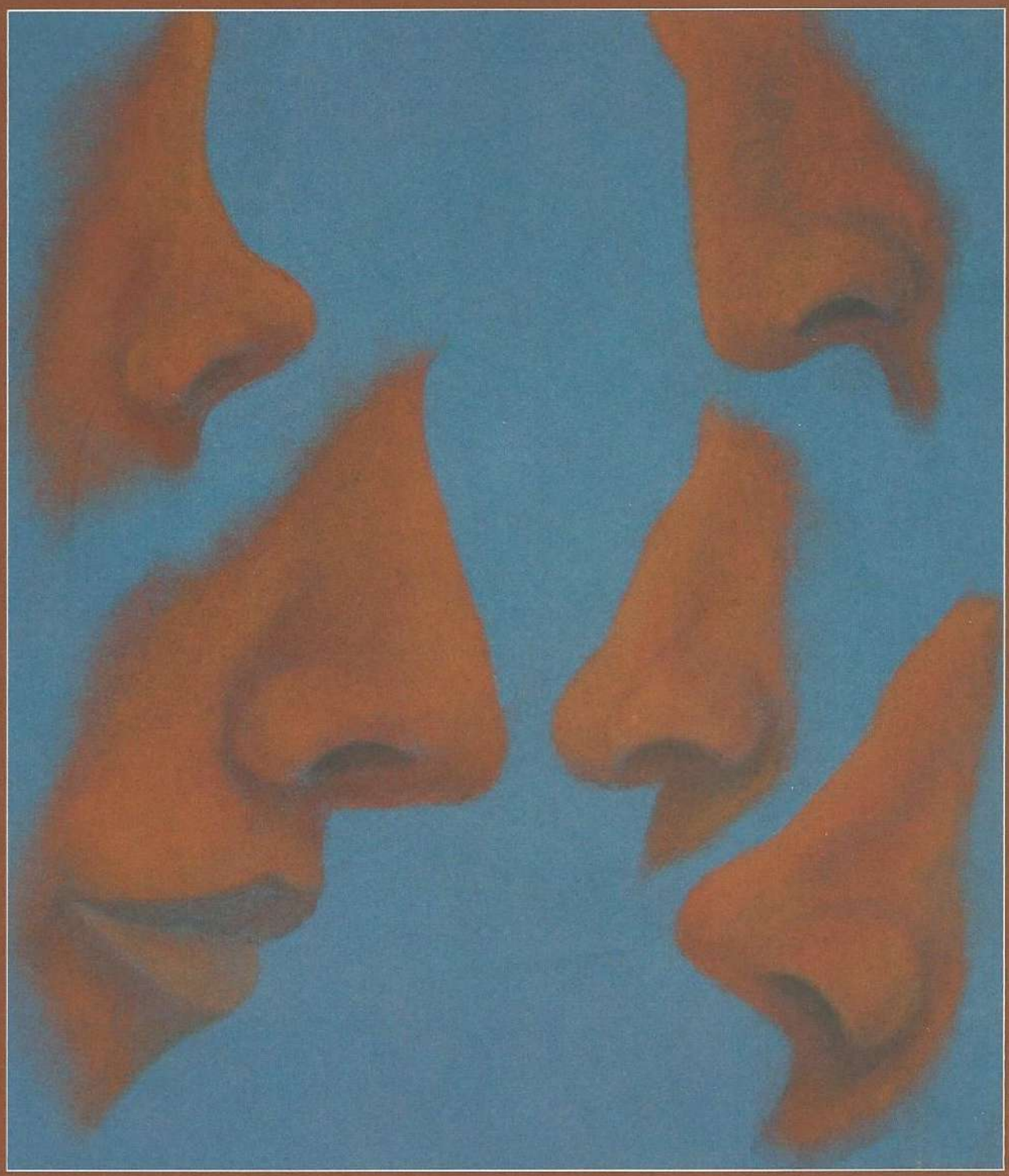
CONTROVERSIES IN CHRONIC RHINOSINUSITIS



Fenna Afien Ebbens

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Promotor:	Prof. dr. W.J. Fokkens
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Faculteit der Geneeskunde

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

INTRODUCTION

Chronic rhinosinusitis (CRS) with or without nasal polyposis is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by the presence of distinctive symptoms (e.g. nasal blockage, nasal discharge, facial pain and/or reduced sense of smell) and either endoscopic signs or computed tomography (CT)-changes characteristic of the disease[1;2]. CRS has a negative impact on quality of life and can substantially impair daily functioning. It contributes to a significant amount of health care expenditure as a result of direct costs arising from physician visits and medical therapies, as well as indirect costs related to loss of productivity and absence from work[3;4].

NORMAL PARANASAL SINUS ANATOMY AND (PATHO)PHYSIOLOGY

The paranasal sinuses constitute a collection of air filled spaces within the anterior skull and are named after the skull bones in which they are located (frontal, ethmoid, (anterior and posterior), maxillary, and sphenoid sinuses). All sinuses contain air and are lined by a thin layer of respiratory mucosa composed of ciliated, pseudostratified, columnar epithelial cells with goblet mucous cells interspersed among the columnar cells. All sinuses communicate with the nasal cavity through small apertures[5]. Despite the presence of accessory ostia, the ciliated mucosa of the maxillary and frontal sinuses transport mucus in specific patterns towards their natural ostia[6]. No precise description is available as to the pattern of mucociliary transport within the ethmoid and sphenoid sinuses.

The function of the paranasal sinuses is controversial. Theories include providing resonance for speech, providing a supply of conditioned air to diffuse with inhaled air, assist with olfaction, provide protection to the skull, secrete mucus to keep the nose moist, reduce weight of the skull and to provide thermal insulation to the brain[7]. However, none of these theories is supported by objective evidence.

Like the paranasal sinuses, the entire nasal cavity is lined by a thin layer of respiratory mucosa composed of ciliated, pseudostratified, columnar epithelial cells with goblet mucous cells interspersed among the columnar cells[5]. A deep layer of

mucus covers the entire nasal cavity. This mucus is slightly acidic and consists of two layers: a thin, low viscosity, periciliary layer (sol phase) that envelops the cilia of the columnar cells, and a thick, more viscous, layer (gel phase) riding on the periciliary layer[5]. The distal tips of the ciliary shafts contact the gel layer and move particulate matter and inhaled bacteria that are caught in this gel layer towards the nasopharynx.

A fundamental role in the pathogenesis of CRS is played by the ostiomeatal complex, a functional unit comprising the maxillary sinus ostium, the anterior ethmoid cells and their ostia, the ethmoid infundibulum, the hiatus semilunaris and the middle meatus. Crucial in normal sinus function is the maintenance of ostial patency since ostial patency significantly affects mucus composition and mucus secretion. An open ostium allows mucociliary clearance to easily remove particulate matter and bacteria from healthy sinuses[8]. Problems occur if the orifice is too small for the amount of mucus, if mucus production is increased, or if ciliary function is impaired. Stasis of secretions follows and bacterial export ceases, causing exacerbating inflammation, whilst aeration is decreased, causing even more ciliary dysfunction. This vicious cycle can be difficult to break, and, if the condition persists, can result in the development of CRS[1].

DEFINITION AND CLASSIFICATION OF CHRONIC RHINOSINUSITIS WITH AND WITHOUT NASAL POLYPOSIS

So far, attempts to classify chronic inflammatory diseases of the nose and paranasal sinuses based on clinical signs and symptoms have resulted in the term "chronic rhinosinusitis" and could therefore cover a spectrum of disease entities with potential different underlying pathophysiological mechanisms. It is clear that the overlapping pattern of symptoms (e.g. nasal blockage, nasal discharge, facial pain and/or reduced sense of smell) of CRS and nasal polyposis hampers an accurate clinical distinction.

The diagnosis of CRS is made by a wide variety of practitioners, including allergologists, otolaryngologists, pulmonologists, primary care physicians and many others. Therefore, an accurate, efficient, and accessible definition of CRS is required. Various authors have published reports on CRS and its definition[1;2;9;10].

The most recent report, the European position paper on rhinosinusitis and nasal polyps (EP^3OS) offers two definitions: one to be used clinically and one, more detailed definition, for research purposes[1;2].

For clinical purposes, the definition of CRS includes nasal polyposis. It is defined as an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by the presence of two or more symptoms characteristic of CRS (i.e. nasal blockage, nasal discharge, facial pain and/or reduced sense of smell) one of which should be either nasal blockage or nasal discharge (anterior and/or posterior) and either endoscopic signs (i.e. nasal polyps, mucopurulent discharge primarily from the middle meatus and/or edema primarily in the middle meatus) or CT-changes characteristic of the disease (i.e. mucosal changes within the ostiomeatal complex and/or sinuses) [1;2].

For research purposes, although clinically CRS is defined as above, CRS without nasal polyposis and CRS with nasal polyposis are considered distinct disease entities. A differentiation between CRS without nasal polyposis and CRS with nasal polyposis should be made based on out-patient nasal endoscopy. Those patients with bilateral nasal polyps in the middle meatus are considered to have CRS with nasal polyposis, those patients without bilateral nasal polyps in the middle meatus are considered to have CRS without nasal polyposis[1;2].

EPIDEMIOLOGY

Rhinosinusitis in its many forms constitutes one of the most common conditions encountered in medicine. However, to give an accurate estimate of the prevalence of CRS with or without nasal polyposis remains speculative since clear disease definitions are lacking in most publications. A US based survey estimated that as much as 13.5% of the total population is affected by CRS without nasal polyposis if CRS was defined as having sinus complaints for more than 3 months in the year before the interview, ranking CRS second in prevalence among all chronic conditions[11]. Although similar results have been observed in a second US based survey[12], the prevalence rate of doctor diagnosed CRS without nasal polyposis is around 2%[13], suggesting that the diagnosis of CRS without nasal polyposis is often overestimated.

To estimate the prevalence of CRS with nasal polyposis is even more difficult since nasal endoscopy is necessary to adequately identify those patients suffering from CRS with nasal polyposis from those CRS patients without nasal polyposis. As a result, few studies focusing on the prevalence of CRS with nasal polyposis have been published. In 2003, Johansson et al reported a prevalence rate of nasal polyposis of 2.7% in the general population based on data obtained from a survey in Skövde, Sweden[14]. In this study, the diagnosis of CRS with nasal polyposis was established via nasal endoscopy. In a second study from Scandinavia, in which the presence of nasal polyposis was established via a questionnaire asking whether polyps had ever been identified in an individual's nose, a prevalence rate of 4.3% was reported[15]. Of the individuals diagnosed with CRS with nasal polyposis, men are twice as likely to be affected[14;16;17]. In addition, an increase in disease incidence is observed in those patients of increasing age[14;18-20]. The average age of onset of CRS with nasal polyposis is approximately 39 years of age[16]. When diagnosed under the age of 20, nasal polyps are most likely related to cystic fibrosis (CF)[21;22].

ASSOCIATED DISEASES

Asthma

Recent evidence suggests that allergic inflammation in the upper (e.g. rhinitis) and lower airways (e.g. asthma) usually co-exist with inflammation in one part of the airway influencing its counterpart at distance. The arguments and consequences of these observations are summarized in the allergic rhinitis and its impact on asthma (ARIA) guidelines[23]. Besides being associated with allergic rhinitis, asthma is also frequently associated with CRS, especially in those patients suffering from CRS with nasal polyposis[20]. The exact nature of this interrelationship is poorly understood. In patients suffering from CRS with nasal polyposis, wheezing and respiratory discomfort are present in 32% and 43% respectively and 26% of CRS patients with nasal polyposis report the presence of asthma[24]. Alternatively, 7% of asthmatic patients have nasal polyps, with a prevalence of 13% in non-atopic asthmatics and 5% in atopic asthmatics[18;25]. The presence of nasal polyps in asthma patients is often associated with an increase in asthma severity, poorer asthma control, and more frequent hospital admissions[26].

Acetyl salicylic acid (ASA) intolerance

Thirty-six to 96% of patients with ASA intolerance (intolerance to aspirin and other non-steroidal anti-inflammatory drugs) are diagnosed as having CRS with or without nasal polyposis[25;27-30] and up to 96% demonstrate radiographic changes characteristic of paranasal sinus disease[29]. Although only 4% of patients with bronchial asthma suffer from ASA intolerance[27], the prevalence of CRS with nasal polyposis in ASA intolerant asthmatics may be as high as 70%[18]. The combination of bronchial asthma, the presence of ASA intolerance and nasal polyposis is called Samter's triad[31]. This trias is often difficult to treat, with high recurrence rates of nasal polyps, frequent need for endoscopic sinus surgery (ESS), and poor asthma control[32]. Although the exact mechanism of ASA intolerance remains to be elucidated, a disorder in eicosanoid biosynthesis is likely involved[33].

Atopy

Various review articles have suggested that atopy is a predisposing factor for the development of CRS in susceptible individuals[34;35]. This idea is based on the postulation that mucosal edema in the region of the ostiomeatal complex may compromise sinus ventilation. Decreased sinus ventilation, in turn, may lead to sinus obstruction, mucus retention, and, ultimately, sinus infection[1].

Prevalence rates of atopy have been reported to range up to 84%[36] in CRS patients without nasal polyposis and up to 36-56%[18;37] in CRS patients with nasal polyposis. However, critical analysis of the papers linking atopy to CRS has revealed that, although many studies suggest a higher prevalence of atopy in patients presenting with symptoms consistent with CRS, a selection bias is involved in most studies[18;36;37]. Since no increase in the incidence of rhinosinusitis is observed during the pollen season in pollen sensitized patients[38], the role of atopy in CRS without nasal polyposis remains controversial. Similarly, although the presence of elevated levels of IgE and eosinophils in both tissue and blood suggest an allergic origin of CRS with nasal polyposis, Settipani et al demonstrated that only 1.5% of allergic rhinitis patients have nasal polyps in their nose and paranasal sinuses compared to 4.7% of subjects without allergic rhinitis[18], rendering a role for atopy in the pathogenesis CRS with nasal polyposis unlikely.

Ciliary impairment

Normal mucociliary transport is essential for the maintenance of healthy sinuses and prevents chronic sinus inflammation. Most patients with CRS demonstrate a decrease in mucociliary clearance rate and abnormalities in ciliary morphology. Mucostasis, hypoxia, microbial products, and toxic proteins generated during the chronic inflammation probably all contribute to the diminished mucociliary function observed in most CRS patients (i.e. secondary ciliary dyskinesia)[39]. Upon disease resolution, mucociliary clearance is likely to improve[5]. In primary ciliary dyskinesia, a genetic defect results in abnormal ciliary morphology and function[40]. Affected patients suffer from derangements of mucociliary transport and experience, amongst other manifestations, recurrent episodes of CRS, which are often difficult to treat[5].

Cystic fibrosis

Cystic fibrosis (CF) is one of the most common autosomal recessive disorders in the Caucasian population and is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene encodes the cystic fibrosis transmembrane conductance regulator, a transmembrane ion channel[41]. Dysfunction of this ion channel results in blockage of ion and water transport into and out of cells. As a result, cells that line the passageways of, amongst others, the nose, paranasal sinuses and lungs, produce abnormally thick, viscous mucus. Cilia of cystic fibrosis patients are unable to transport this thick, viscous mucus and, as a consequence, ciliary malfunction and CRS follow. Up to 97% of CF patients above the age of 5 have nasal polyps in their nose and paranasal sinuses (ranking CF highest as a risk factor for the development of CRS with nasal polyposis)[42-46].

DIAGNOSIS AND STAGING

Symptoms

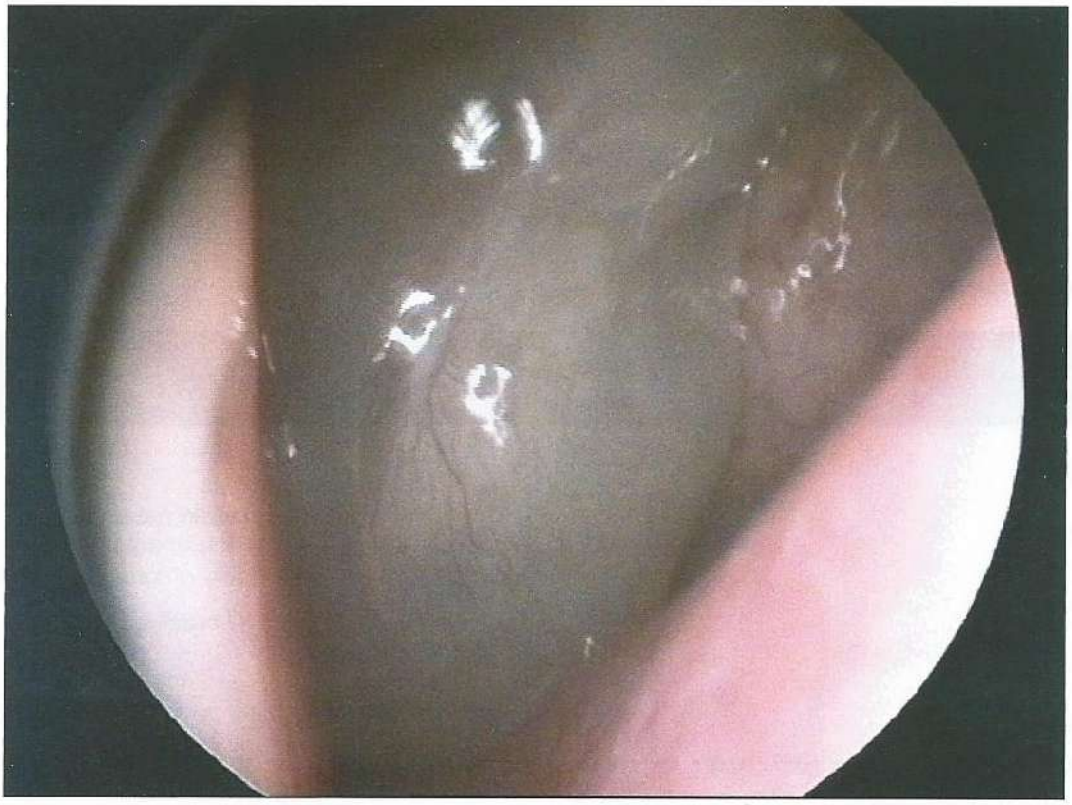
As mentioned earlier, CRS with or without nasal polyps is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by the presence of distinctive symptoms.

These symptoms include nasal blockage, nasal discharge (anterior and/or posterior), facial pain and/or facial pressure and/or reduced sense of smell. Symptoms are principally the same in those CRS patients without nasal polyposis and those with nasal polyposis, but the pattern of symptoms and intensity of symptoms may vary. In general, disorders of smell are more prevalent in those CRS patients with nasal polyposis. The degree or strength of symptoms can be estimated using various grading tools. The Visual Analogue Scale (VAS) is most widely used for this purpose[47].

Nasal endoscopy

Anterior rhinoscopy alone is inadequate to accurately diagnose CRS with or without nasal polyposis. Nasal endoscopy is advised in every patient suspected to

FIGURE 1. Endoscopic image of CRS with nasal polyposis (left nasal cavity)



Note the presence of a transparent, pale grey, edematous mass that originates from the anterior ethmoid cells and descends between the middle turbinate and the lateral nasal wall into the nasal cavity. This polyp just touches the superior border of the inferior turbinate.

suffer from the disease to identify nasal polyps, mucopurulent discharge and/or edema. Nasal polyps appear as grape-like structures in the upper nasal cavity and originate from within the ostiomeatal complex (figure 1). In case of severe congestion, decongestion may be necessary to adequately visualize the area of the ostiomeatal complex. For research purposes, semi-quantitative scores may be obtained to stage disease severity: i.e. the severity of nasal polyps, edema, discharge, post-operative crusting and scarring (table 1)[48].

Assessment of nasal patency

Peak nasal inspiratory flow (PNIF) is an inexpensive, quick and easy to perform test that is a useful to assess nasal patency. Although it measures both sides of the nose together, it is able to measure gross reduction in polyp size over time and compares well with rhinomanometry[49]. In CRS without nasal polyposis, in which changes in nasal patency are more subtle, the role of PNIF is limited.

