001- 2003: Doctoral training in the Department of Othorhinolaryngology. Grant from the Faculty of Medicine (2000- 2003), Ghent University Hospital, Belgium.

2003- 2005: Doctoral in the Department of Othorhinolaryngology, Ghent University Hospital, Belgium. BOF Grant from the University of Ghent (2003-2005).

Congresses, workshops and meetings (Posters and oral presentations)

- Biotechnology Congress Havana' 94. December 1994. Havana City, Cuba *(Poster)*.

- X Central American of Microbiology and X National Congress of Microbiology, Parasithology, and Clinical Pathology. November 1995. San José Costa Rica *(Poster)*.

- International Symposia of Chemistry: "LABOR 96". November 1996, Santiago de Cuba. Cuba *(oral presentation)*.

- X National Forum. December 1996, Havana City, Cuba *(Poster)*.

- Biotechnology Congress Havana' 97. December 1996, Havana City. Cuba *(Poster)*.

- 9th Annual Symposium of the European Charcot- Marie-Tooth Consortium, June 30- July 1, 2000, Antwerp, Belgium.

- XXIst Congress of the European Academy of Allergology and Clinical Immunology. June 1-5, 2002, Naples, Italy *(Poster)*.

- Keystone Symposia: Eicosanoid Lipid Mediators: from Molecular Discovery to Clinical Application", March 11-16, 2003, California, USA *(Poster)*.

- EAACI- Section E.N.T. Meeting. 5th International Symposium on Experimental Rhinology and Immunology of the Nose. November 15- 19, 2003, Ghent, Belgium *(oral presentation)*.

- XXIIIst Congress of the European Academy of Allergology and Clinical Immunology. June 12- 16, 2004, Amsterdam, Netherlands *(Poster)*.

- Wetenschappelijk Imfodag, january 22, 2004, Ghent University Hospital, Ghent, Belgium *(Poster)*.

- Wetenschappelijk Imfodag, February 20, 2005, Ghent University Hospital, Ghent, Belgium *(Poster)*.

- Wetenschapsdag Heelkunde UZ Gent, Juny 24, 2005, Het Pand, Ghent, Belgium *(Poster)*.

- University Forum, University Ghent- UCB Institute of Allergy. September 29- 30, 2005, Ghent University Hospital, Belgium *(oral presentation)*.

- Superantigens in airway disease. Immunology and clinics of superantigen-driven inflammation. November 25- 26, 2005, Ghent, Belgium *(oral presentation)*.

- 6th Symposium on Experimental Rhinology and Immunology of the Nose (SERIN), February 9- 11, 2006, Barcelona, Spain.

International courses and seminars

- XIth EuroCellPath Course. Molecular Mechanisms in Chronic Inflammatory Disease. June 13-18, 2001. Ghent, Belgium.

- Quantitative PCR seminar (Bio-Rad), March 14, 2002 Affligem, Belgium.

- Interuniversitary PhD training program: Identification of genetic defects in cancer. Ghent Universiy Hospital, February 28, 2003, Ghent, Belgium.

- Quantitative PCR seminar (Bio-Rad), March, 2003, Ghent, Belgium.

- Minisymposium and workshop: Large scale gene expression analysis using DNA microarrays. Turku Centre for Biotechnology, May 10- 15, 2004, Turku, Finland.

- Training course: Practical Microarray Software. Westburg Co., May 3- 4, 2004, Leusden, Netherlands

- Quantitative PCR and Protein Expression Seminar (BioRad), Leuven, 2005.

Professional Awards

- Institutional Award from the Center for Genetic Engineering and Biotechnology, 1996 and Special Award from the Academy of Sciences of Cuba 1997 Havana City, Cuba: "Establishment of a technology production of a recombinant protein TAB-9, the first vaccine candidate against VIH-1".

- Institutional Award from the Center for Genetic Engineering and Biotechnology, 1996, Havana City, Cuba: "Establishment of a system to detect host DNA (E. coli W 3110 trpA 905) in recombinant protein TAB-9".

- Institutional Award from the Center for Genetic Engineering and Biotechnology, 1996, Havana City, Cuba: Establishment of a technology production for recombinant protein P64K, for clinical assays.