Imaging

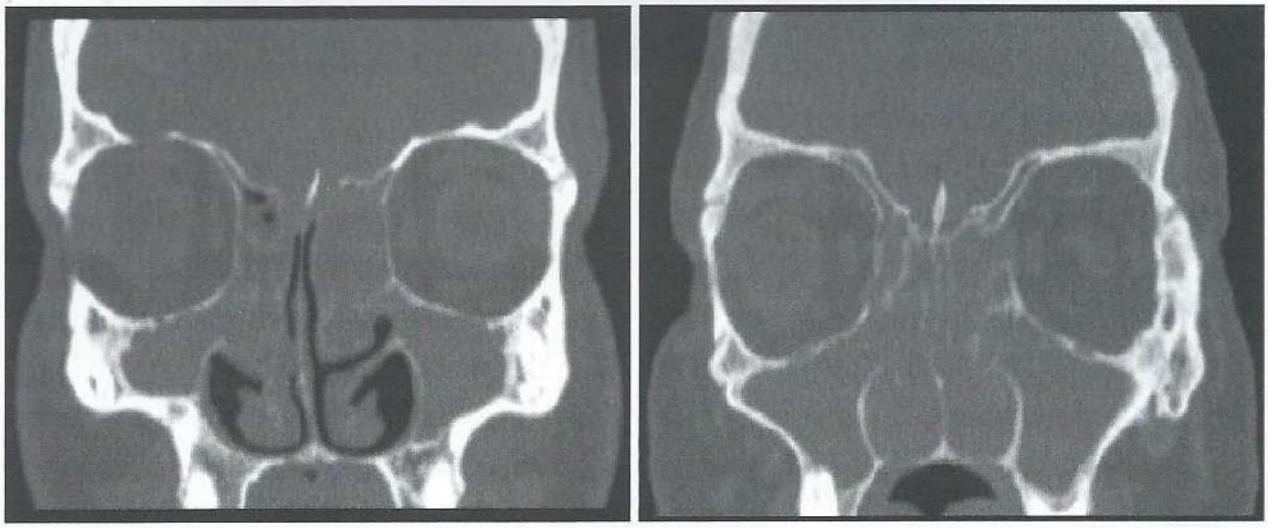
Due to the large number of false positive and false negative results, plain sinus X-

TABLE 1. Endoscopic appearance score[46]

Characteristic	Left	Right	
Polyp (0,1,2,3)			Polyps: 0: absence of polyps 1: polyps in the middle meatus 2: polyps beyond the middle meatus 3: polyps completely obstructing the nose
Edema (0,1,2)			Edema / scarring / crusting: 0: absent 1: mild 2: severe
Discharge (0,1,2)			
Scarring (0,1,2)			
Crusting (0,1,2)			
Total			Discharge: 0: no discharge 1: clear, thin discharge 2: thick, purulent discharge

N.B. Scarring and crusting only to be assessed post-operatively (outcome assessment score)

FIGURE 2. Coronal reconstruction of Computed Tomography (CT) images of two patients: one with moderate chronic rhinosinusitis with nasal polyposis (left) and one with massive nasal polyposis (right)



rays are insensitive and of limited use in the diagnosis of CRS with or without nasal polyposis[50]. Computed tomography (CT) is the imaging modality of choice to confirm the extent of pathology (figure 2) and to assess the complex sinonasal anatomy pre-operatively. Of several CT-staging systems, the Lund-Mackay system is most widely used[51]. It relies on a simple numerical score of 0 to 2 dependent on the absence or presence of opacification of each sinus system and/or the ostiomeatal complex (table 2). It has been used for many years to quantify the extent of sinus inflammation prior to sinus surgery and has been validated[52]. CT and endoscopy scores have been shown to correlate well, but the correlation between CT findings and symptom scores is poor[53,54]. Incidental abnormalities are found on scanning in up to 39% of the normal population[55]. For ethical reasons, postoperative sinus CT imaging should be performed in those cases with persistent problems only.

Quality of life questionnaires

Since a few years, doctors pay more attention to not only the patient's symptoms, but also to the patient's quality of life. Generic health status instruments enable us

TABLE 2. Lund-Mackay CT scoring system[51]

Sinus system	Left	Right
Maxillary sinus (0,1,2)		
Anterior ethmoid cells (0,1,2)		
Posterior ethmoid cells (0,1,2,)		
Sphenoid sinus (0,1,2)		
Frontal sinus (0,1,2)		
Ostiomeatal complex (0,2)*		
Total		

0: no abnormalities; 1: partial opacification; 2: total opacification
*0: not occluded; 2: occluded

to compare quality of life of patient suffering from CRS with other patient groups. Disease specific health status instruments enable us to establish treatment effect on quality of life. Of the generic health status instruments, the Medical Outcomes Study Short Form 36 (SF-36) is by far the most widely used[56;57]. It is well validated and has been used to assess quality of life in CRS patients[58;59]. Various disease specific health status instruments are currently available. Of these, the Rhinosinusitis Outcome Measure 31 (RSOM-31)[60], Sinonasal Outcome Test 20 (SNOT-20)[61] and Rhinosinusitis Disability Index (RSDI)[62] are most widely used. All are well validated.

INFLAMMATORY MECHANISMS

CRS is a heterogeneous group of diseases with potentially different underlying pathophysiological mechanisms. It is currently not understood whether acute recurrent rhinosinusitis precedes CRS and finally polyp growth or whether these disease entities develop independently. So far, CRS is clinically divided in CRS with and CRS without nasal polyposis based on the presence of nasal polyps on

endoscopy. Various studies have attempted to classify the disease and its severity based on the presence of various inflammatory markers.

Tissue specimens obtained from patients suffering from CRS without nasal polyposis are generally characterized by basement membrane thickening, goblet cell hyperplasia, fibrosis, subepithelial edema, and influx of inflammatory cells[63]. These inflammatory cells are predominantly neutrophils[64;65], but eosinophils[66;67], macrophages[66], lymphocytes[65] and mast cells[67] are also regularly observed. A range of mediators and cytokines, namely interleukin 1 (IL-1), IL-3, IL-6, IL-8, tumor necrosis factor α (TNF-α), granulocyte macrophage colony stimulating factor (GM-CSF), intercellular adhesion molecule 1 (ICAM-1), myeloperoxidase (MPO), matrix metalloproteinase 9 (MMP-9) and eosinophilic cationic protein (ECP), has been described as being increased in tissue specimens of patients suffering from CRS without nasal polyposis[1;68-72]. Vascular cell adhesion molecule 1 (VCAM-1), an adhesion molecule involved in selective eosinophil recruitment, and IL-5, a cytokine involved in eosinophil recruitment, are not increased[68;70]. Except for the increase in ECP, mediator profiles in CRS patients without nasal polyposis are similar to those observed in patients suffering from viral rhinitis and are said not to resemble mediator profiles observed in CRS patients with nasal polyposis.

Tissue specimens of patients suffering from CRS with nasal polyposis reveal frequent epithelial damage, a thickened basement membrane, large quantities of extracellular edema and a dense inflammatory cell infiltrate localized around so called 'emtpy' pseudocyst formations (figure 3)[68;73;74]. Reduced numbers of vessels and glands and virtually no neural structures are characteristic as well. Among the inflammatory cells, EG2+ (activated) eosinophils are prominent in about 80% of Caucasian nasal polyps[75;76]. Although more prominent in CF nasal polyp tissue specimens, non-Caucasian nasal polyp tissue specimens and CRS without nasal polyposis tissue specimens, increased numbers of neutrophils are observed in most tissue specimens of CRS patients with nasal polyposis[66;77;78]. In addition, activated T-cells, plasma cells, monocytes, fibroblasts and mast cells are regularly found[66]. A range of mediators and cytokines, namely IL-3, IL-4, IL-5, IL-8, regulated upon activation normal T cell expressed and secreted (RANTES), eotaxin, eotaxin-2, eotaxin-3, GM-CSF, interferon γ (IFN-γ), macrophage inflammatory protein 1 alpha (MIP-1α), TNF-α, leukotriene C_4 (LTC4), leukotriene

FIGURE 3. Nasal polyps reveal frequent epithelial damage, a thickened basement membrane, large quantities of extracellular edema and a dense inflammatory cell infiltrate localized around so called "empty" pseudocyst formations

D_4 (LTD4), leukotriene E_4 (LTE4), MMP-7, MMP-8, MMP-9, tissue inhibitor of metalloproteinase 1 (TIMP-1), VCAM-1, immunoglobulin E (IgE), and ECP has been described as being increased in tissue specimens of patients suffering from CRS with nasal polyposis[1;72;76;79-87]. As can be observed, most studies focused on the role eosinophil-related mediators in the pathogenesis of CRS with nasal polyposis. However, the idea that nasal polyposis is an eosinophil mediated disease should be challenged by the fact that CF nasal polyps and non-Caucasian nasal polyps, polyps macroscopically remarkably similar to Caucasian nasal polyps, are characterized by abundant neutrophils[66;77;78] and the fact that most Caucasian nasal polyps are obtained from patients suffering from atopy, asthma and/or ASA intolerance, co-morbidities known to be (at least in part) eosinophil mediated. In chapters 2.1, 2.2, 2.3 and 3.1, the levels of various inflammatory cells (a.o. eosinophils and neutrophils) and mediators (a.o. IL-5 and IL-8) are studied in well characterized nasal polyp tissue specimens, results that may help to elucidate discrepancies in previous findings.

TREATMENT

Glucocorticoids

In an extensive review of the literature, the European Position Paper on Rhinosinusitis and Nasal Polyps (EP^3OS) has proposed an evidence based treatment scheme for CRS patients[1;2]. Topical glucocorticoids are important treatment modalities for both CRS patients without nasal polyposis and CRS patients with nasal polyposis. These results are based on various randomized controlled trials in which the effect of topical glucocorticoids in the treatment of patients suffering from both primary and recurrent CRS (with or without nasal polyposis) has been established[88-97].

Traditionally, systemic glucocorticoids are frequently prescribed in patients suffering from CRS with nasal polyposis. However, it was not until recently that the clinical impression that systemic glucocorticoids are indeed effective was confirmed in two randomized placebo-controlled studies[98;99]. No studies are currently available showing efficacy of systemic glucocorticoids in the treatment of patients suffering from CRS without nasal polyposis.

The biological action of glucocorticoids is mediated through activation of intracellular glucocorticoid receptors[100]. Two isoforms of these receptors have been identified: glucocorticoid receptor α (GRα) and glucocorticoid receptor β (GRβ)[101]. Upon hormone binding, GRα enhances anti-inflammatory gene transcription and/or represses pro-inflammatory gene transcription. GRβ does not bind glucocorticoids, but may interfere with GRα function. To date, the exact mechanisms underlying the anti-inflammatory and immunoregulatory effect of glucocorticoids remains to be fully explained.

Clinical efficacy of topical glucocorticoids in CRS patients depends in part on the ability of glucocorticoids to reduce airway eosinophil infiltration (either directly via a reduction in eosinophil viability and activation[102-104] or indirectly via a reduction in the secretion of cytokines[105-108]) and in part on the ability of glucocorticoids to interfere with prostanoid synthesis[109]. In chapter 3.1 the effect of topical glucocorticoids on the expression levels of cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2), two key enzymes in the generation of prostanoids, are described.

Although a substantial number of CRS patients is refractory to glucocorticoid treatment[110], the exact cause of glucocorticoid resistance is unknown. Overexpression of GRβ and/or a downexpression of GRα are two of the mechanisms that have been proposed[111;112]. Ideally, those patients resistant to glucocorticoids are identified prior to starting glucocorticoid therapy so that alternative treatment can be initiated as soon as possible. In chapter 3.2 we describe our search for a cellular marker predicting the response to surgery and/or post-operative fluticasone propionate aqueous nasal spray in a mixed group of CRS patients with and without nasal polyposis.

Antibiotics

Antibiotics, both short term courses and long term treatment regimens, are frequently prescribed in the treatment of patients suffering from CRS with or without nasal polyposis. Data supporting the short term (less than 14 days) use of antibiotics are limited and lacking in terms of proper randomized placebo controlled trials. The available studies comparing antibiotics do not show significant differences between ciprofloxacin and amoxicillin/clavulanic acid[113] or amoxicillin/clavulanic acid and cefuroxim[114] but do show effect on symptoms in 56% to 95% of CRS patients[113;114]. Although suggestive of a positive effect, regression to the mean cannot be ruled out since proper randomized placebo-controlled studies are lacking.

The efficacy of long term treatment with macrolide antibiotics in diffuse panbronchiolitis, a chronic inflammatory condition affecting the bronchioles, has inspired the Asians to treat patients suffering from CRS with long term macrolide antibiotics. Although few studies are currently available, they all suggest that long-term (8 to 12 weeks), low dose treatment with macrolide antibiotics is effective in treating those CRS patients incurable by surgery or glucocorticoids[58;115-118], results that are likely caused by immunomodulatory effects of macrolide antibiotics[119-121]. It is unlikely that antimicrobial effects are involved in the beneficial effect of macrolide antibiotics.

Antifungals

Recently, a fungal etiology has been proposed to play a prominent role in the pathogenesis of CRS with or without nasal polyposis (chapter 4.3), an idea based on the premise that an altered local immune response to sinonasal fungi results in the generation of disease in susceptible individuals[122]. Based on this premise, topical amphotericin B nasal lavages have been advocated in the treatment of CRS with or without nasal polyposis. Although safe to use and despite initial evidence of benefit in two uncontrolled trials[123;124], one subsequent uncontrolled prospective trial[125] and four subsequent double-blind placebo-controlled studies (one of which is presented in chapters 4.1 and 4.2) investigating the effect of topical amphotericin B nasal lavages[126;128;129] and nasal sprays[127] either failed to show benefit[127-129] or showed, at best, only modest radiological benefit without symptomatic improvement[126].

Although several uncontrolled reports have suggested that oral antifungals are effective in the treatment of CRS[130], a recent double-blind placebo-controlled study treated 53 CRS patients with high-dose oral terbinafine for a period of 6 weeks and demonstrated no improvement in subjective and objective outcome measures[131].

Other medical treatments

Although frequently prescribed in patients suffering from CRS without nasal polyposis[132], no evidence-based data are currently available supporting the use of oral antihistamines in the treatment of CRS without nasal polyposis. However, in patients suffering from CRS with nasal polyposis, three months of treatment with cetirizine in a dose of 20 mg/day significantly reduced post-operative sneezing, rhinorrhoea and nasal obstruction in a randomized placebo-controlled trial[133].

Open studies suggest that antileukotrienes may be of benefit in patients suffering from CRS with or without nasal polyposis. Added to standard treatment, antileukotriene treatment significantly improved symptom scores for headache, facial pain and pressure, ear discomfort, dental pain, purulent nasal discharge, postnasal drip, nasal congestion and obstruction, olfaction, and fever in 36 patients

suffering from CRS with or without nasal polyposis[134]. Larger randomized controlled trials are needed to reveal the true role of antileukotrienes in the treatment of CRS with and without nasal polyposis.

Surgery

As a consequence of technical advances in endoscopic systems and the recognition that patent anatomical ostia are important for normal sinus function, over the past two decades management of CRS patients has changed dramatically. Functional endoscopic sinus surgery (FESS), comprising a set of minimally invasive techniques, is now a well-established strategy in the treatment of patients refractory to medical therapy. FESS is not a standardized set of surgical steps as the extent and specific nature of surgery is determined by those sinuses affected and how extensively they are involved. The goal of FESS is to restore sinus ventilation and normal sinus function by opening the affected sinus air cells and sinus ostia under direct visualization[135].

Enthusiasm for FESS, mainly based on reports of short term success, has been justified more recently by the publication of long term postoperative results showing a continued trend towards subjective improvement of symptoms in most patients[136]. However, there is still a paucity of evidence regarding the effectiveness of FESS when compared to medical treatment and more conventional sinus surgery techniques. A systematic review of the literature has demonstrated that the vast majority of studies on FESS are cohort studies or case series. Only a handful of trials qualify as level I (meta-analysis or randomized controlled trial) or level II (controlled study without randomization) evidence. Only three randomized controlled trials have met the inclusion criteria of a recent Cochrane review on FESS for CRS[137]. Although these studies have important differences in their study populations, the interventions being compared and the methods that are used for outcome assessment, the authors conclude that FESS is equal to medical treatment with or without sinus irrigation in patients with CRS with or without nasal polyposis[137].

Conclusions

CRS with or without nasal polyposis is a chronic inflammatory condition involving the mucosa of the upper airways. It constitutes a major health problem and often involves a significant impact on quality of life. Although a fungal etiology has been proposed (chapter 4.3), the pathogenesis of CRS with or without nasal polyposis is believed to be multifactorial. Since differentiation between CRS with nasal polyposis and CRS without nasal polyposis is considered to be difficult, the two disease entities are often taken together as one. Adequate differentiation of patient subgroups, taking associated diseases such as atopy, asthma, ASA intolerance and CF into account, may aid in trying to understand the inflammatory mechanisms involved in CRS with and without nasal polyposis (chapters 2.1, 2.2, 2.3 and 3.1).

Treatment of CRS patients usually requires of a combination of glucocorticoids (topical and/or oral), antibiotics and/or surgery. Although frequently prescribed, the exact mechanisms underlying the anti-inflammatory and immunoregulatory effects of (topical) glucocorticoids remain to be fully explained (chapter 3.1). To date, the role of glucocorticoid resistance in the disease escalation remains unclear (chapter 3.2). Although some authors have advocated the use of topical antifungals in the treatment of patients suffering from CRS with or without nasal polyposis, recent evidence suggests that topical antifungals are ineffective (chapters 4.1 and 4.2).

CHAPTER 2

EFFECTOR CELLS AND MEDIATORS IN CRS WITH NASAL POLYPOSIS

CHAPTER 2.1

INCREASED NEUTROPHIL CHEMOATTRACTANT IL-8 IS CHARACTERISTIC OF ALL NASAL POLYP TISSUE SPECIMENS

Ebbens FA, Rinia AB, Luiten S, Adriaensen GFJPM, van Egmond D, van Drunen CM, and Fokkens WJ

ABSTRACT

Background: Most data on Caucasian nasal polyps are based on tissue specimens obtained from patients suffering from atopy, asthma, and/or ASA intolerance.

Objective: In this study we aimed to examine the effect of atopy, asthma, and/or ASA intolerance on the presence of various cytokines, chemokines, and growth factors in tissue specimens of patients suffering from CRS with nasal polyposis.

Methods: Using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA), we assessed tissue specimens of 54 CRS patients with nasal polyps with or without concurrent atopy, asthma, and/or ASA intolerance for a variety of mediators (i.e. IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin, RANTES, MCP-1, VEGF, EGF, FGF-basic, and HGF) and compared these levels to their levels in inferior turbinates of healthy controls (n = 9) and allergic rhinitics (n = 8)

Results: Compared to control nasal mucosa, the concentrations of IL-5, IL-6, IL-7, IL-8, IL-10 and IL-17 are increased in all nasal polyps. After Bonferroni adjustment for multiple comparisons, the increase in IL-8 was shown to be highly significant. The concentrations of IL-4, VEGF, EGF and HGF are significantly decreased in all nasal polyps. Atopy, asthma and/or ASA intolerance were shown not to influence outcome.

Conclusion: Elevated levels of the neutrophil chemoattractant IL-8 are characteristic of all nasal polyps. Atopy, asthma and/or ASA intolerance were shown not to influence the level of any of the tested cytokines, chemokines of growth factors.

INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by distinctive symptoms (e.g. nasal blockage, nasal discharge, facial pain, and/or reduced sense of smell) and either endoscopic signs or computed tomography (CT) changes characteristic of the disease[1]. CRS is a heterogeneous group of diseases with potentially different underlying etiologies and pathomechanisms. So far, CRS is clinically divided in CRS with nasal polyposis and CRS without nasal polyposis based on the presence of nasal polyps upon nasal endoscopy[1]

Tissue specimens of patients suffering from CRS with nasal polyposis reveal frequent epithelial damage, a thickened basement membrane, large quantities of extracellular edema, and a dense inflammatory cell infiltrate localized around so called 'emtpy' pseudocyst formations (figure 1)[68;73;74]. Among the inflammatory cells, EG2+ (activated) eosinophils are prominent in about 80% of Caucasian nasal polyps[75;76]. Increased numbers of neutrophils, activated T-cells, plasma cells, and mast cells are, however, also observed[66;77;78].

FIGURE 1. Nasal polyps reveal frequent epithelial damage, a thickened basement membrane, large quantities of extracellular edema and a dense inflammatory cell infiltrate localized around so called "empty" pseudocyst formations

Cytokines, chemokines, and growth factors are potent biologic factors involved in the regulation of inflammation, immune defense, and wound healing. A range of cytokines, chemokines, and growth factors, namely interleukin (IL)-3, IL-4, IL-5, IL-8, RANTES (regulated on activation normal T cell expressed and secreted), eotaxin, eotaxin-2, eotaxin-3, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ), macrophage inflammatory protein 1 alpha (MIP-1α), and tumor necrosis factor α (TNF-α), has been described as being increased in tissue specimens of patients suffering from CRS with nasal polyposis[1;72;76;79-87]. As can be observed, most studies focus on the role of eosinophil-related mediators in the pathogenesis of CRS with nasal polyposis. However, the idea that nasal polyposis is an eosinophil mediated disease only needs to be challenged. Cystic fibrosis nasal polyps and non-Caucasian nasal polyps, polyps macroscopically remarkably similar to Caucasian nasal polyps, are characterized by abundant tissue neutrophilia and not tissue eosinophilia[66;77;78].

Most data on Caucasian nasal polyps are based on tissue specimens obtained from patients suffering from atopy, asthma and/or acetyl salicylic acid (ASA) intolerance. Possibly, the presence of one or more of these co-morbid diseases is explanatory for the observed eosinophil predominance. In this study we aimed to examine the effect of atopy, asthma, and/or ASA intolerance on the presence of various cytokines, chemokines, and growth factors in tissue specimens of patients suffering from CRS with nasal polyposis. Using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA), we assessed tissue specimens of 54 CRS patients with nasal polyposis in the absence or presence of atopy, asthma, and/or ASA intolerance for a variety of mediators and compared the level of these mediators to their levels in inferior turbinates of healthy controls (n = 8) and in allergic rhinitics (n = 9).

METHODS

Subjects

Nasal polyps (n = 54) were obtained from patients undergoing Endoscopic Sinus Surgery (ESS). Prior to surgery a full ENT history was taken. Diagnosis of asthma was based on clinical features and when necessary on pulmonary function tests.

Diagnosis of ASA intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAID's). Diagnosis of atopy was based on Skin Prick Test (SPT) positivity for 18 of the most common aeroallergens. Those patients treated with glucocorticoids (both oral and topical nasal) in the four weeks prior to inclusion were excluded. In addition, those patients that smoked were excluded. Inferior turbinate biopsies of healthy nasal mucosa (n = 8) and allergic nasal mucosa (n = 9) were obtained from patients undergoing corrective surgery for turbinate hypertrophy or septoplasty. Again, diagnosis of atopy was based on Skin Prick Test (SPT) positivity. None of the patients in these two control groups suffered from asthma and/or ASA intolerance and none of them was treated with glucocorticoids in the four weeks prior to inclusion. In addition, none of the patients in these two control groups smoked.

Tissue handling

All specimens, obtained at the time of surgery, were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Detection of pro-inflammatory mediators in control inferior turbinates, inferior turbinates of allergic rhinitis and nasal polyps

Upon analysis, all tissue samples were weighed, defrosted to room temperature, and dissociated in 300 µl Nucleospin® RA1 lysisbuffer (Macherey-Nagel, Düren, Germany) using a gentleMACS™ dissociator (Miltenyi Biotec B.V., Utrecht, the Netherlands). Following dissociation, all samples were centrifuged at 1400 g to collect supernatants for subsequent analysis. Determination of protein expression level of the pro-inflammatory cytokines (i.e. IL-1β, IL-6, IL-8, and TNF-α), eosinophil-related mediators (i.e. GM-CSF, RANTES, and eotaxin), T_H2-cytokines (i.e. IL-4, IL-5, and IL-13), T cell-related cytokines (i.e. IL-2, IL-7, IL10, and IL-17), and growth factors (i.e. basic fibroblast growth factor (FGFbasic), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF)) were performed using a sandwich immunoassay containing 30 analytes that, in addition to the above mentioned mediators, also included IL-1 receptor antagonist (IL-1RA), IL-2 receptor (IL-2R), IL-12 (p40/p70 subunits), IFN-α, IFN-γ, granulocyte colony-stimulating factor (G-CSF), MIP-1α, MIP-1β,

interferon-inducible protein of 10 kDa (IP-10), monokine induced by IFN-γ (MIG), and monocyte chemoattractant protein 1 (MCP-1) (Biosource™, Invitrogen, Breda, the Netherlands). All assays were performed as described by the manufacturer. Plates were analyzed using a Luminex 100™ reader (Luminex BV, Oosterhout, the Netherlands).

Statistical analysis

Statistical analysis was carried out using SPSS 16.0 (Chicago, IL, USA). Cytokine, chemokine, and growth factor values reported as below threshold were recoded to the lowest measurable value. Mediator concentrations are reported as median mediator concentration in pg/mL per mg tissue. Kruskal-Wallis non-parametric tests were performed to check for significant in between-group variability. In case of significant in between-group variability, Mann-Whitney-U non-parametric tests were performed for between-group comparisons. Using the Bonferroni adjustment for multiple comparisons, the level of statistical significance was set to 0.002 (2-sided).

RESULTS

Increased levels of IL-8 characterize nasal polyp tissue specimens

In the first part of this study, mediator concentrations in control inferior turbinates from healthy individuals (n = 8) and nasal polyps from a mixed group of CRS patients (n = 54) were compared. Compared to control inferior turbinates, the concentrations of the pro-inflammatory cytokines IL-6 and IL-8 are increased and the concentration of the pro-inflammatory cytokine TNF-α is decreased in all nasal polyp tissue specimens. The increase in IL-8 is extremely large (6-fold) and highly significant, even after Bonferroni adjustment for multiple comparisons. A trend towards significance is observed for the decrease in TNF-α. Although the concentration of all eosinophil-related mediators is slightly lower in all nasal polyps, differences are statistically not significant. Of the T_H2-cytokines, the concentration of IL-5 is increased slightly and the concentrations of IL-4 and IL-13 are decreased. A 4-fold decrease in IL-4 is observed in all nasal polyps, a highly significant decrease, even after Bonferroni adjustment for multiple comparisons.

TABLE 1. Comparison of median mediator concentrations in control inferior turbinates and nasal polyps

Concentration in pg/mL per mg tissue	Control inferior turbinate (n = 8)	Nasal polyps (n = 54)	*p* value
IL-1β	7.44	4.53	0.053
IL-4	4.41*	0.20*	0.001
IL-5	4.68	5.89	0.600
IL-6	1.34	2.17	0.176
IL-7	2.66	3.41	0.437
IL-8	5.25*	29.40*	0.002
IL-10	2.73	3.39	0.600
IL-13	5.56	2.05	0.032
IL-17	4.94	5.02	0.437
TNF-α	1.33	0.09	0.003
Eotaxin	2.58	1.61	0.059
IFN-γ	4.63	3.49	0.425
GM-CSF	4.53	1.55	0.016
RANTES	82.12	39.95	0.017
MIP-1α	7.44	4.73	0.133
VEGF	5.81*	0.91*	0.001
EGF	6.89*	0.07*	0.001
HGF	25.57*	4.53*	0.002

* Significant between group difference ($p < 0.002$)

Although the concentration of most T cell-related cytokines (i.e. IL-7, IL-10, and IL-17) is slightly increased in all nasal polyps, no significant differences are observed between nasal polyps and control inferior turbinate specimens. The concentration of all but one of the tested growth factors (i.e. FGFbasic) is decreased significantly in all nasal polyps. The decrease in VEGF, EGF, and HGF is highly significant. In table 1, relevant data are summarized.

Atopy is not involved in CRS with nasal polyposis pathogenesis

In the second part of this study, mediator concentrations in non-atopic nasal polyps and atopic nasal polyps were compared. All patients with concurrent asthma and/or ASA intolerance were excluded. Although mediator concentrations in atopic nasal polyps are similar to those observed in non-atopic nasal polyps, median mediator concentration are slightly decreased in atopic nasal polyps in comparison to non-atopic nasal polyps. To test whether similar observations occur in allergic rhinitics,

median mediator concentrations were determined in inferior turbinate specimens of 9 allergic rhinitics. Except for IL-8, a similar decrease in all median mediator concentrations is observed in inferior turbinates of allergic rhinitics compared to healthy control inferior turbinates. Although similar results were recently observed by others[138], we currently have no explanation for the slight decrease in median mediator concentration in both atopic nasal polyps and inferior turbinates of allergic rhinitics. In table 2, all relevant data are summarized.

Asthma and/or ASA intolerance may result in changes in median mediator concentrations

In the third part of this study, mediator concentrations in nasal polyps of patients suffering from various co-morbid diseases were studied. Polyps were grouped

TABLE 2. Comparison of median mediator concentrations in inferior turbinate tissue specimens of healthy controls, inferior turbinate specimens of allergic rhinitics, non-atopic nasal polyps and atopic nasal polyps

Concentration in pg/mL per mg tissue	Inferior turbinate atopy (-) (n = 8)	Nasal polyps atopy (-) (n = 15)	Inferior turbinate atopy (+) (n = 9)	Nasal polyps atopy (+) (n = 11)	*p* value
IL-1β	7.44	4.67	3.37	3.70	0.086
IL-4	4.41	0.27	2.24	0.08	0.015
IL-5	4.68	7.05	2.55	6.38	0.085
IL-6	1.34	2.59	0.73	1.99	0.011
IL-7	2.66	4.97	1.39	3.45	0.022
IL-8	5.25	33.24	7.94	31.55	0.003
IL-10	2.73	4.06	1.47	3.67	0.055
IL-13	5.56	3.80	2.08	1.54	0.178
IL-17	4.94	6.93	2.52	5.30	0.123
TNF-α	1.33	0.19	0.73	0.19	0.036
Eotaxin	2.58	1.62	1.17	1.28	0.059
IFN-γ	4.63	4.86	2.49	3.35	0.057
GM-CSF	4.53	3.29	3.24	1.63	0.236
RANTES	82.12	54.42	59.68	31.03	0.068
MIP-1α	7.44	6.02	4.43	4.48	0.245
VEGF	5.81	1.57	2.63	0.33	0.012
EGF	6.89	0.15	3.12	0.02	0.007
HGF	25.57	7.32	10.27	4.77	0.128

TABLE 3. Comparison of median mediator concentrations in classified nasal polyps

Conc. in pg/mL per mg tissue	atopy (-) asthma (-) ASA (-) (n = 15)	atopy (+) asthma (-) ASA (-) (n = 11)	atopy (-) asthma (+) ASA (-) (n = 8)	atopy (+) asthma (+) ASA (-) (n = 12)	atopy (-) asthma (+) ASA (+) (n = 5)	atopy (+) asthma (+) ASA (+) (n = 3)	*p* value
IL-1β	4.67	3.70	5.19	4.23	2.34	3.26	0.593
IL-4	0.27	0.08	0.15	0.06	0.24	0.66	0.759
IL-5	7.05	6.38	7.59	5.97	4.18	6.16	0.612
IL-6	2.59	1.99	2.17	2.51	1.19	2.35	0.758
IL-7	4.97	3.45	4.11	3.44	2.26	3.53	0.370
IL-8	33.24	31.55	19.31	20.74	32.19	56.27	0.472
IL-10	4.06	3.67	4.37	3.44	2.40	3.54	0.613
IL-13	3.80	1.54	2.77	1.76	0.92	1.36	0.545
IL-17	6.93	5.30	6.76	5.07	3.47	5.11	0.624
TNF-α	0.19	0.19	0.01	0.03	0.18	0.21	0.934
IFN-γ	4.86	3.35	4.91	3.17	2.20	3.24	0.274
GM-CSF	3.29	1.63	2.29	1.22	0.86	0.96	0.822
Eotaxin	1.62	1.28	1.94	1.72	0.81	1.13	0.386
RANTES	54.42	31.03	35.71	47.00	27.65	25.33	0.246
MIP-1 α	6.02	4.48	5.69	4.74	3.74	2.52	0.331
VEGF	1.57	0.33	0.90	0.38	0.96	0.85	0.966
EGF	0.15	0.02	0.05	0.03	1.14	1.01	0.881
HGF	7.32	4.77	4.08	3.96	1.24	1.84	0.145

according to the presence or absence of atopy, asthma and/or ASA intolerance. Median mediator concentrations are similar in all groups with no significant differences between the 6 nasal polyp subgroups (table 3). When comparing grouped allergic nasal polyps (n = 26) with grouped non-allergic nasal polyps (n = 28), no significant differences are observed. However, when comparing grouped asthmatic nasal polyps (n = 28) with grouped non-asthmatic nasal polyps (n = 26), a trend towards lower levels of MIG (p = 0.033) and HGF (p =0.025) is observed. Finally, when comparing grouped ASA intolerant nasal polyps (n = 8) with grouped ASA tolerant nasal polyps (n = 46), a trend towards lower levels of IL-1RA (p = 0.022), IL-2R (p = 0.026), IL-7 (p = 0.038), IL-12 (p = 0.017), IFN-α (p = 0.006), IFN-γ (p = 0.040), MIP-1α (p = 0.033), MIG (p = 0.040), eotaxin (p = 0.038), RANTES (p = 0.046), G-CSF (p = 0.021) and HGF (p = 0.012) is observed. However, after Bonferroni adjustment for multiple comparisons, none of these differences is statistically significant.

DISCUSSION

Compared to control inferior turbinates, the concentrations of most mediators are decreased in all nasal polyp tissue specimens. Tissue specimens of nasal polyps are known to contain large quantities of extracellular edema (so called 'emtpy' pseudocyst formations) that are surrounded by a dense inflammatory cell infiltrate (figure 1)[73;74]. Possibly, a relative reduction of inflammatory cells (and thus mediators) as a consequence of nasal polyp edema explains the observed reduction in many mediator concentrations.

Although the concentrations of most mediators are decreased, the concentrations of the pro-inflammatory cytokines IL-6 and IL-8 are increased. The observed increase in IL-8 is extremely large (6-fold) and highly significant. Although an increase in IL-8 in nasal polyp tissue specimens has been reported previously[86;87;138], most authors focus on the role of the eosinophil-related cytokine IL-5 in CRS with nasal polyposis pathogenesis and suggest that it is the increase in IL-5 that is characteristic of nasal polyp inflammation[66;139-141]. IL-8 is a potent neutrophil recruiting and activating factor, produced by macrophages, epithelial cells and endothelial cells. Unpublished data by our group demonstrate that nasal polyp epithelial cells produce the vast majority of IL-8. In nasal polyps, the presence of elevated levels of IL-8 has been associated to collagen breakdown and tissue remodeling via activation of matrix metalloproteinase 8 (MMP-8)[87].

Based on previous observations that eosinophils and neutrophils are present in equal amounts in most nasal polyps[142] and the observation that high concentrations of IL-8 characterize all CRS with nasal polyposis tissue specimens, we conclude that both neutrophils and IL-8 are likely to play an important role in the pathogenesis of CRS with nasal polyposis. Although high levels of IL-8 suggest that neutrophils are involved in CRS with nasal polyposis pathogenesis, high levels of IL-8 do not exclude the involvement of eosinophils since IL-8 has been shown to be chemotactic for eosinophils in patients with high levels of blood eosinophils[143].

In asthma, in about 50% of asthmatic patients, a neutrophil-mediated inflammatory mechanisms is likely involved in producing enhanced bronchial reactivity and

reversible airflow obstruction[144] and recent data from Asia suggest that non-Caucasian nasal polyps, nasal polyps macroscopically remarkably similar to Caucasian nasal polyps, are characterized not by abundant tissue eosinophils but by abundant tissue neutrophils[66;77;78]. As the presence of neutrophils characterizes nasal polyps of (Caucasian) patients suffering from CF (unpublished results by our group) as well, we suggest that neutrophils are likely involved in CRS with nasal polyposis pathogenesis.

Of the T_H2-cytokines, the concentration of IL-5 is increased and the concentrations of IL-4 and IL-13 are decreased. A 4-fold decrease in IL-4 is observed in all nasal polyps, a highly significant decrease, even after Bonferroni adjustment for multiple comparisons. Since high levels of IL-4 and IL-13 are linked to allergic inflammation, based on our results, one may conclude that atopy is not involved in the pathogenesis of CRS with nasal polyposis, results confirmed by observations in the second part of our study and observations from population studies[18]. Although a significant increase in the concentration of IL-5 and eotaxin was expected in those nasal polyps of patients also suffering from concurrent atopy, asthma and/or ASA intolerance[145-147], we currently show that IL-5 (a key cytokine for eosinophil maturation, differentiation and survival) and eotaxin (a selective CC chemokine involved in eosinophil recruitment) concentrations are similar in all nasal polyp subgroups. In addition, although a slight increase in IL-5 is observed in all nasal polyps, no significant differences were observed between nasal polyp tissue specimens and healthy control inferior turbinate tissue specimens. Although in contrast to observations by others, our results are in line with observations in both non-Caucasian nasal polyps[148] and non-atopic, non-asthmatic, non-ASA intolerant Caucasian nasal polyps (unpublished results by our group). Future studies are necessary to confirm our results, especially in those patients with concurrent ASA intolerance.

All nasal polyps are characterized by a decrease in the concentrations of VEGF, EGF, and HGF, a decrease that is highly significant, even after Bonferroni adjustment for multiple comparisons. VEGF is a growth factor involved in angiogenesis, EGF is a growth factor involved in the regulation of cell growth, proliferation and differentiation, and HGF is a growth factor involved in wound healing. Although the mechanisms leading to nasal polyp growth are unclear,

growth factors have been suggested to play an important role. Although increased mRNA levels of VEGF and HGF were recently demonstrated in tissue specimens of (small) nasal polyps[149;150], in our study VEGF and HGF protein levels are decreased significantly. We postulate that it is the low level of VEGF protein that results in the reduced vascularity characteristic of CRS with nasal polyposis tissue specimens. Reduced levels of HGF may result in a decreased tendency for wound healing. Similar to results at the mRNA level[149], we currently demonstrate that in nasal polyps EGF protein levels are low. As mRNA expression levels and protein expression levels not necessarily correlate, the observed dichotomy mat be explained by differences in mRNA and protein VEGF and HGF levels.

In conclusion, we currently show that elevated levels of IL-8 are characteristic of all nasal polyp tissue specimens. Although elevated, the precise role of IL-8 in the pathogenesis of CRS with nasal polyposis remains speculative. Most likely, not only eosinophils, but also neutrophils are involved in CRS with nasal polyposis pathogenesis. Human trials with IL-8 inhibitors are necessary to reveal the true role of IL-8 in the formation of nasal polyps in susceptible individuals.

CHAPTER 2.2

ENDOTHELIAL L-SELECTIN LIGAND EXPRESSION IN NASAL POLYPS

Ebbens FA, Toppila-Salmi SK, Renkonen JA, Renkonen RLO, Mullol J, van Drunen CM, and Fokkens WJ, *Allergy (accepted for publication)*

ABSTRACT

Background: L-selectins on leukocytes and their counterreceptors on endothelial cells have been shown to be involved in leukocyte recruitment in chronic rhinosinusitis without nasal polyps (NP).

Objectives: The purpose of this study was to evaluate the expression level of functionally active endothelial L-selectin ligands in NP obtained from patients with NP of different etiology (simple NP, antrochoanal polyps (ACP) and cystic fibrosis (CF) NP) and inferior turbinate specimens of healthy controls and to compare these levels to the presence of various leukocyte subsets.

Methods: NP specimens and healthy nasal mucosa specimens were obtained from patients undergoing surgery and were immunohistochemically stained with monoclonal antibodies detecting CD34, sialyl Lewis X of sulfated extended core 1 lactosamines and various leukocyte subsets.

Results: All NP are characterized by a decrease in the number of CD34+ vessels. The number of eosinophils and the percentage of vessels expressing endothelial sulfated sialyl Lewis X epitopes are upregulated in all groups of simple NP. Tissue eosinophilia is increased in those patients with increased disease severity (ASA intolerance), but the percentage of endothelial sulfated sialyl Lewis X epitopes is not. Results on CF NP are similar to those observed for simple NP. ACP are characterized by low numbers of tissue eosinophils and relatively few vessels expressing endothelial sulfated sialyl Lewis X epitopes.

Conclusion: Our results suggest that functionally active L-selectin ligands may be involved in guiding leukocyte traffic into NP in patients with simple NP and CF NP but not ACP. Their presence, however, is unlikely to be rate-limiting.

INTRODUCTION

Nasal polyposis is a multifactorial disease, associated with asthma, cystic fibrosis (CF), primary ciliary dyskinesia, acetyl salicylic acid (ASA) intolerance and possibly allergy, affecting between 1 and 4% of the general population[15;25], up to 60% of patients with ASA intolerance[15;25] and up to 90% of patients suffering from Cystic Fibrosis (CF)[42-45;151;152]. Although in general occurring bilaterally and originating from the ethmoid cells, nasal polyps do exist unilaterally. Originating from the maxillary sinus mucosa and protruding into the choana, these polyps, frequently observed on only one side of the nasal cavity, are called antrochoanal polyps (ACP) and constitute a distinct disease entity[153].