- Special award for the best scientific and economic result of the year, Academy of Sciences of Cuba (CITMA). December 1997. Cuba: "Development of new fermentation, immunoidentification and ionic interchange methods to raise the production capacity of surface antigen Hepatitis B for the production of the HeberBiovac HB vaccine".

- First ENT poster prize, Junior Member Poster section, XXIIIst Congress of the European Academy of Allergology and Clinical Immunology. June 12- 16, 2004, Amsterdam, Netherlands.

Travel grants

- Travel & Extended Scholarship Award (1000 euros) by the UCB Institute of Allergy, Brussels, Belgium.

- Travel grant for the 6th Symposium on Experimental Rhinology and Immunology of the Nose (SERIN), February 2006, Barcelona, Spain.

Work as project reviewer

- Biomedical Research Council & National Medical Research council Joint Grant, Singapore, June 2004. Title: Role of Staphylococcus aureus superantigens (enterotoxin) in the pathogenesis of chronic rhinosinusitis and nasal polyposis.

- Competitive Programme Grant (CPG) from the National Medical Research council, Singapore, April 2005. Title: Investigating the genomics of new strains of virulent community-acquired *Staphylococcus aureus* isolated in Singapore.

PUBLICATIONS

1. Gevaert P, Bachert C, Holtappels G, **Novo CP**, Van der Heyden J, Fransen L, Depraetere S, Walter H, van Cauwenberge P, Tavernier J. Enhanced soluble interleukin-5 receptor alpha expression in nasal polyposis. *Allergy* 2003; 58: 371- 379.

2. **Pérez C**, Vandesompele J, Vandenbroucke I, Holtappels G, Speleman F, Gevaert P, Van Cauwenberge P, Bachert C. Quantitative Real Time Polymerase Chain Reaction for measurement of human Interleukin - 5 receptor alpha spliced isoforms mRNA. *BMC Biotechnology* 2003; 3:17- 26.

3. Watelet JB, Claeys C, **Pérez-Novo C,** Gevaert P, Van Cauwenberge P, Bachert C. Transforming growth factor beta1 in nasal remodeling: differences between chronic rhinosinusitis and nasal polyposis. *American Journal of Rhinology* 2004; 18: 267- 72.

4. **Pérez-Novo CA**, Kowalski ML, Kuna P, Ptasinska A, Holtappels G, van Cauwenberge P, Gevaert P, Johannson S, Bachert C. Aspirin sensitivity and IgE antibodies to Staphylococcus aureus enterotoxins in nasal polyposis: studies on the relationship. *International Archives of Allergy and Immunology* 2004; 133: 255- 60.

5. **Pérez- Novo CA**, Claeys C, Speleman F, Van Cauwenberge P, Bachert C, Vandesompele J. Impact of RNA quality on reference gene expression stability. *Biotechniques* 2005; 39: 52- 56.

6. **Pérez- Novo CA,** Watelet JB, Claeys C, Van Cauwenberge P, Bachert C. Prostaglandin, Leukotriene and Lipoxin balance in Chronic Rhinosinusitis with and without Nasal Polyposis. *Journal of Allergy and Clinical Immunology* 2005; 115: 1189- 1196.

7. Zhang N, Gevaert P, Van Zele T, **Pérez- Novo C**, Patou J, Holtappels G, Van Cauwenberge P, Bachert C. An update on the impact of staphylococcus aureus enterotoxins in chronic rhinosinusitis with nasal polyposis. *Rhinology* 2005; 43: 162- 168.

8. **Pérez- Novo CA,** Claeys C, Van Zele T, Van Cauwenberge P, Bachert C. Eicosanoid metabolism and eosinophilic inflammation in nasal polyp patients with immune response to *Staphylococcus aureus* enterotoxins. *American Journal of Rhinology*, 2005.

COLLABORATION PROJECTS

- Bilateral Scientific Cooperation Project between Flanders and priority countries, BOF (50000 euros). Collaboration with the Medical University of Lodz, Poland (2004- 2006).

- Scientific Project with Merck Sharp & Dohme (MSD) Company, Germany (47000 euros, 2003- 2005)

MEMBERSHIP OF SCIENTIFIC SOCIETIES

- Member of the European Academy of Allergology and Clinical Immunology, Sweden (2001- 2006)

- Member of the Belgian Society of Molecular Biology, Belgium (2002- 2006)

- Member of the Farmaceutical Society, Cuba (1998- 2006)