Although many hypotheses have been suggested, the pathogenesis of all nasal polyps is still largely unknown. Macroscopically, nasal polyps are characterized by the presence of edematous masses of inflamed mucosa, predominantly localized in the middle meatus and prolapsing into the nose, leading to nasal obstruction, secretion, loss of smell, headache/facial pain and a reduced quality of life. Histologically, nasal polyps are characterized by a cover of respiratory epithelium, large quantities of extracellular edema and a dense inflammatory cell infiltrate consisting of mast cells, eosinophils, lymphocytes, neutrophils, and plasma cells, which release a variety of pro-inflammatory mediators, including cytokines, histamine, prostanoids and leukotrienes.

Although macroscopically and clinically remarkably similar, careful analysis of the literature reveals important histopathological differences between subtypes of nasal polyps[77;153-155]. In the majority of bilaterally occurring NP from patients without CF (in this study called simple NP), eosinophils are the most abundant inflammatory cell type. In contrast, CF NP, occurring bilaterally, and ACP, generally occurring unilaterally, are classically thought of as being neutrophilic[153;154;156-158].

Leukocyte recruitment in acute and chronic inflammation is characterized by sequential cell adhesion and activation events[159]. Low affinity adhesive interactions between selectins and their counter-receptors (glycosylated ligands) initially tether leucocytes to the vessel wall and cause tethered leukocytes to roll along endothelial cells lining the inside of the vessels, thereby facilitating activation

of leukocytes by chemokines, leading to increased levels of expression of leukocyte integrins, resulting in firm adhesion of leukocytes to the vessel wall, arrest of rolling and, ultimately, the process of transmigration[159-163]. Selectins are a family of type I membrane-spanning glycoproteins that are essential for leukocyte recruitment. Each selectin recognizes related but distinct counter-receptors displayed by leucocytes and/or endothelial cells. E-selectin is expressed by endothelial cells, P-selectin is expressed by endothelial cells and platelets and L-selectin is constitutively expressed by most types of circulating leukocytes. L-selectin differs from E- and P-selectins in that it, besides mediating low-affinity interactions leading to rolling, recognizes glycan-dependent endothelial cell-derived counter-receptors and glycan-dependent counter-receptors expressed by leucocytes that have previously adhered to the endothelium. L-selectin counter-receptors include CD34, sulphated glycoprotein of 200 kD (Sgp200), endomucin, glycolsylation dependent cell adhesion molecule 1 (GlyCAM-1), podocalyxin, endoglycan, mucosal addressin cell-adhesion molecule 1 (MAdCAM-1), and P-selectin glycoprotein ligand 1 (PSGL-1), expressed by endothelial cells of High Endothelial Venules (HEV) of lymph nodes and Peyers patches, activated endothelial cells and other leukocytes[164-167]. All of these ligands are sialomucin in nature and contain a predominance of *O*-linked carbohydrate chains (*O*-glycans). The adhesive 'activities' of counter-receptors require specific glycan-based post-translational modifications, determined largely by glycosyl-transferases. The result is decoration with specific sialylated, sulphated, and fucosylated oligosaccharides, situated on C-6 positions of galactose and *N*-acetylglucosamine residues on numerous *O*-glycans[168-175]. Most L-selectin ligands such as CD34 present sulfation of the sialyl Lewis x (sLe^x) tetrasaccharide (Siaα2,3Galβ1,4-[Fucα1,3]GlcNAc) on the C-6 position of GlcNAc (6-sulfated sialyl Lewis x or 6-sulfo-sLe^x) as a recognition determinant within their heavily glycosylated mucin domains[176]. Under normal conditions, properly glycosylated L-selectin ligands are expressed along HEV in lymphatic tissues only[177]. However, in rodents and humans undergoing allograft rejection as well as in patients suffering from chronic inflammatory diseases such as asthma and chronic rhinosinusitis (CRS), induction of sLe^x and 6-sulfo-sLe^x decorated L-selectin ligands is seen in postcapillary endothelial cells in tissues other than lymphatic tissue[177-181].

Previously we demonstrated that, in CRS patients without nasal polyps, the expression level of functionally active L-selectin ligands clearly correlates with the extent of tissue eosinophilia and severity of inflammation[181]. Based on these observations, we postulate that it is the level of functionally active L-selectin ligands that determines the extent of tissue eosinophilia in simple NP as well. Elaborating on this, we suggest that increased levels of functionally active L-selectin ligands explain the increase in tissue eosinophilia observed in simple NP patients with asthma and/or ASA intolerance (classically thought of as a more severe phenotype of (simple) nasal polyposis) when compared to simple NP patients without asthma and/or ASA intolerance.

MATERIAL AND METHODS

Subjects

Nasal polyps were obtained from patients undergoing Endoscopic Sinus Surgery (ESS). Prior to surgery a full ENT history was taken. Diagnosis of CF was established on the basis of clinical features, a positive sweat test and/or characteristic genotype abnormalities. Diagnosis of ACP was established by nasal endoscopy and computed tomography (CT)-imaging. Diagnosis of asthma was based on clinical features and when necessary on pulmonary function tests. Diagnosis of ASA intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAID's).

TABLE 1. Patient characteristics

Group	Number of patients	Male sex, n (%)	Age, median (IQ range)
Antrochoanal nasal polyps	8	3 (38 %)	51.0 (11.0 – 65.0)
Cystic fibrosis nasal polyps	8	6 (75 %)	13.0 (9.5 – 16.5)
Control inferior turbinate	22	17 (71 %)	29.0 (26.5 – 40.5)
Simple NP (asthma- ASA intolerance-)	9	4 (40 %)	46.0 (41.5 – 55.5)
Simple NP (asthma+ ASA intolerance-)	2	1 (50 %)	68.5 (66.0 – 71.0)
Simple NP (asthma+ ASA intolerance+)	8	6 (75 %)	47.0 (37.0 – 52.0)

Diagnosis of atopy was based on Skin Prick Test (SPT) positivity. Those patients with a positive SPT against one or more common aeroallergens were excluded. In addition, those patients treated with glucocorticoids (both oral and topical nasal) in the four weeks prior to inclusion were excluded. Control nasal mucosa (NM) samples (inferior turbinate) were obtained from patients undergoing corrective surgery for turbinate hypertrophy or septoplasty. None of the patients in this control group suffered from allergy, asthma or ASA intolerance and none of them was treated with glucocorticoids. Patient characteristics are summarized in table 1.

Tissue handling

All specimens, obtained at the time of surgery, were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemistry

Alkaline phosphatase staining of CD68, CD117, MBP, elastase, functionally active L-selectin ligands and CD34

5-µm thick serial sections of all snap-frozen specimens were cut on a Microm HM560 frigocut cryostat and transferred to APES (amino-phosphate-ethylsilane) coated slides (Starfrost), dried and stored at -70°C until analysis. Upon analysis, tissue sections were defrosted to room temperature (RT), dried and fixed in acetone (cold as ice) for 10 minutes at RT. After fixation, tissue sections were rinsed with phosphate-buffered saline (PBS, pH 7.8), placed in a semi-automatic stainer (Sequenza, Shandon) and incubated with 10% normal goat serum (CLB, Amsterdam, the Netherlands) in blocking reagent (Roche 10961760) for 10 minutes. Following the blocking procedure, sections were incubated with primary antibody for 60 minutes at RT. Mouse anti-human monoclonal antibodies directed against CD68, CD117, major basic protein (MBP) and elastase, functionally active L-selectin ligands (as detected with mAbs HECA-452 and MECA-79) and endothelial cells (CD34) were used (table 2). All primary antibodies were diluted in PBS containing 1% (w/v) blocking reagent (Roche 10961760) to block endogenous avidin and biotin activity. Following incubation with primary antibody, sections were rinsed with PBS for 5 minutes and incubated with biotinylated goat anti-mouse antiserum (Klinipath, Duiven, the Netherlands, 1:50) for 30 minutes at RT. Next,

sections were rinsed with PBS and incubated with streptavidin alkaline phosphatase (ss-AP, Biogenics, Klinipath, Duiven, the Netherlands, 1:50) for 30 minutes at RT. Sections were then rinsed with PBS containing tris hydroxymethylaminoethane (TRIS) buffer (0.2mol/L, pH8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20 minutes at RT. Sections were counterstained with Gill's hematoxylin (CD68, CD117, MBP, elastase) or Mayer's Hämalaun (CD34, mAbs HECA-452 and MECA-79), rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with PBS in case of CD68, CD117, MBP, elastase and CD34. mAbs 7C7 (mouse IgM) and TIB-146 (rat IgM) were used as negative controls for mAbs HECA-452 and MECA-79 respectively.

Tyramide signal amplification (TSA) staining for basogranulin

A sensitive protocol was used based on the alkaline phosphatase method described above. Sections were cut and defrosted as described above. Sections were fixed in acetone (cold as ice) for 10 minutes, placed in the semi-automatic stainer and incubated with normal goat serum as described above. Slides were incubated with mouse anti-human monoclonal antibody directed against basogranulin, diluted in PBS containing 1% (w/v) blocking reagent (table 2). After incubation with biotinylated goat anti-mouse antiserum, endogenous peroxidase was blocked using 0.2% (w/v) azide, 0.02% (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Following the blocking procedure, slides were incubated with streptavidin conjugated peroxidase (NEN, USA) for 30 minutes at RT, rinsed with PBS and incubated with biotinyl tyramide in TRIS buffer for 10 minutes in order to amplify the signal. Next, slides were rinsed once again in PBS and incubated with alkaline phosphatase conjugated goat-anti-biotin antiserum (Sigma, Zwijndrecht, the Netherlands). Sections were then rinsed with PBS containing TRIS buffer (0.2mol/L, pH8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20 minutes at RT. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with PBS.

TABLE 2. Monoclonal antibodies

Antibody	Specificity	Cell type	Titer	Source
KI-M6	CD68	Macrophages	1:200	DAKO,Glostrup, Denmark
YB5.B8	CD117	Mastcells	1:65	Becton Dickinson, the Netherlands
BB1	Basogranulin	Basophils	1:150	A.F. Walls, South Hampton, UK
AHN-10	Elastase	Neutrophils	1:32000	Chemicon, Temecula, CA, USA
BMK-13	MBP	Eosinophils	1:100	Sanbio, Uden, the Netherlands
HECA-452	2,3-sialylation & 1,3- fucosylation of lactosamine	L-selectin ligand	1:100	BD Biosciences Pharmingen, CA, USA
MECA-79	extended sulphate core 1 lactosamine	L-selectin ligand	1:100	BD Biosciences Pharmingen, CA, USA
CD34	CD34	Endothelial cells	1:100	DAKO, Glostrup, Denmark

Light microscopic evaluation

All sections were examined with an Olympus BX51 light microscope by 2 independent observers blinded to the experimental conditions, as described previously. Positively stained leukocytes and vessels were counted in the lamina propria at a final magnification of 200x. Results are expressed as the mean number of positive leukocytes or vessels (CD34+) per mm^2. Mean numbers of mAb HECA-452 positive and mAb MECA-79 positive vessels were divided by the mean number of CD34+ vessels from the same specimen to yield the percentage of sLe^x- or sulfated lactosamine reactive vessels[181].

Data analysis

Statistical analysis was carried out by using SPSS 12.01 (Chicago, IL, USA). Data are expressed as median and interquartile range. Kruskal-Wallis non-parametric tests were performed to check for significant in between-group variability. In case of significant in between-group variability, Mann-Whitney-U non-parametric tests were performed for between-group comparisons. Spearman rank-order correlation was used to assess bivariate association. The correlation value (rho) indicates the strength of correlation and varies between 0 and 1, in which 1 means a perfect

correlation. Two-tailed p values of less than 0.05 were considered statistically significant.

RESULTS

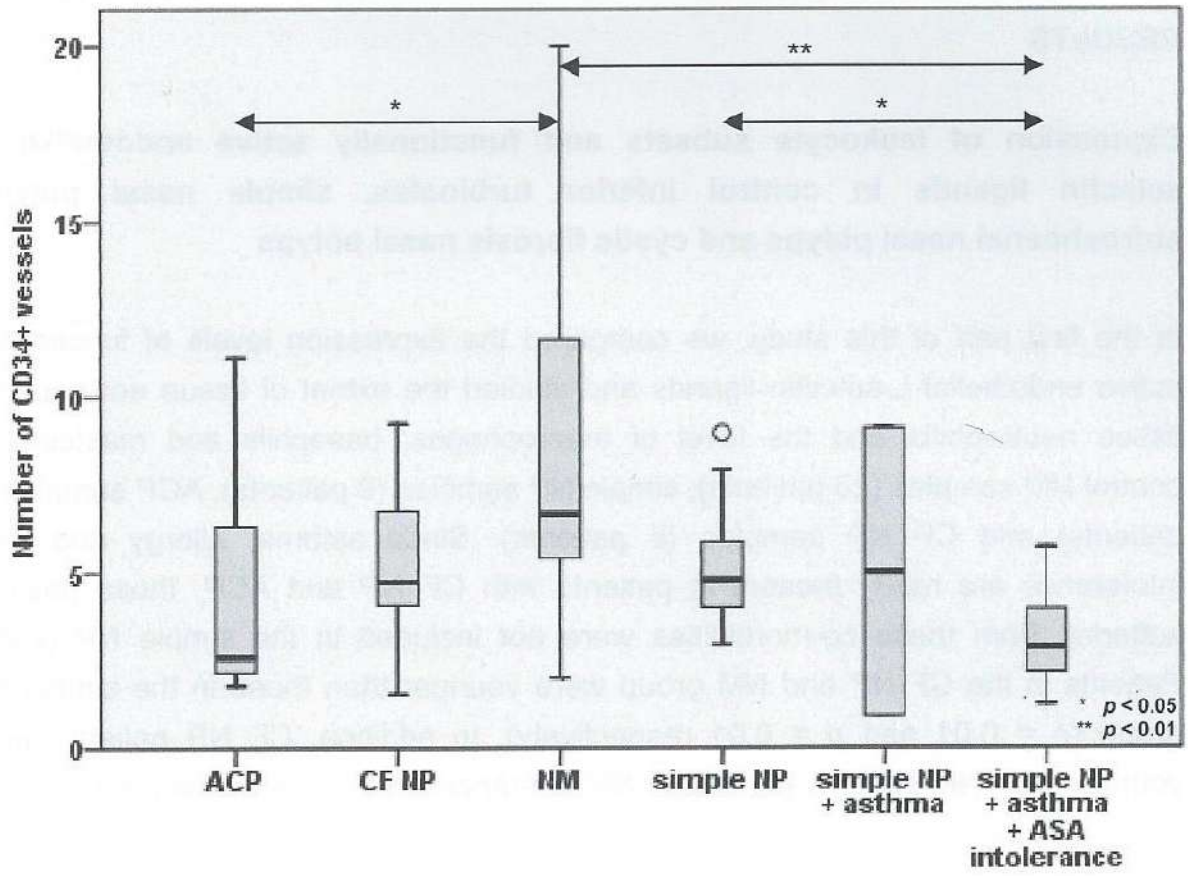
Expression of leukocyte subsets and functionally active endothelial L-selectin ligands in control inferior turbinates, simple nasal polyps, antrochoanal nasal polyps and cystic fibrosis nasal polyps

In the first part of this study, we compared the expression levels of functionally active endothelial L-selectin ligands and studied the extent of tissue eosinophilia, tissue neutrophilia and the level of macrophages, basophils and mastcells in control NM samples (20 patients), simple NP samples (9 patients), ACP samples (8 patients) and CF NP samples (8 patients). Since asthma, allergy and ASA intolerance are rarely present in patients with CF NP and ACP, those patients suffering from these co-morbidities were not included in the simple NP group. Patients in the CF NP and NM group were younger than those in the simple NP group (p = 0.01 and p = 0.01 respectively). In addition, CF NP patients were younger than NM patients (p = 0.05). No differences in age were observed

TABLE 3. Number of leukocytes per mm^2

Leukocyte subset	Control NM *(median, min-max)*	Simple NP *(median, min-max)*	ACP *(median, min-max)*	CF NP *(median, min-max)*
Eosinophils	4,0 (0,0-140,9)	41,6 (1,6-137,6)	4,8 (0,8-26,6)	9,4 (0,5-36,1)
Neutrophils	23,4 (4,3-297,4)	25,6 (4,0-251,8)	104,9 (40,8-157,8)	124,5 (63,0-267,6)
Macrophages	47,8 (17,3-129,1)	35,5 (16,1-138,9)	152,5 (65,7-239,4)	82,8 (51,3-158,9)
Mastcells	28,4 (7,1-63,2)	11,9 (2,3-41,0)	11,4 (9,6-18,3)	26,0 (17,9-83,1)
Basophils	1,1 (0,0-7,6)	0,4 (0,0-5,6)	0,6 (0,5-0,7)	1,5 (0,4-3,0)

FIGURE 1. Number of CD34+ vessels/mm^2 in antrochoanal polyps (ACP), CF nasal polyps (CF NP), control nasal mucosa (NM), simple nasal polyps (simple NP), simple nasal polyps of patients with asthma (simple NP + asthma) and simple nasal polyps of patients with asthma and ASA intolerance (simple NP + asthma + ASA intolerance).

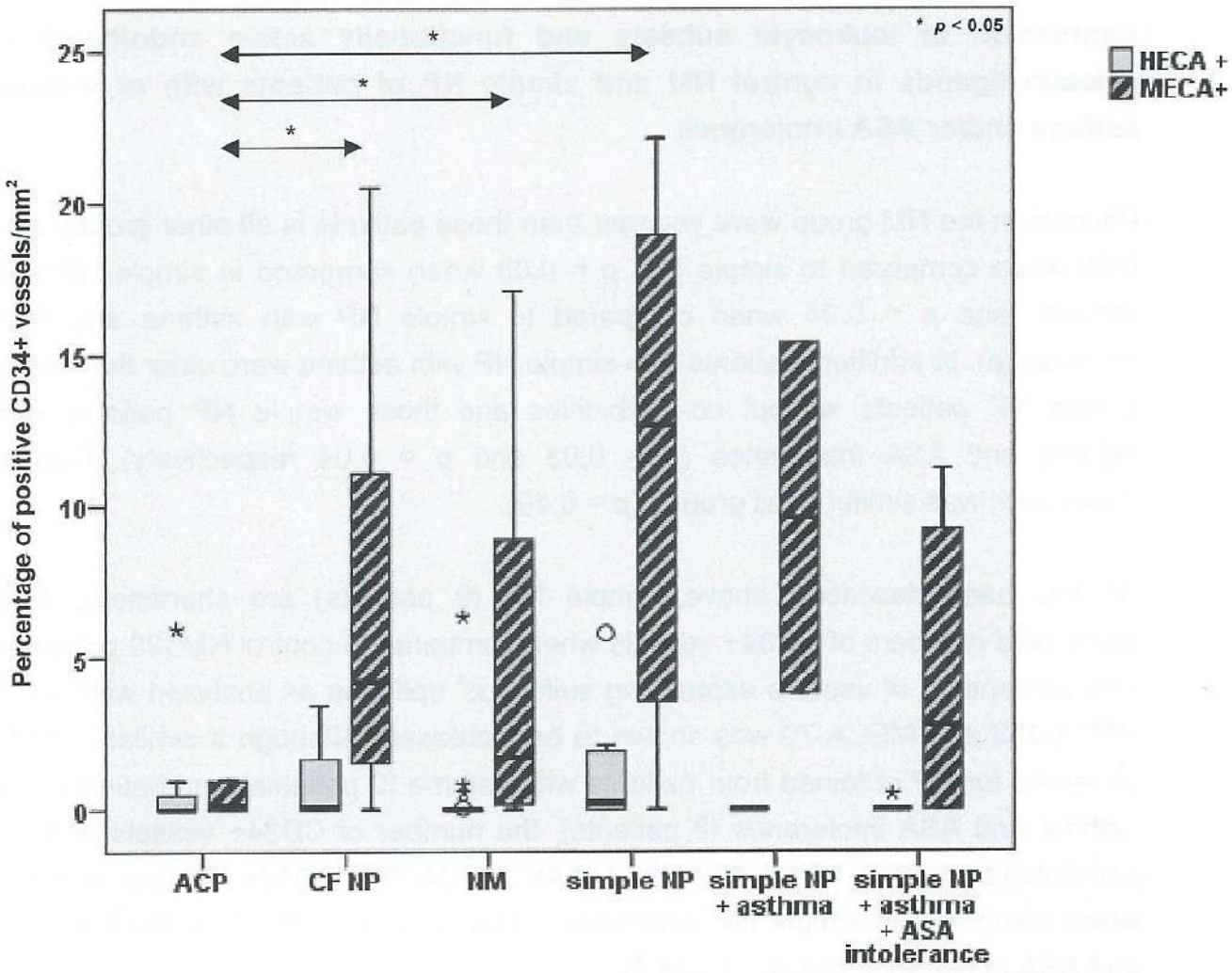


between patients suffering from ACP and all other patients. Gender distribution was similar in all groups ($p = 0.49$).

Compared to control NM, all three groups of NP are characterized by decreased numbers of CD34+ vessels. For ACP, this difference reached significance (figures 1 and 4 (appendix 1)). Compared to control NM, simple NP as well as CF NP are characterized by an increased percentage of vessels expressing sulfated sLe^x epitopes (functionally active L-selectin ligands) as analyzed with mAbs HECA-452 and MECA-79 (figures 2 and 4 (appendix 1)). In contrast, ACP are characterized by decreased levels of functionally active L-selectin ligands when compared to control NM, simple NP and CF NP ($p < 0.05$).

Although the number of eosinophils is clearly increased in simple NP (figures 3 and 4 (appendix 1)), this increase is not significant when compared to control NM, CF NP and ACP. When compared to control NM, CF NP are characterized by a significant increase in the number of neutrophils and macrophages (table 3). Although numbers are not significant, similar results are observed for ACP (table 3). Simple NP as well as ACP are characterized by a significant decrease in the

FIGURE 2. Percentage of mAb HECA-452+ and mAb MECA-79+ CD34+ vessels in control nasal mucosa (NM), simple nasal polyps (simple NP), simple nasal polyps of patients with asthma (simple NP + asthma) and simple nasal polyps of patients with asthma and ASA intolerance (simple NP + asthma + ASA intolerance).



number of mast cells (table 3). No differences were observed in the number of basophils present.

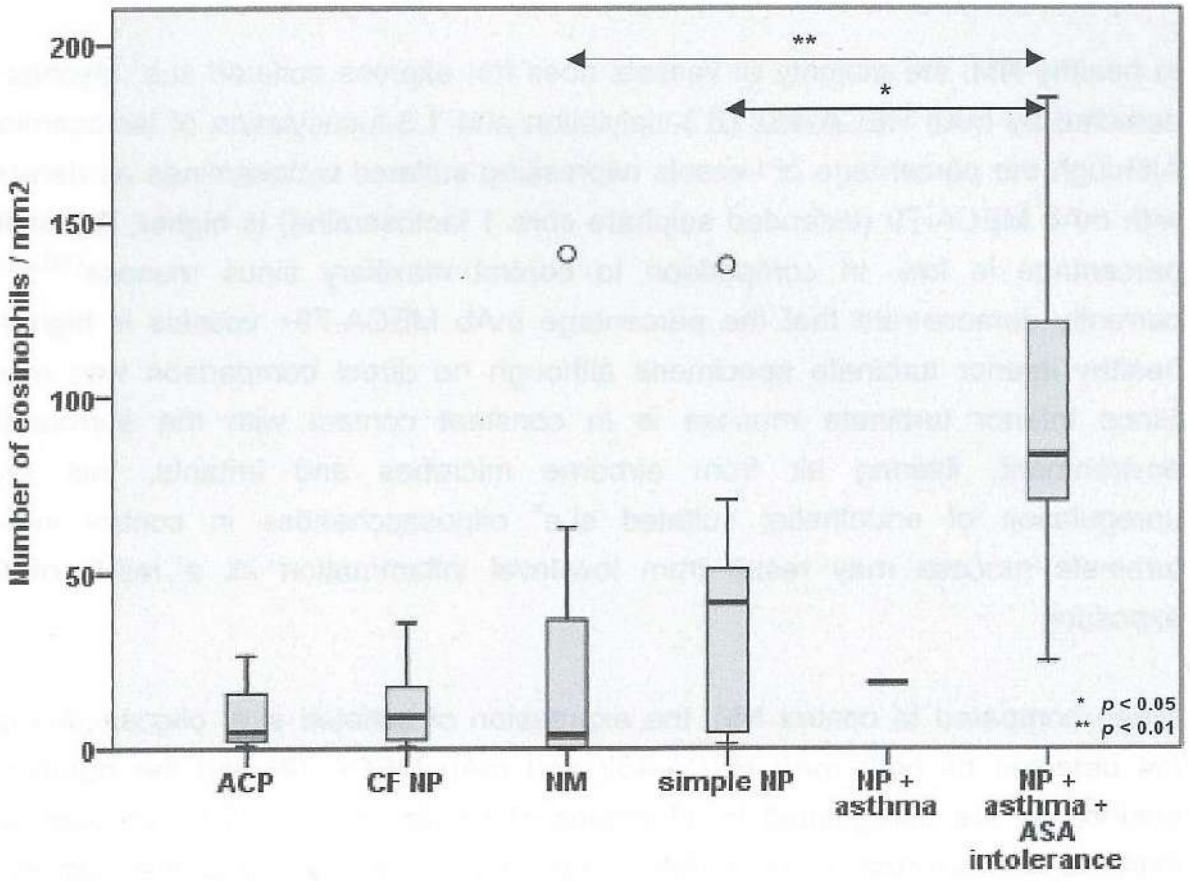
When studying the correlation between the percentage of mAb MECA-79+ vessels and the presence of leukocytes in this group of tissue specimens, a clear correlation between mAb MECA-79 positivity and the presence of eosinophils was observed ($p = 0.02$, $r = 0.39$, data not shown), thereby suggesting a possible role for sulphated endothelial sLe^x oligosaccharides to preferentially guide eosinophils to diseased sinonasal mucosa. No correlation was observed between the percentage of mAb MECA-79+ vessels and all other leukocyte subsets and no correlation was observed for mAb HECA-452+ vessels (data not shown).

Expression of leukocyte subsets and functionally active endothelial L-selectin ligands in control NM and simple NP of patients with or without asthma and/or ASA intolerance

Patients in the NM group were younger than those patients in all other groups ($p = 0.05$ when compared to simple NP, $p = 0.03$ when compared to simple NP with asthma, and $p = 0.04$ when compared to simple NP with asthma and ASA intolerance). In addition, patients with simple NP with asthma were older than those simple NP patients without co-morbidities and those simple NP patients with asthma and ASA intolerance ($p = 0.03$ and $p = 0.04$ respectively). Gender distribution was similar in all groups ($p = 0.49$).

As has been described above, simple NP (9 patients) are characterized by decreased numbers of CD34+ vessels when compared to control NM (20 patients). The percentage of vessels expressing sulfo sLe^x epitopes as analyzed with mAbs HECA-452 and MECA-79 was shown to be increased. Although a similar trend is observed for NP obtained from patients with asthma (2 patients) and patients with asthma and ASA intolerance (8 patients), the number of CD34+ vessels and the percentage of mAb HECA-452+ and mAb MECA-79+ CD34+ vessels is lower when compared to simple NP specimens obtained from patients without asthma and ASA intolerance (figures 1 and 2).

FIGURE 3. Absolute number of eosinophils per mm^2 in control nasal mucosa (NM), simple nasal polyps (simple NP), simple nasal polyps of patients with asthma (simple NP + asthma) and simple nasal polyps of patients with asthma and ASA intolerance (simple NP + asthma + ASA intolerance).



Although the number of eosinophils is significantly increased in polyps from patients with NP with asthma and ASA intolerance (figure 3), no correlation between mAb MECA-79 positivity and the presence of eosinophils in nasal polyp tissue is observed (data not shown). The percentage of mAb MECA-79+ vessels did not correlate with other subsets of leukocytes and no correlation was observed for mAb HECA-452+ vessels (data not shown).

DISCUSSION

All nasal polyps are characterized by a decrease in the number of CD34+ vessels per mm^2. This lower density of CD34+ vessels clearly reflects the edema observed

in all nasal polyps and is most obvious in those polyps obtained from simple NP patients with asthma and ASA intolerance as was shown previously by others[182].

In healthy NM, the majority of vessels does not express sulfated sLe^x glycans as detected by mAb HECA-452 (2,3-sialylation and 1,3-fucosylation of lactosamine). Although the percentage of vessels expressing sulfated lactosamines as detected with mAb MECA-79 (extended sulphate core 1 lactosamine) is higher, the overall percentage is low. In comparison to control maxillary sinus mucosa[181], we currently demonstrate that the percentage mAb MECA-79+ vessels is higher in healthy inferior turbinate specimens although no direct comparison was made. Since inferior turbinate mucosa is in constant contact with the surrounding environment, filtering air from airborne microbes and irritants, this slight upregulation of endothelial sulfated sLe^x oligosaccharides in control inferior turbinate mucosa may result from low-level inflammation as a result of this exposure.

When compared to control NM, the expression of sulfated sLe^x oligosaccharides (as detected by both mAb HECA-452 and mAb MECA-79) and the number of eosinophils are upregulated in all groups of simple NP. Similarly, although less extreme, the expression of sulfated sLe^x oligosaccharides and the number of eosinophils are upregulated in CF NP. All together, these data confirm previous data from our group, in which we showed *de novo* induction of sulfated sLe^x oligosaccharides in bronchial mucosa of asthmatic patients and maxillary sinus mucosa of patients suffering from chronic rhinosinusitis[179;181]. From these observations one may conclude that simple NP and CF NP share (even though tissue eosinophilia is more profound in simple NP) a common pathophysiological pathway.

In contrast to simple NP and CF NP, the expression of sulfated sLe^x oligosaccharides is extremely low in ACP. In addition, low numbers of eosinophils are observed. The number of neutrophils is however high. Based on these result, one may conclude that ACP are a distinct histopathological entity separate from both simple NP and CF NP.

Previously it was suggested that the amount of sulfated sLe^x oligosaccharides correlates with the presence of specific leukocyte subsets, especially eosinophils, in inflamed sinus mucosa. Although both eosinophils and sulfated sLe^x oligosaccharides are increased in simple NP, especially in those patients without asthma and ASA intolerance, thereby suggesting a correlation between functionally active L-selectin ligands and the presence of tissue eosinophilia, the percentage of functionally active L-selectin ligands decreases with disease severity in simple NP whereas the number of eosinophils clearly increases. In addition, although increased numbers of eosinophils are present in CF NP (as are sulfated sLe^x oligosaccharides), it is the tissue neutrophilia that is characteristic of CF NP. Taken together, these data suggest that functionally active L-selectin ligands are not a major determinant of tissue eosinophilia in nasal polyps. Since no correlation was observed between the amounts of sulfated sLe^x oligosaccharides and all other leukocytes subsets studied, it is highly unlikely that functionally active L-selectin ligands are key players in guiding a single leukocyte subset to diseased sinus mucosa. Sulfated sLe^x oligosaccharides may however play some role in guiding leukocytes (including eosinophils) to diseased sinonasal mucosa in nasal polyp patients. Their presence is however not rate-limiting, as can be concluded from the decrease in sulfated sLe^x oligosaccharides in simple NP obtained from patients with asthma and ASA intolerance.

CHAPTER 2.3

CYSTIC FIBROSIS NASAL POLYPS: INCREASED NUMBERS OF INTERLEUKIN-5 EXPRESSING CELLS WITHOUT MARKED TISSUE EOSINOPHILIA

Ebbens FA, Maldonado M, de Groot EJJ, Alobid I, van Drunen CM, Picado C, Fokkens WJ, and Mullol J, *Allergy (submitted for publication)*

ABSTRACT

Background: Nasal polyps (NP) frequently occur in patients with cystic fibrosis (CF). Although macroscopically remarkably similar to non-CF NP, controversy exists as to whether these polyps should be considered distinct histopathological entities.

Methods: Immunohistochemical analysis of various leukocytes (eosinophils, neutrophils, basophils, mastcells, and macrophages), mediators (IL-4, IL-5, IL-6, and eotaxin) and adhesion molecules (VCAM-1) was performed on healthy nasal mucosa, non-CF NP and CF NP.

Results: Increased numbers of macrophages and neutrophils and an increase in the number of IL-6 expressing cells are striking features of CF NP. Although eosinophils are more abundant in non-CF NP, differences between both groups are statistically not significant. In contrast to what was expected, an upregulation of IL-5 expressing cells is characteristic of CF NP only.

Conclusion: Eosinophils are clearly present in higher numbers in non-CF NP than in CF NP but large variations are observed. Differentiating non-CF NP from CF NP based on the level of tissue eosinophils alone may thus be hard. We suggest that the level of neutrophils is more suitable for discriminating non-CF NP from CF NP. Interestingly, we show that CF NP are characterized by elevated levels of IL-5 expressing cells and that this increase is not linked to a similar increase in tissue eosinophils.

INTRODUCTION

Nasal polyposis (NP) is a multifactorial disease, often associated with asthma, acetyl salicylic acid (ASA) intolerance and possibly allergy, affecting between 1 and 4% of individuals in the general population[15;25] and up to 90% of patients suffering from Cystic Fibrosis (CF)[42-45;151;152]. Although many hypotheses have been suggested, the pathogenesis of NP is still largely unknown.

Macroscopically, nasal polyps are characterized by the presence of edematous masses of inflamed mucosa that are predominantly localized in the middle meatus and prolaps into the nose, leading to nasal obstruction, secretion, loss of smell, headache/facial pain, and reduced quality of life[1;2]. Histologically, NP are characterized by a cover of respiratory epithelium, large quantities of extracellular edema and a dense inflammatory cell infiltrate consisting of eosinophils, neutrophils, mast cells, lymphocytes, macrophages and plasma cells, that release a variety of pro-inflammatory mediators, including cytokines, histamine, prostanoids and leukotrienes[1;142;183].

Although macroscopically remarkably similar, careful analysis of the literature reveals important histopathological differences between non-CF NP and CF NP. The majority of non-CF NP are characterized by high numbers of tissue eosinophils[1], whereas CF NP are classically said to be neutrophilic[154;156;157]. However, various authors have recently demonstrated that, besides neutrophils, a fair amount of eosinophils is present in CF NP[154;184]. As a consequence, the statement of non-CF NP being typically eosinophilic and of CF NP being typically neutrophilic may be false.

With the objective of clarifying the role of eosinophils and neutrophils in the pathogenesis of both non-CF NP and CF NP we studied the number of eosinophils and neutrophils in both polyp subgroups and compared these to the number of eosinophils and neutrophils in control inferior turbinate specimens. Besides eosinophils and neutrophils, the number of macrophages, basophils and mast cells was determined in all tissue specimens. In addition, the number of cells producing the pro-inflammatory cytokine interleukin (IL) 6, the T_H2-related cytokines IL-4 and

IL-5, the chemokine eotaxin, and vascular adhesion molecule-1 (VCAM-1) was determined.

MATERIAL AND METHODS

Subjects

Non-CF NP (n = 9) and CF NP (n = 8) were obtained from patients undergoing Endoscopic Sinus Surgery (ESS). Prior to surgery a full ENT history was taken. None of the patients suffered from asthma, ASA intolerance and/or allergy and none was treated with either (nasal) topical and/or oral glucocorticoids. Diagnosis of CF was established on the basis of clinical features, a positive sweat test, and/or characteristic genotype abnormalities. Diagnosis of asthma was based on clinical features and when necessary on pulmonary function tests. Diagnosis of ASA intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAID's). Diagnosis of allergy was based on Skin Prick Test (SPT) positivity. Samples of healthy nasal mucosa (NM, n = 18) were obtained from patients undergoing corrective nasal surgery for turbinate hypertrophy or septoplasty. None of the patients of this control group suffered from allergy, asthma or ASA intolerance and none was treated with glucocorticoids.

Tissue handling

All specimens, obtained at the time of surgery, were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemistry

Immunohistochemical staining of CD68, c-KIT (CD117), MBP, VCAM-1 (CD106), tryptase, elastase and IgE

6-µm thick serial sections of all snap-frozen specimens were cut on a Microm HM560 frigocut cryostat and transferred to APES (amino-phosphate-ethylsilane) coated slides (Starfrost, MarketLab, Caledonia, MI, USA), dried, and stored at -70°C until analysis. Upon analysis, tissue sections were thawed to room

temperature, dried and fixed in acetone (cold as ice) for 10 minutes. After fixation, tissue sections were rinsed with phosphate-buffered saline (PBS, pH 7.8), placed in a semi-automatic stainer (Shandon Sequenza, Waltham, MA, USA) and incubated with normal goat serum (CLB, Amsterdam, the Netherlands) for 10 minutes. Following the blocking procedure, sections were incubated with primary antibody for 60 minutes at room temperature. Mouse anti-human monoclonal antibodies directed against CD68, c-KIT (CD117), MBP, VCAM-1 (CD106), tryptase, elastase, and IgE were used (table 1). All primary antibodies were diluted in PBS containing 1% (w/v) blocking reagent (10961760, Roche, Basel, Switzerland) to block endogenous avidin and biotin activity. Following incubation with primary antibody, sections were rinsed with PBS for 5 minutes and incubated with biotinylated secondary goat anti-mouse antiserum (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Next, sections were rinsed with PBS and incubated with streptavidin alkaline phosphatase (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Sections were then rinsed with PBS containing TRIS (tris(hydroxymethyl)-aminomethane) buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous alkaline phosphatase (AP) enzyme activity) for 20 minutes at room

TABLE 1. Monoclonal antibodies

Antibody	Specificity	Cell type/ cytokine	Titer	Source
KI-M6	CD68	Macrophages	1:200	DAKO, Glostrup, Denmark
G3	Tryptase	Mast cells	1:200	Chemicon, Temecula, CA, USA
YB5.B8	CD117	Mast cells	1:65	Becton Dickinson, the Netherlands
BB1	Basogranulin	Basophils	1:150	A.F. Walls, South Hampton, UK
AHN-10	Elastase	Neutrophils	1:32000	Chemicon, Temecula, CA, USA
MH25-1	IgE	IgE	1:750	CLB, Amsterdam, the Netherlands
BMK-13	MBP	Eosinophils	1:100	Sanbio, Uden, the Netherlands
IL-4 1-1	IL-4	IL-4	1:150	Novartis, Basel, Switzerland
IL-5	IL-5	IL-5	1:100	Tavernier, Ghent, Belgium
IL-6	IL-6	IL-6	1:50	Sigma, Zwijndrecht, the Netherlands
Eotaxin	Eotaxin	Eotaxin	1:500	R&D Systems, Abingdon, Oxon, UK
VCAM-1	VCAM-1	VCAM-1	1:100	Sanbio, Uden, the Netherlands

temperature. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with the appropriate isotype control antibody.

Tyramide signal amplification (TSA) staining for basogranulin, IL-4, IL-5, IL-6, and eotaxin

A sensitive protocol was used based on the alkaline phosphatase method described above. After the first blocking step with normal goat serum, slides were incubated with mouse anti-human monoclonal antibodies directed against basogranulin, IL-4, IL-5, IL-6, and eotaxin diluted in PBS containing 1% (w/v) blocking reagent (table 1). After incubation with biotinylated goat anti-mouse antiserum, endogenous peroxidase was blocked using 0.2% (w/v) azide, 0.02% (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Following the blocking procedure, slides were incubated with streptavidin conjugated peroxidase (NEN, Waltham, MA, USA) for 30 minutes at room temperature, rinsed with PBS and incubated with biotinyl tyramide in TRIS buffer for 10 minutes in order to amplify the signal. Next, slides were rinsed once again in PBS and incubated with alkaline phosphatase conjugated goat-anti-biotin antiserum (Sigma, Zwijndrecht, the Netherlands). Sections were then rinsed with PBS containing TRIS buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20 minutes at room temperature. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with the appropriate isotype control antibody.

Light microscope evaluation

All sections were examined with an Olympus BX51 light microscope by 2 independent observers blinded to the experimental conditions. The number of positively stained cells was counted in the epithelium (per mm) and adjacent lamina propria (per mm^2) at a final magnification of 200x. Results are expressed as the mean number of positive cells per mm or mm^2.

Data analysis

Statistical analysis was carried out by using SPSS 12.01 (Chicago, IL, USA). Data are expressed as median and interquartile range (median, interquartiles 25-75). The Kruskal-Wallis non-parametric test was performed to check for statistically significant in between-group variability. In case of statistically significant in between-group variability, the Mann-Whitney-U non-parametric test was performed for between-group comparisons. Spearman rank order correlation was used to assess bivariate associations. The correlation value (rho) indicates the strength of correlation and varies between 0 and 1 in which 1 means a perfect correlation. Two-tailed p values of less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

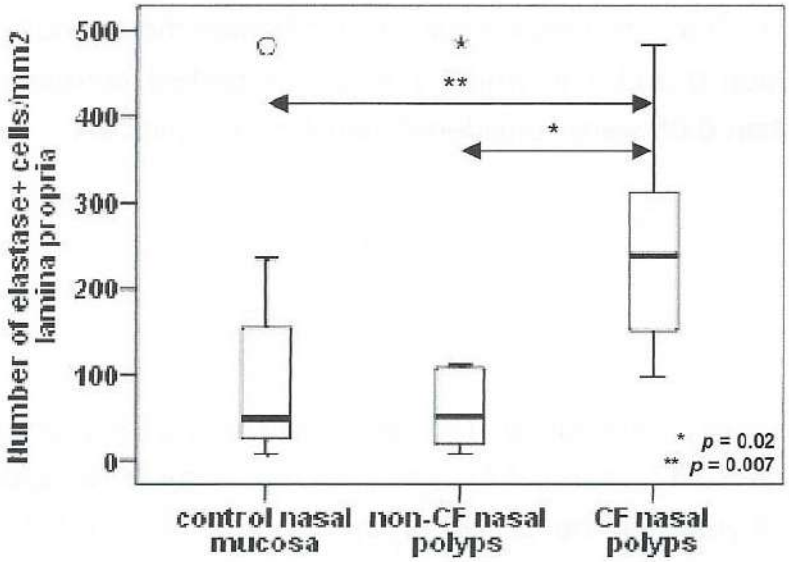
Thirty-five patients were enrolled in this study. The mean age was 31.6 years for the healthy NM group (range 20-47 years), 47.6 years for the non-CF NP group (range 36-56 years) and 12.9 years for the CF NP group (range 7-18 years). Male subjects were more common in the control group (80%) and CF NP group (75%) as compared to the non-CF NP group (44%). None of the patients in the NM, non-CF NP, and CF NP groups suffered from allergy, asthma or ASA intolerance and none was treated with (nasal) topical and/or oral corticosteroids for at least 4 weeks prior to inclusion.

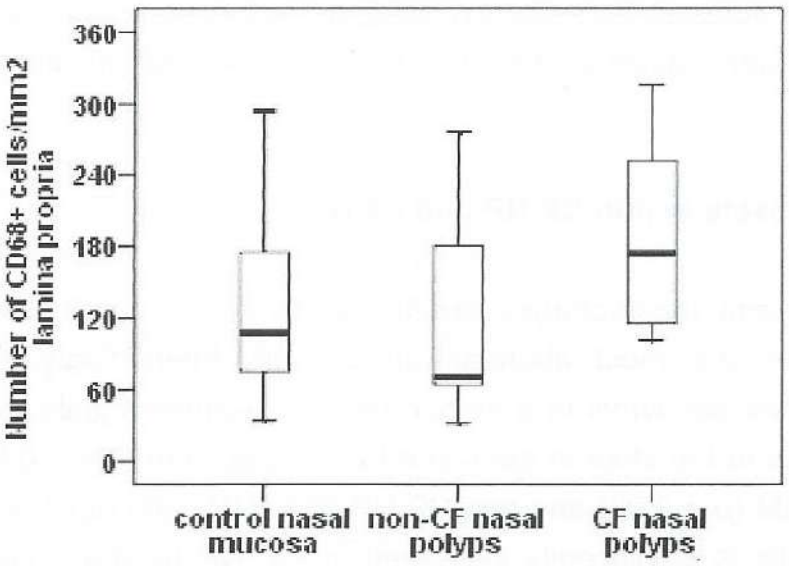
Presence of leukocyte subsets in non-CF NP and CF NP

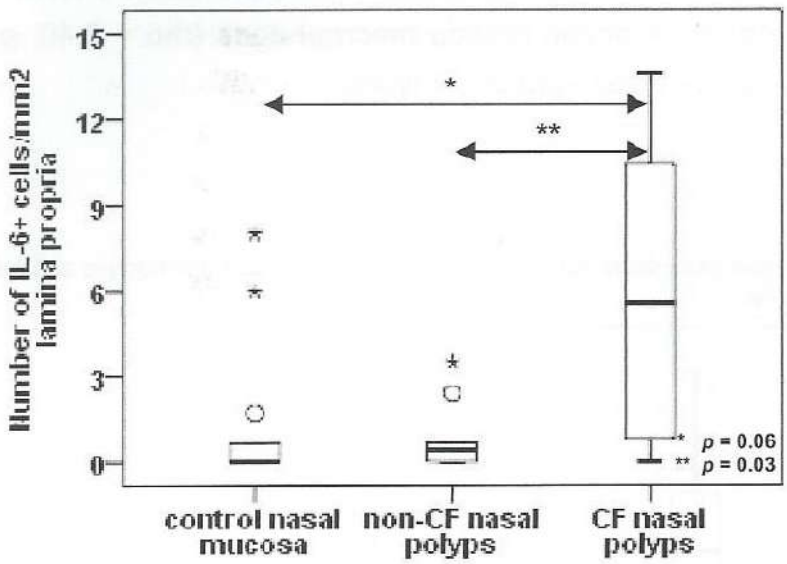
Although both neutrophils and macrophages are frequently found in all tissue specimens, both cell types are most abundant in CF NP. Interestingly, the presence of one of these two cell types in a certain tissue specimen significantly correlated with the presence of the other in the same tissue specimen (rho = 0.62, $p < 0.001$). Compared to NM ($p = 0.02$) and non-CF NP ($p = 0.01$), the number of elastase positive neutrophils is significantly increased in CF NP lamina propria (figures 1 (appendix 2) and 2). MBP+ eosinophils are present in both non-CF NP and CF NP. When compared to NM, their numbers are increased in both polyp

subgroups. Although MBP+ eosinophils are most abundant in non-CF NP, no statistically significant differences are observed between non-CF NP and CF NP in the number of MBP+ eosinophils (figures 1 (appendix 2) and 3). Mast cells

FIGURE 2. Increased numbers of neutrophils, macrophages and IL-6 positive cells are characteristic findings in CF NP inflammation







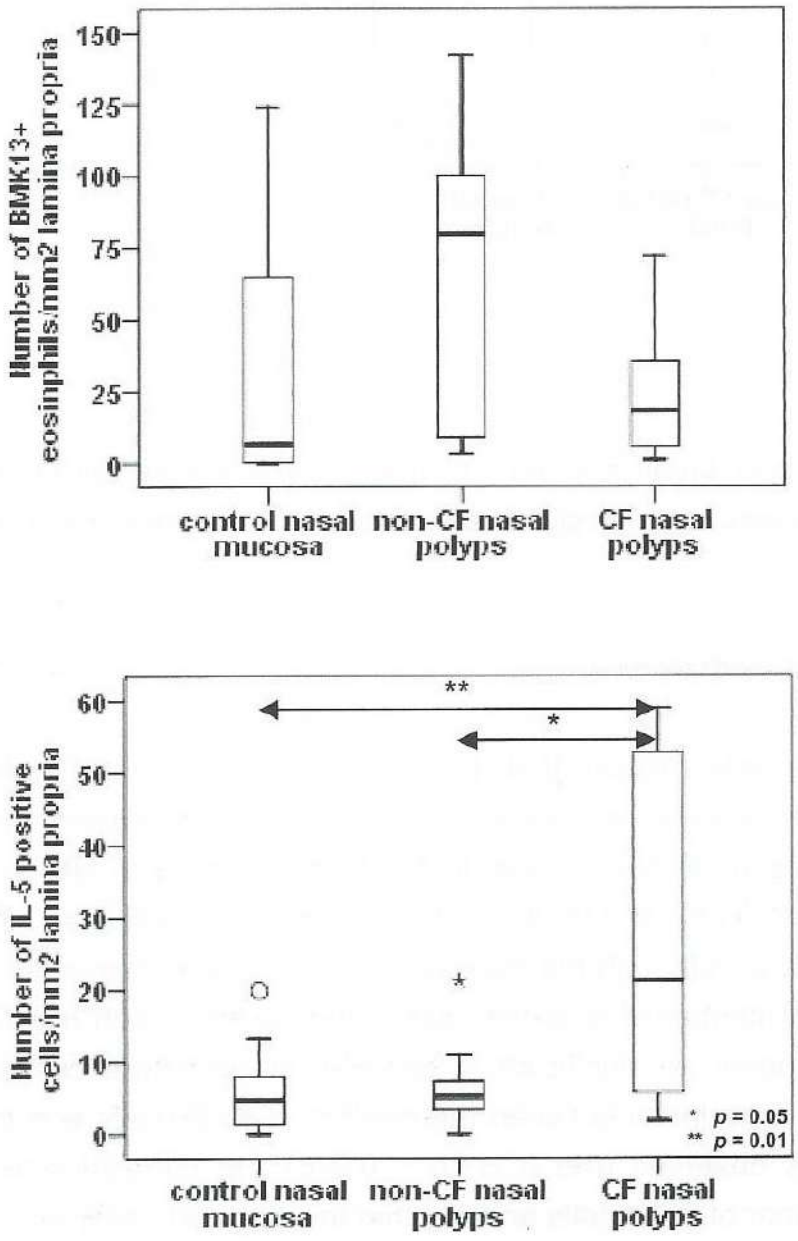
(tryptase+ CD117+ cells) and small numbers of basophils (basogranulin+) are present in all tissue specimens with no significant differences between the three groups.

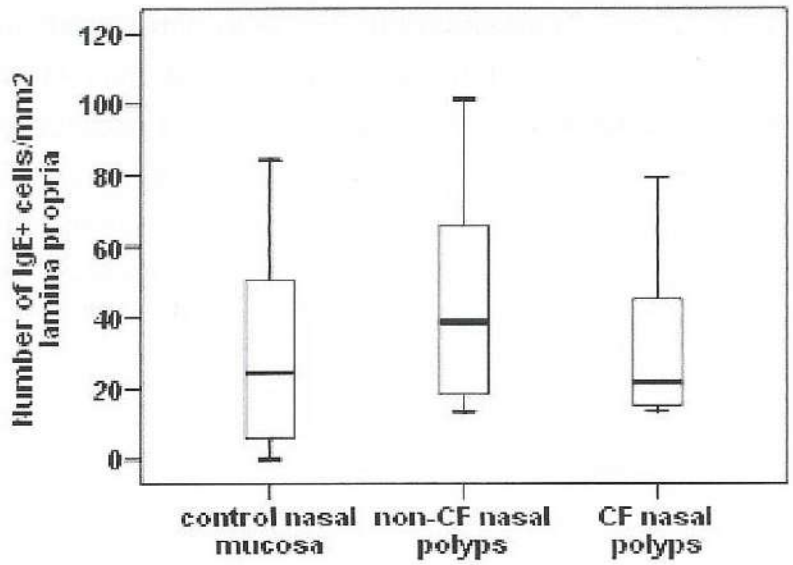
Presence of inflammatory mediators

In the epithelium, very few cells express IL-4, IL-5, IL-6, and/or eotaxin. For NP lamina propria, the number of cells positive for IL-5 and IL-6 is increased. This observation is most obvious in CF NP for both IL-5, when compared to NM (p = 0.01) and to non-CF NP (p = 0.05), and for IL-6, when compared to NM (p = 0.03) and to non-CF NP (p = 0.06). Although the number of IL-4+ cells is increased in NM lamina propria and the number of eotaxin+ cells is increased in both non-CF NP and CF NP lamina propria, no significant in between group differences are observed. A trend towards correlation between the number of IL-5+ cells and the number of neutrophils was observed (rho 0.31, p = 0.078). No correlation was observed between the number of IL-5+ cells and all other investigated cell types,

notably not for eosinophils. Increased numbers of IL-6+ cells were shown to correlate with increased numbers of lamina propria macrophages (rho = 0.40, p = 0.023) but not with any of the other investigated cell types.

FIGURE 3. The number of IL-5 positive cells does not correlate with the number of eosinophils and IgE+ cells in CF nasal polyp lamina propria





Presence of VCAM-1 positive vessels

On average, 7.2 VCAM-1 positive vessels are present in NM lamina propria per mm^2 (median 7.2, IQ range 3.38-15.20). Although the number of VCAM-1 positive vessels was decreased in both non-CF NP (median 3.80, IQ range 2.60-10.00) and CF NP (median 2.20, IQ range 0.90-3.50), differences were statistically not significant.

DISCUSSION

In our present study we demonstrate that high levels of neutrophils and macrophages are present in all tissue specimens. When compared to both NM and non-CF NP, a significant increase in the number of elastase positive neutrophils is observed for CF NP. Besides a significant increase in the number of neutrophils, CF NP are characterized by an increase in the number of IL-6 positive cells. IL-6 is a multifunctional cytokine that is, amongst others, involved in acute and chronic inflammation[185]. As has been shown previously, increased numbers of neutrophils, macrophages, and IL-6 are important features of chronic airway inflammation and are characteristic findings in CF lung inflammation[186] as well as in CF NP inflammation[77].

Besides neutrophils and macrophages, we demonstrate that both non-CF NP and (to a lesser extent) CF NP contain a fair number of eosinophils in their lamina propria. However, in contrast to most studies[156;157;187], although tissue eosinophilia was most profound in non-CF NP, we did not observe a statistically significant difference in the number of eosinophils in NP lamina propria between both polyp groups. This lack of significance may result from the fact that only 9 non-CF NP patients and 8 CF NP patients were included in this study.

Although similar in some respects, several clear distinctions between CF NP and non-CF NP were observed. The number of IL-5 positive cells was increased significantly in the lamina propria of CF NP but not of non-CF NP when compared to NM. This increase in IL-5 positive cells (seen without a significant increase in tissue eosinophilia) has not been demonstrated previously. Although many authors have reported an increase in IL-5 and eosinophils as being characteristic of non-CF NP, based on our data one may conclude that previous results may have been partially biased by the inclusion of patients with allergy, asthma and/or ASA intolerance [66;77;139;141;188;189]. Increased IL-5 has long been associated with allergy and asthma. Both allergy and asthma in turn are linked to an increase in the number of circulating, airway tissue, and induced sputum eosinophil numbers. Thus, the presence of allergy and/or asthma may obscure histological results (i.e. high levels of tissue eosinophils) presented in many non-CF NP studies.

Although lower levels of eosinophils are observed in CF NP, high levels of IL-5 seen without a marked tissue eosinophilia do not exclude a causative role for eosinophils in the pathogenesis of CF NP. As was shown by Koller et al, high levels of eosinophil granule proteins are present in serum and sputum of most CF patients but these proteins are relatively absent in peripheral blood and in the lung[190]. Although less abundant, these CF eosinophils were shown to have an increased propensity to release their toxic granule proteins[191]. Thus, relatively few eosinophils may ultimately lead to the formation of NP in CF patients as well.

Besides being a cytokine involved in eosinophil recruitment, maturation and activation, recent studies suggest that IL-5 may also be involved in the recruitment of neutrophils[192]. Although IL-5 is unable to directly act on neutrophils (neutrophils lack an IL-5 receptor on their surface), anti-IL-5 treatment has been shown to block

neutrophil accumulation *in vivo*[192]. This blockage of neutrophil accumulation by anti-IL-5 was shown to involve granulocyte colony stimulating factor (G-CSF), tumor necrosis factor alpha (TNF-α), and IL-8 (all neutrophil chemotactic cytokines)[192]. Thus, IL-5 may be an essential cytokine for neutrophil-mediated inflammation in CF NP. In support of this hypothesis is the lack of increase in VCAM-1, an endothelial adhesion molecule involved in lymphocyte, monocyte, and eosinophil, but not neutrophil adhesion to activated endothelium, and only slight increase in eotaxin, a chemokine that has potent chemotactic activity for eosinophils, basophils, mastcells, T_H2 lymphocytes, but not neutrophils in both non-CF NP and CF NP. Also in support, although not significant, is a trend towards correlation between the presence of lamina propria neutrophils and the level of IL-5.

In conclusion, this study confirms the suggestion by Rowe-Jones et al[154] that the statement of non-CF NP being typically eosinophilic and CF NP being typically neutrophilic may need to be reconsidered. Although eosinophils are clearly present in higher numbers in non-CF NP than in CF NP, we did not observe a statistically significant difference between both NP groups. Differentiating non-CF NP from CF NP based on the level of tissue eosinophil may thus be incorrect. Based on our results we suggest that the level of lamina propria neutrophils is more suitable in discriminating non-CF NP from CF NP. Interestingly, we show that CF NP are characterized by a significant elevation of lamina propria IL-5 positive cells which is not linked to a similar increase in tissue eosinophils, questioning a crucial role for IL-5 in the pathogenesis of non-CF NP.

CHAPTER 3

THE ROLE OF TOPICAL GLUCOCORTICOIDS

CHAPTER 3.1

TOPICAL GLUCOCORTICOIDS DOWNREGULATE COX-1 POSITIVE CELLS IN NASAL POLYPS

Ebbens FA, Maldonado M, de Groot EJJ, Alobid I, van Drunen CM, Picado C, Fokkens WJ, and Mullol J, *Allergy 2009;64:96-103*

ABSTRACT

Rationale: Influx of inflammatory cells is one of the hallmarks of nasal polyposis. Since glucocorticoids (GC) are known to exhibit strong anti-inflammatory effects, these drugs are frequently used in the treatment of the disease. Part of the anti-inflammatory effects of GC is attributed to their interference with prostanoid synthesis. Since cyclo-oxygenases (COX) are key enzymes in the synthesis of both pro- (COX-1, COX-2) and anti-inflammatory prostanoids (COX-2), we investigated the role of topical GC on COX-1, COX-2 and inflammatory markers in nasal polyps (NP).

Methods: Immunohistochemical analysis of inflammatory markers (CD68, CD117, MBP, elastase, IgE, basogranulin, IL-4, IL-5 and IL-6), COX-1 and COX-2 was performed on control nasal mucosa (NM) (n = 18), non-GC treated NP (n = 27) and topical GC treated NP (n = 12). NP groups were matched for allergy, asthma and ASA intolerance.

Results: Increased numbers of eosinophils, IL-5+ cells and IgE+ cells and decreased numbers of mastcells are striking features of NP inflammation ($p < 0.05$). In addition, increased numbers of COX-1+ cells are observed in NP epithelium when compared to NM ($p < 0.05$). No significant reduction in the number of eosinophils is observed in GC treated NP. Unexpectedly, the number of IL-5+ cells is increased significantly upon GC treatment ($p < 0.05$). Topical GC significantly downregulate the number of COX-1+ cells (but not the number of COX-2+ cells) in NP epithelium.

Conclusion: Inflammation in NP is characterized by an influx of eosinophils, increased numbers of IL-5+ and IgE+ cells and is associated with an increase in the number of COX-1+ cells. Topical GC significantly downregulate the number of COX-1+ cells in NP epithelium, thereby possibly restoring the balance between pro- and anti-inflammatory prostanoids, resulting in reduced levels of inflammatory cells and ultimately resolution of disease.

INTRODUCTION

Nasal polyposis is a multifactorial disease, associated with asthma, primary ciliary dyskinesia, acetyl salicylic acid (ASA) intolerance and possibly allergy, affecting between 1 and 4% of the general population[15;25]. Although many hypotheses have been suggested, the pathogenesis of nasal polyposis is still largely unknown.

Macroscopically, nasal polyps are characterized by the presence of edematous masses of inflamed mucosa that are predominantly localized in the middle meatus and prolaps into the nose, leading to nasal obstruction, secretion, loss of smell, headache/facial pain, and reduced quality of life[1;2].

Histologically, nasal polyps are characterized by a cover of respiratory epithelium, large quantities of extracellular edema and a dense inflammatory cell infiltrate consisting of mast cells, eosinophils, lymphocytes, neutrophils and plasma cells, that release a variety of pro-inflammatory mediators, including cytokines, histamine, prostanoids and leukotrienes[1].

In recent years, various randomized controlled trials have demonstrated the effectiveness of topical GC in the treatment of primary and recurrent nasal polyposis[88-96]. However, the mechanisms underlying the anti-inflammatory and immunoregulatory effects of these drugs *in vivo* remain to be fully explained.

GC are known to exhibit anti-edematous and strong anti-inflammatory effects that are in part attributed to their interference with prostanoid synthesis[109]. Cyclo-oxygenases are key enzymes in the generation of prostanoids from arachidonic acid and exist in at least two isoforms: cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2). COX-1 is considered to be expressed constitutively in all cells, whereas COX-2 is considered to be induced under inflammatory conditions. Since both isoforms are expressed in nasal polyposis and since prostanoids are known to control chemotaxis of inflammatory cells, vascular tone, vascular permeability and mucous secretion (all hallmarks of nasal polyposis)[193;194], their possible involvement in the pathogenesis of nasal polyposis has long been studied.

Since COX-2 is presumed to be induced under pro-inflammatory conditions, most studies focused on the role of this enzyme in the pathogenesis of nasal polyposis. Recent studies indicate however, that mediators generated by COX-2 can both promote and inhibit inflammation[195]. Since COX-1 is expressed in nasal polyps as well, we postulate that elevated levels of COX-1 (and not COX-2) are responsible for the relative predominance of pro-inflammatory prostanoids in nasal polyposis. In this study we investigated the role of both enzymes in nasal polyp inflammation and studied the effect of topical glucocorticoid treatment on nasal polyp inflammation.

MATERIAL AND METHODS

Subjects

NP (n = 39) were obtained from patients undergoing Endoscopic Sinus Surgery (ESS). Prior to surgery a full ENT history was taken. Diagnosis of asthma was based on clinical features and when necessary on pulmonary function tests. Diagnosis of ASA intolerance was made on the basis of a clear-cut history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs (NSAID's). Diagnosis of allergy was based on Skin Prick Test (SPT) positivity. Presence or absence of treatment with topical and/or oral glucocorticoids was recorded. Those patients treated with oral glucocorticoids were excluded. Those patients (n = 12) treated with a topical glucocorticoid spray (either fluticasone or budesonide 200-

TABLE 1. Characteristics of the study population

	Control nasal mucosa (n = 18)	Non-steroid treated NP (n = 27)	Steroid treated NP (n = 12)
Age (y), mean (SD)	31.6 (8.6)	45.6 (10.5)	48.1 (15.6)
Male (n, %)	14 (78.8%)	14 (52%)	5 (42%)
Asthma (n, %)	0 (0%)	13 (48%)	6 (50%)
ASA intolerance (n, %)	0 (0%)	11 (41%)	4 (33%)
Allergy (n, %)	0 (0%)	9 (33%)	5 (42%)

800 µg/day) for a period of at least four weeks prior to inclusion were included in the GC treated NP group. Those patients not treated with topical glucocorticoids in the four weeks prior to inclusion were included in the non-GC treated NP group (n = 27) (tables 1 and 2). Samples of healthy nasal mucosa (n = 18) were obtained from patients undergoing corrective surgery for turbinate hypertrophy or septoplasty. None of the patients in the control group had a history of allergy, asthma, ASA intolerance, or glucocorticoid treatment (table 1).

Tissue handling

All specimens, obtained at the time of surgery, were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemistry

Immunohistochemical staining of CD68, CD117, MBP, elastase, IgE and COX-1
6-µm thick serial sections of all snap-frozen specimens were cut on a Microm HM560 frigocut cryostat and transferred to APES (amino-phosphate-ethylsilane) coated slides (Starfrost, MarketLab, Caledonia, MI, USA), dried and stored at -70°C until analysis. Upon analysis, tissue sections were defrosted to room temperature, dried and fixed in acetone (cold as ice) for 10 minutes at room temperature. After fixation, tissue sections were rinsed with phosphate-buffered saline (PBS, pH 7.8), placed in a semi-automatic stainer (Shandon Sequenza, Waltham, MA, USA) and incubated with normal goat serum (CLB, Amsterdam, the Netherlands) for 10 minutes. Following the blocking procedure, sections were incubated with primary antibody for 60 minutes at room temperature. Mouse anti-human monoclonal antibodies directed against CD68, CD117, major basic protein (MBP), elastase, IgE and COX-1 were used (table 3). All primary antibodies were diluted in PBS containing 1% (w/v) blocking reagent (10961760, Roche, Basel, Switzerland) to block endogenous avidin and biotin activity. Following incubation with primary antibody, sections were rinsed with PBS for 5 minutes and incubated with biotinylated goat anti-mouse antiserum (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Next, sections were rinsed with

TABLE 2. Scheme of topical glucocorticoid spray used*

Dosage	Fluticasone	Budesonide
200 µg once daily	3	2
200 µg twice daily	1	5
400 µg twice daily	0	1

* used for at least one month prior to biopsy (n = 12)

PBS and incubated with streptavidin alkaline phosphatase (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Sections were then rinsed with PBS containing TRIS (tris(hydroxymethyl)aminomethane) buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous alkaline phosphatase (AP) enzyme activity) for 20 minutes at room temperature. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with PBS.

Tyramide signal amplification (TSA) staining for basogranulin, IL-4, IL-5, IL-6 and COX-2

A sensitive protocol was used based on the alkaline phosphatase method described above. Sections were cut and defrosted as described above. Sections were fixed in acetone (cold as ice) for 10 minutes, placed in the semi-automatic stainer and incubated with normal goat serum as described above. Slides were incubated with mouse anti-human monoclonal antibodies directed against basogranulin, IL-4, IL-5, IL-6 or COX-2 diluted in PBS containing 1% (w/v) blocking reagent (table 3). After incubation with biotinylated goat anti-mouse antiserum, endogenous peroxidase was blocked using 0.2% (w/v) azide, 0.02% (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Following the blocking procedure, slides were incubated with streptavidin conjugated peroxidase (NEN, Waltham, MA, USA) for 30 minutes at room temperature, rinsed with PBS and incubated with biotinyl tyramide in TRIS buffer for 10 minutes in order to amplify the signal. Next, slides

TABLE 3. Monoclonal antibodies

Antibody	Specificity	Cell type/ cytokine	Titer	Source
KI-M6	CD68	Macrophages	1:200	DAKO, Glostrup, Denmark
YB5.B8	CD117	Mastcells	1:65	Becton Dickinson, the Netherlands
BB1	Basogranulin	Basophils	1:150	A.F. Walls, South Hampton, UK
MH25-1	IgE	IgE	1:750	CLB, Amsterdam, the Netherlands
BMK-13	MBP	Eosinophils	1:100	Sanbio, Uden, the Netherlands
AHN-10	Elastase	Neutrophils	1:320	Chemicon, Temecula, CA, USA
IL-4 1-1	L-4	IL-4	1:150	Novartis, Basel, Switzerland
IL-5	IL-5	IL-5	1:100	Tavernier, Ghent, Belgium
IL-6	IL-6	IL-6	1:50	Sigma, Zwijndrecht, the Netherlands
MH25-1	IgE	IgE	1:750	CLB, Amsterdam, the Netherlands
COX-1	COX-1	COX-1	1:150	Sanvertech, the Netherlands
COX-2	COX-2	COX-2	1:50	Sanvertech, the Netherlands

were rinsed once again in PBS and incubated with alkaline phosphatase conjugated goat-anti-biotin antiserum (Sigma, Zwijndrecht, the Netherlands). Sections were then rinsed with PBS containing TRIS buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20 minutes at room temperature. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with PBS.

Light microscope evaluation

All sections were examined with an Olympus BX51 light microscope by 2 independent observers blinded to the experimental conditions. The number of positively stained cells was counted in the epithelium (per mm) and adjacent lamina propria (per mm^2) at a final magnification of 200x. Results are expressed as the mean number of positive cells per mm or mm^2.

Data analysis

Statistical analysis was carried out by using SPSS 12.01 (Chicago, IL, USA). Data are expressed as median and interquartile range. Kruskal-Wallis non-parametric tests were performed to check for significant in between-group variability. In case of significant in between-group variability, Mann-Whitney-U non-parametric tests were performed for between-group comparisons. *P* values of less than 0.05 were considered statistically significant.

RESULTS

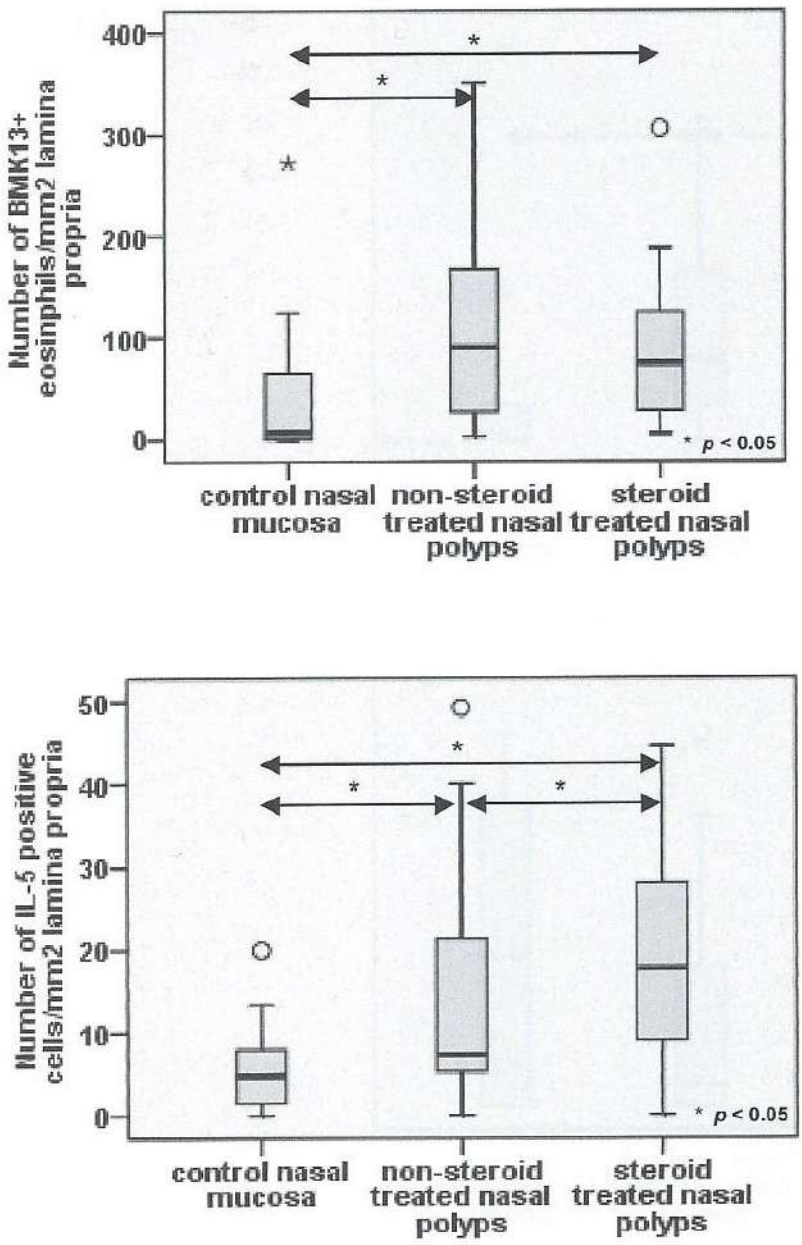
Patient characteristics

No significant differences in patient characteristics were observed between non-GC treated and GC treated NP patients. Treatment regimens of those patients treated with topical GC are described in table 2. The prevalence of asthma, allergy and ASA intolerance was similar in both treatment arms (table 1). None of the patients in the NM group suffered from asthma, allergy and/or ASA intolerance and none was treated with GC.

Normal nasal mucosa compared to non-GC treated nasal polyps

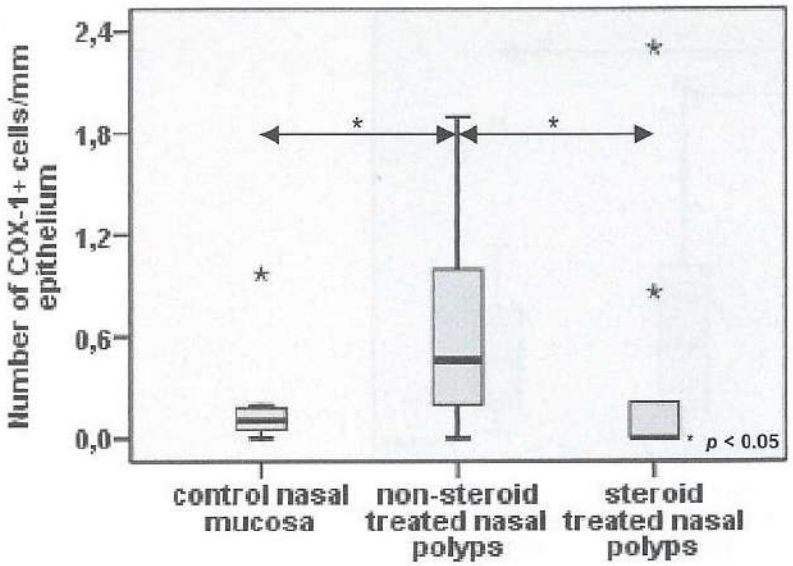
When comparing the presence of allergic mediators (IgE, IL-4) between NM and non-GC treated NP, it is clear that the number of IgE+ cells, but not IL-4+ cells, is significantly increased in NP epithelium and increased, although not significantly, in NP lamina propria. Compared to NM lamina propria, the number of cells positive for IL-5 and IL-6 (cytokines involved in eosinophil and neutrophil recruitment respectively) is increased in non-GC treated NP (figure 1). As one would expect, the number of eosinophils is markedly increased in both the epithelium and lamina propria of non-GC treated NP when compared to NM. For lamina propria, this increase is significant (figure 1, figure 2b (appendix 3)). The number of neutrophils is slightly increased in non-GC treated NP lamina propria, but this increase is not significant. The number of mast cells is decreased in both epithelium and lamina propria of non-GC treated NP when compared to NM. For lamina propria, this decrease is significant. No differences are observed in the number of macrophages

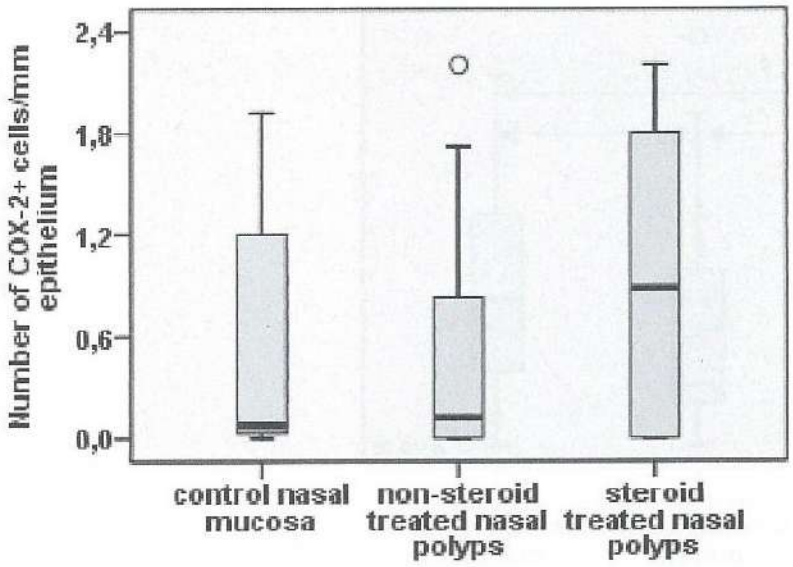
FIGURE 1. Influence of glucocorticoids on expression levels of eosinophils (BMK13+) and interleukin 5 (IL-5) in the lamina propria of normal nasal mucosa and nasal polyps



and basophils present. Compared to NM, the number of COX-1+ cells, but not COX-2+ cells, is significantly increased in non-GC-treated NP epithelium (figures 2a and 2b (appendix 3)). No significant differences are observed in the number of COX-1+ and COX-2+ cells in NM and non-GC treated NP lamina propria (tables 4a and 4b).

FIGURE 2a. Influence of glucocorticoids on expression levels of COX-1 and COX-2 in the epithelium of normal nasal mucosa and nasal polyps





Non-GC treated nasal polyps compared to topical GC treated nasal polyps

When comparing GC-treated NP with non-GC-treated NP, no significant differences are observed in the number of IgE+ cells and IL-4+ cells in both NP

TABLE 4a. Number of positive cells in the per mm

	Control nasal mucosa (n = 18)	Non-steroid treated NP (n = 27)	Steroid treated NP (n = 12)
Macrophages (CD68+)	2.3 (1.6-2.6)	1.3 (0.5-2.2)	1.7 (1.2-2.4)
Mast cells (CD117+)	1.8 (0.3-3.8)	0.9 (0.0-3.1)	4.0 (0.12-6.1)
Eosinophils (MBP+)	0.0 (0.0-2.1)	1.0 (0.1-1.9)	0.0 (0.0-1.8)
Basophils (basogranulin+)	0.0 (0.0-0.3)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Neutrophils (elastase+)	3.1 (0.3-32.5)	1.7 (0.0-13.1)	9.3 (0.7-18.1)
IL-4	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
IL-5	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
IL-6	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.0 (0.0-0.1)
IgE	0.0 (0.0-0.5)[a]	2.4 (0.4-9.1)[a]	2.5 (0.0-11.1)
COX-1	0.1 (0.1-0.2)[a]	0.5 (0.2-1.0)[a,c]	0.0 (0.0-0.4)[c]
COX-2	0.1 (0.0-1.5)	0.1 (0.0-1.0)	1.0 (0.0-2.0)

TABLE 4b. Number of positive cells in the lamina propria per mm^2

	Control nasal mucosa (n = 18)	Non-steroid treated NP (n = 27)	Steroid treated NP (n = 12)
Macrophages (CD68+)	107.5 (74.5-191.6)	115.9 (70.3-169.0)	146.0 (112.0-156.0)
Mast cells (CD117+)	50.1 (32.4-67.5)[a,b]	22.0 (15.1-44.0)[a]	31.2 (20.8-46.2)[b]
Eosinophils (MBP+)	6.6 (0.3-69.4)[a,b]	90.8 (26.5-176.9)[a]	79.1 (23.9-131.8)[b]
Basophils (basogranulin+)	2.4 (0.0-6.8)	1.8 (0.5-4.6)	1.7 (0.0-9.7)
Neutrophils (elastase+)	48.0 (22.3-163.9)	82.6 (26.9-149.7)	92.1 (16.7-185.2)
IL-4	5.0 (0.5-12.2)	1.0 (0.0-3.4)	1.7 (0.2-6.2)
IL-5	4.8 (1.3-8.8)[a,b]	7.4 (4.7-22.3)[a,c]	25.0 (14.5-31.0)[b,c]
IL-6	0.0 (0.0-0.9)	0.5 (0.0-3.1)	1.2 (0.0-2.9)
IgE	24.3 (5.3-50.9)	31.6 (16.4-62.8)	62.2 (29.5-102.8)
COX-1	16.6 (13.7-26.5)	18.0 (12.6-26.3)	19.9 (17.8-26.1)
COX-2	0.5 (0.0-5.0)	0.4 (0.0-2.0)	1.0 (0.1-1.6)

Data are expressed as median (25^{th}-75^{th} percentile). Note a) significant difference ($p < 0.05$) between normal nasal mucosa and non-steroid treated NP, b) significant difference ($p < 0.05$) between normal nasal mucosa and steroid treated NP and c) significant difference ($p < 0.05$) between non-steroid treated NP and steroid treated NP

epithelium and NP lamina propria. The number of IL-5+ cells, on the contrary, is significantly increased in GC treated NP lamina propria (figure 1). No differences are observed for NP epithelium. No differences are observed in the number of IL-6+ cells in both NP epithelium and NP lamina propria, although a tendency towards

increased numbers of IL-6+ cells in GC treated NP lamina propria is observed. As one would expect, the level of tissue eosinophilia is decreased in both NP epithelium and NP lamina propria upon GC treatment, but this decrease is statistically not significant (figure 1, figure 2b (appendix 3)). A tendency towards increased numbers of neutrophils is observed upon GC treatment in both NP epithelium and NP lamina propria. As was shown for non-GC treated NP, the number of mast cells present in GC treated NP lamina propria is significantly decreased compared to NM and similar to levels present in non-GC treated NP lamina propria. No differences were observed in the number of macrophages and basophils present. As was described in the previous section, the number of COX-1+ cells is significantly increased in non-GC treated NP when compared to NM. As is shown in figure 2a, treatment with GC results in a significant decrease in the level of COX-1+ cells in NP epithelium back to levels similar to NM. This striking difference between non-GC treated NP and GC treated NP is not observed for NP lamina propria. Compared to non-GC treated NP, a tendency towards increased levels of COX-2+ cells is observed for both NP epithelium and NP lamina propria of GC treated patients (figures 2a and 2b (appendix 3), tables 4a and 4b).

DISCUSSION

Similar to findings by other groups, we demonstrate that NP are characterized by the presence of large amounts of eosinophils and increased numbers of IL-5+ cells and IgE+ cells[141;196]. Although other authors previously showed that treatment with oral GC resulted in a significant decrease in tissue eosinophilia and IL-5 protein levels[141], we show that treatment with topical GC does not result in a significant reduction in the number of eosinophils and IL-5+ cells in NP. Although tissue eosinophilia is reduced somewhat upon treatment with topical GC, the number of IL-5+ cells is not. Contrasting to what one would expect, the number of IL-5+ cells is instead increased significantly in topical GC treated NP. An explanation for this contradictory finding may be the fact that protein levels as assessed with ELISA (Enzyme Linked Immunosorbent Assay) do not necessarily correlate with the number of positive cells as assessed with immunohistochemistry (i.e. the total number of cells producing IL-5 may be increased, despite reduced IL-5 protein levels). Since duration of topical GC treatment, type of drug formulation and dosage used varied among our included patients, this may also explain the lack of

evidence to support the assumption that topical GC downregulate the number of eosinophils and IL-5+ cells in NP tissue specimens. Since all NP were obtained from patients with persistent disease, we cannot exclude the possibility that some of our patients are resistant to topical GC. As was shown by Hamilos et al, fluticasone insensitive NP patients have a greater percentage of inflammatory cells expressing GC receptor β (GRβ), a splice variant of the GR that is said to fail to bind GC. Hamilos et al showed that in inflammatory cells expressing this GRβ, the percentage of cells producing IL-5 is increased at baseline and is even further increased upon fluticasone treatment[111].

Compared to NM, the number of COX-1+ cells is significantly increased in non-GC-treated NP epithelium (figures 2a and 2b (appendix 3)). Treatment with topical GC results in a significant reduction in the number of COX-1+ cells in NP epithelium back to the level observed in NM epithelium. Although results are not significant, an opposite effect is observed for COX-2+ cells. The number of COX-2+ cells in non-GC treated NP is similar to NM, but is upregulated upon treatment with topical GC (figures 2a and 2b (appendix 3)).

Several authors previously reported that GC downregulate COX-2 mRNA and protein levels *in vitro*[194;197;198]. Consistent with previous findings from our group, in which we observed no *in vivo* effect of GC (topical and/or oral) on COX-2 mRNA expression[199], we do not show a significant effect of topical GC on COX-2 protein expression in NP cells *in vivo*. In the studies by Picado et al and Pujols et al, we investigated the expression levels of COX-1 and COX-2 mRNA in NM and NP *in vivo*. COX-1 mRNA levels were shown to be elevated in NP when compared to NM[199;200]. Upon GC (topical and/or oral) treatment, a slight downregulation of COX-1 mRNA was observed[199]. In our present study, the effect of topical GC on COX-1 and COX-2 protein expression levels was studied. In 2000 we reported that the effect of oral GC on COX-1 mRNA expression levels in inflammatory nasal mucosa is only marginal[194]. In our current study we demonstrate that topical GC (in contrast to oral GC) significantly downregulate COX-1 protein expression levels in nasal polyp cells *in vivo*. A possible explanation for this dichotomy, as suggested by Hoff et al 1993, is the lack of correlation between expression levels of COX-1 mRNA and expression levels of COX-1 protein[197]. Post-transcriptional regulation may be one of the mechanisms involved. A second explanation for the observed

dichotomy may follow from observations by Kitzler et al, who showed that COX-1 mRNA exists in two splice variants: non-functional and functional COX-1 mRNA. Differentially or abnormally spliced, non-functional COX-1 mRNA may overestimate the level of functional COX-1 mRNA. As a consequence, the induction of functional COX-1 mRNA may be underestimated[198], thereby suggesting a lack of induction of COX-1 protein expression. Although the observed downregulation of COX-1 protein has been reported previously for GC treated cultured fetal pulmonary-artery endothelial cells and rheumatoid arthritis derived synovial cells[201;202], we report for the first time that COX-1 protein levels are significantly downregulated in NP epithelium upon topical GC treatment.

In a previous study, we showed that nasal mucosa (including NP mucosa) expresses COX-2 mRNA rather constitutively[199;200], data confirmed at the immunohistochemical level by Gosepath et al[203]. Upon GC (topical and/or oral) treatment, we demonstrated a slight upregulation of COX-2 mRNA in NP tissue specimens[199]. In our present study we show that COX-2 protein levels are also increased (although not significantly) in topical GC treated NP tissue specimens (figure 2a). This finding is in contrast with *in vitro* data by Aksoy et al who showed a clear inhibitory effect of topical GC on COX-2 eicosanoid synthesis in cultured BEAS-2B airway epithelial cells[204]. Based on our results, we conclude that one cannot predict the effect of topical GC on COX-2 protein expression levels *in vivo* based on the suppressability of COX-2 by topical GC *in vitro*. Since it remains unclear whether data obtained from cultured airway epithelial cells can be extrapolated to NP epithelial cells, the results by Aksoy et al should be interpreted with care. Having said this, in 1999, an interesting study by Gilroy et al was published, showing that COX-2 may be pro-inflammatory during the early polymorphonuclear leukocyte-dominated phase (a.o. eosinophils), but may aid to the resolution of inflammation in the later, mononuclear-cell dominated phase (a.o. macrophages) by generating an alternative set of anti-inflammatory prostaglandins, mainly prostaglandin E_2, in a carrageen-induced pleurisy model in rat[195]. Thus, the relative upregulation of COX-2 upon topical GC treatment may skew prostaglandin production in NP towards a more anti-inflammatory profile and this, in turn, may result, together with the observed downregulation of upregulated NP epithelial COX-1, in the resolution of disease.

In summary, we report that topical GC significantly downregulate the number of COX-1+ cells and possibly upregulate the number of COX-2+ cells in NP epithelium. We postulate that differences in tissue GC concentration between oral and topical GC or binding to different subsets of GC receptors account for this differential effect. Dexamethasone has been shown to increase COX-2 protein levels at low concentrations and to decrease COX-2 protein levels at higher concentrations in a mesangial cell culture system[205]. Differences in GC receptor expression have been shown to account for the differential effects of dexamethasone on COX-2 expression in various cell types[206] and therefore it may well be that topical GC and oral GC stimulate different subsets of GC receptors, explaining the differential effect on cyclo-oxygenase (COX-1 and COX-2) expression. Future studies are necessary to confirm these hypotheses.

CHAPTER 3.2

PREDICTORS OF POST-OPERATIVE RESPONSE TO TREATMENT: A DOUBLE BLIND PLACEBO CONTROLLED STUDY IN CHRONIC RHINOSINUSITIS PATIENTS

Ebbens FA, Toppila-Salmi S, de Groot EJJ, Renkonen J, Renkonen R, van Drunen CM, Dijkgraaf MGW, and Fokkens WJ, *Allergy (submitted for publication)*

consistent with CRS with nasal polyposis or CRS without nasal polyposis (i.e. a sinus CT-scan score of at least 2 (e.g. obstruction of at least one ostiomeatal complex) according to the Lund & Mackay scoring system[51]) were eligible to enroll provided that none of the exclusion criteria was present (table 1). Nasal endoscopy was performed using a 30° Storz nasendoscope (Karl Storz GmbH, Tuttlingen, Germany) after local anesthesia and decongestion with novesin and xylomethazoline. All patient-related study data were recorded in Case Report Forms and internally monitored for accuracy and completeness.

Intervention

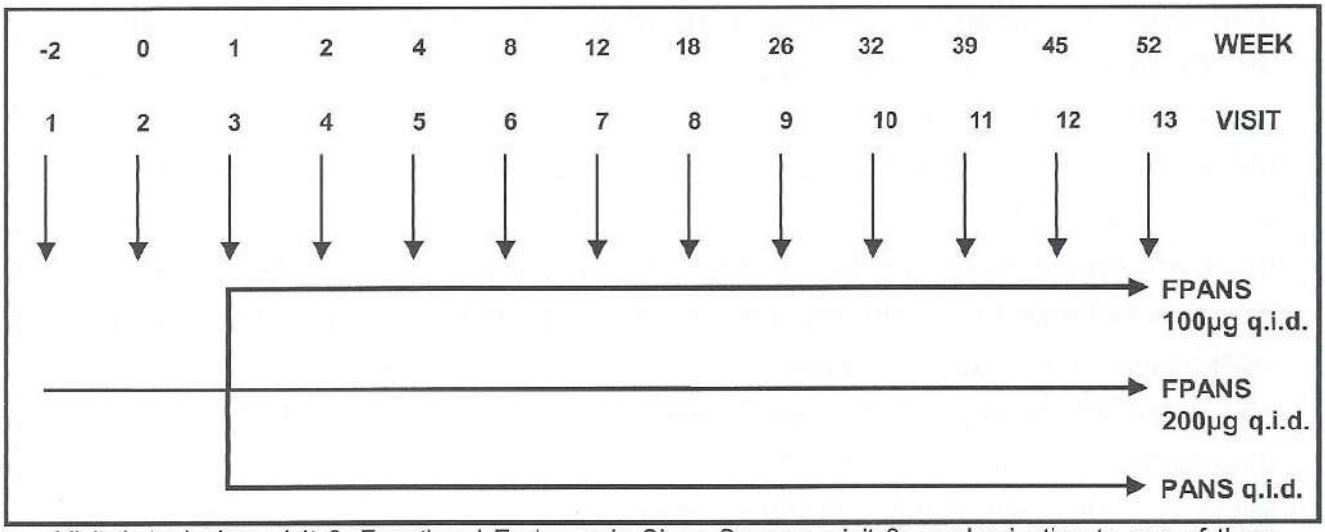
Each patient underwent a FESS procedure according to the extent of his or her disease[135]. In all cases, the ostiomeatal complex was opened. If necessary, the procedure included opening to the ethmoidal, frontal and/or sphenoidal sinuses. In case a severe septal deviation was present, septoplasty was performed. All patients received a dexamethasone booster course starting with 1 mg q.i.d. at the day of surgery and tapering off with 0.5 mg/day every second day. The use of systemic steroids was not allowed in the remainder of this study. Following FESS (visit 2) and removal of the ethmoidal packing (visit 3), patients applied either FPANS 100 µg (i.e. 2 actuations) q.i.d., FPANS 200 µg (i.e. 2 actuations) q.i.d., or PANS (2 actuations) q.i.d. into each nostril. Randomized allocation to one of the three treatment groups took place after removal of the ethmoidal packing on visit 3 (one week after surgery). Follow-up visits were scheduled at 2, 4, 8, 12, 18, 26, 32, 39, 45, and 52 months after surgery (figure 1). Upon randomization and at each clinic visit, the hospital pharmacist provided each participating patient with trial medication. Trial medication was instructed to be stored in a secure area between 2°C and 25°C, free of environmental extremes. All groups continued current treatment regimens for conditions other than CRS with or without nasal polyposis. Dosages of these treatment regimens were kept constant throughout the period of participation in this study whenever possible and patients were instructed to record and report any changes. The use of systemic steroids was not allowed except for a dexamethasone booster course starting with 1 mg q.i.d. at the day of surgery and tapering off with 0.5 mg/day every second day. Antibiotics (e.g. amoxicillin/clavulanic acid 500/125 mg t.i.d.) were allowed upon exacerbation of disease when necessary. In case of severe symptoms, those patients suffering

from concurrent allergic rhinitis were allowed to use terfenadine 60 mg as rescue medication should their symptoms of allergy become troublesome (maximum 2 tablets a day). Upon randomization, the use of the nasal spray pump was explained to each participating patient. Patients were instructed to actuate the spray twice into each nostril in the morning before breakfast and twice into each nostril after breakfast. Patients were instructed to actuate the spray into each nostril in a similar way and dose before and after dinner. Compliance was checked by looking at the amount of residual spray solution returned to the pharmacist at each clinic visit.

Objectives and outcome

Clinical outcomes of this study were recently published in *Clinical and Experimental Allergy*[208]. The purpose of our present study was to obtain one or more baseline immunohistochemical parameters (thus prior to the start of treatment with FPANS or PANS) that could predict the recurrence or persistence of CRS with or without nasal polyposis. Nonresponders were identified in case of recurrent or persistent disease, which was defined as a progressive regrowth of nasal polyps, recurrent

FIGURE 1. Flow diagram of the study



Visit 1: inclusion, visit 2: Functional Endoscopic Sinus Surgery, visit 3: randomization to one of three treatment arms and visit 13: end of study.

complaints of CRS combined with signs of CRS upon endoscopy and abnormalities on a sinus CT-scan, or persistent complaints of CRS with or without nasal polyposis for at least two months after FESS.

Randomization

Following FESS, patients were randomly allocated at visit 3 to one of the three treatment groups (ratio 1:1:1) using a computer generated randomization schedule provided by the Department of Biostatistics, Erasmus University Medical Center, Rotterdam, the Netherlands. Patient numbers were sequentially assigned in time. Numbered aqueous nasal spray bottles were dispensed by an independent pharmacist to each patient upon randomization and at each clinic visit. All study personnel and participants were blinded to experimental conditions for the duration of the study. Randomization codes were revealed to the researchers only when recruitment and data collection were complete.

Statistical analysis

Analysis was based on thirty-five patients that had been randomly assigned to one of three treatment arms. One-way ANOVA and chi-square analyses were performed to check for between-group demographic differences between the three treatment groups. Two-sided T-tests and Mann-Whitney-U non-parametric tests were performed to check for between-group differences in clinical and cellular characteristics at baseline between non-responders and responders. Spearman rank-order correlation was used to assess bivariate association. Immunohistochemical data are represented as median (interquartile range). A binary logistic regression analysis was performed to study the association between cellular markers at baseline and response to therapy. P values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 16.0 (Chicago, IL, USA).

Tissue handling

Samples of inferior turbinate obtained at the time of surgery were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemical staining of MBP, CD14, CD34 and functionally active L-selectin ligands

6-µm thick serial sections of all snap-frozen specimens were cut on a Microm HM560 frigocut cryostat and transferred to APES (amino-phosphate-ethylsilane) coated slides (Starfrost, MarketLab, Caledonia, MI, USA), dried and stored at -70°C until analysis. Upon analysis, tissue sections were defrosted to room temperature, dried and fixed in acetone (cold as ice) for 10 minutes at room temperature. After fixation, tissue sections were rinsed with phosphate-buffered saline (PBS, pH 7.8), placed in a semi-automatic stainer (Shandon Sequenza, Waltham, MA, USA) and incubated with normal goat serum (CLB, Amsterdam, the Netherlands) for 10 minutes. Following the blocking procedure, sections were incubated with primary antibody for 60 minutes at room temperature. Mouse anti-human monoclonal antibodies directed against major basic protein (MBP), CD14, CD34 and functionally active L-selectin ligands (as detected with mAbs HECA-452 and MECA-79) (table 2). All primary antibodies were diluted in PBS containing 1% (w/v) blocking reagent (10961760, Roche, Basel, Switzerland) to block endogenous avidin and biotin activity. Following incubation with primary antibody, sections were rinsed with PBS for 5 minutes and incubated with biotinylated goat anti-mouse antiserum (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Next, sections were rinsed with PBS and incubated with streptavidin alkaline phosphatase (1:50, Biogenics Klinipath, Duiven, the Netherlands) for 30 minutes at room temperature. Sections were subsequently rinsed with PBS containing TRIS (tris(hydroxymethyl)aminomethane) buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous alkaline phosphatase (AP) enzyme activity) for 20 minutes at room temperature. Sections were counterstained with Gill's hematoxylin (MBP,CD14) or Mayer's Hämalaun (CD34, mAbs HECA-452 and MECA-79), rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with the appropriate isotype control.

Tyramide signal amplification (TSA) staining for basogranulin, ECP/EDN, IL-5, CD15 and CD94

A sensitive protocol was used based on the alkaline phosphatase method described above. Sections were cut and defrosted as described above. Sections were fixed in acetone (cold as ice) for 10 minutes, placed in the semi-automatic stainer and incubated with normal goat serum as described above. Slides were incubated with mouse anti-human monoclonal antibodies directed against basogranulin, ECP/EDN, IL-5, CD15 and CD94 diluted in PBS containing 1% (w/v) blocking reagent (table 2). After incubation with biotinylated goat anti-mouse (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Following the blocking procedure, slides were incubated with streptavidin conjugated peroxidase (NEN, Waltham, MA, USA) for 30 minutes at room temperature, rinsed with PBS and incubated with biotinyl tyramide in TRIS buffer for 10 minutes in order to amplify the signal. Next, slides were rinsed once again in PBS and incubated with alkaline phosphatase conjugated goat-anti-biotin antiserum (Sigma, Zwijndrecht, the Netherlands). Sections were then rinsed with PBS containing TRIS buffer (0.2 mol/L, pH 8.5) and incubated with new fuchsine (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20

TABLE 2. Monoclonal antibodies

Antibody	Specificity	Cell type / cytokine	Titer	Source
CD14	CD14	Macrophages	1:600	CLB, the Netherlands
CD15	CD15	Neutrophils	1:50	Immunotech,France
CD34	CD34	Endothelial cells	1:100	DAKO, Denmark
CD94	CD94	NK-cells	1:25	Beckman Coulter, NL
BB-1	Basogranulin	Basophils	1:150	A.F. Walls, UK
BMK-13	MBP	Eosinophils	1:100	CLB, Netherlands
EG2	ECP/EDN	Activated eosinophils	1:100	Sanbio, Netherlands
IL-5	IL-5	IL-5	1:100	Novartis,Switzerland
HECA-452	2,3-sialylation & 1,3-fucosylation of lactosamine	L-selectin ligand	1:100	BD Biosciences, USA
MECA-79	Extended sulphate core 1 lactosamine	L- selectin ligand	1:100	BD Biosciences, USA

minutes at room temperature. Sections were counterstained with Gill's hematoxylin, rinsed with distilled water and mounted in Vecta Mount (Vector, Burlingame, CA, USA). Control staining was performed by substituting primary antibody with the appropriate isotype control.

Light microscope evaluation

All sections were examined with an Olympus BX51 light microscope by 2 independent observers blinded to the experimental conditions. The numbers of positively stained cells were counted in the epithelium (per mm) and adjacent lamina propria (per mm^2) at a final magnification of 200x. Results are expressed as the mean number of positive cells per mm or mm^2.

RESULTS

Participants

Of the 162 patients included in this double-blind placebo-controlled multicenter trial, 59 patients completed the study without signs of recurrent disease (i.e. no signs of progressive regrowth of nasal polyps, recurrent complaints of CRS combined with signs of CRS upon endoscopy and sinus CT scan abnormalities, or persistent complaints of CRS with or without nasal polyposis for at least two months after FESS). Thirty-five patients were randomly selected from the 162 patients included in this trial, including both responders and non-responders (14 from the PANS treated group, 9 from the FPANS 100 µg q.i.d. treated group and 12 from the FPANS 200 µg q.i.d. treated group). Biopsies were obtained at the time of surgery (visit 2). No significant differences in demographic and clinical characteristics were observed at baseline between the three treatment groups (table 3). When comparing demographic and clinical characteristics at baseline between non-responders and responders, similar results were obtained (data not shown).

Cellular markers at baseline

In the first part of our study, patients were divided into responders and non-responders based on the criteria described above. Endothelial expression levels of

TABLE 3. Baseline characteristics of the study population (n = 35)

	Placebo (n = 14)	FPANS 400 µg/day (n = 9)	FPANS 800 µg/day (n = 12)	*p* value
Age (y), mean (SD)	48 (14)	41 (12)	43 (11)	0.38
Male sex, n (%)	6 (42.9)	8 (88.9)	7 (58.3)	0.09
Asthma, n (%)	3 (21.4)	4 (44.4)	2 (16.7)	0.41
Atopy, n (%)	5 (35.7)	3 (33.3)	5 (41.7)	0.92
Previous sinus surgery, n (%)	8 (57.1)	4 (44.4)	6 (50.0)	0.83
Smoking habits				0.48
Current smoker, n (%)	3 (21.4)	2 (22.2)	4 (33.3)	
Ex-smoker, n (%)	1 (7.1)	0 (0)	2 (16.7)	
Non-smoker, n (%)	10 (71.4	7 (77.8)	5 (41.7)	
CT scan score, mean (SD)	12.9 (5.7)	12.7 (6.0)	10.9 (3.8)	0.61
Total VAS score, mean (SD)	227.6 (106.7)	199.8 (96.6)	261.8 (93.7)	0.37
Non-responders, n (%)	9 (64.3)	3 (33.3)	9 (75.0)	0.14
CRS with NP, n (%)	8 (57.1)	5 (55.6)	5 (41.7)	0.83

functionally active L-selectin ligands were analyzed using two anti-glycan antibodies (mAb MECA-79 and mAb HECA-452). In addition, the number of CD34+ endothelial cells was determined in all tissue specimens. Other markers of inflammation that were quantified included the numbers of IL-5+ cells, eosinophils, neutrophils, macrophages, NK-cells and basophils (tables 4 and 5). Compared to non-responders, the number of lamina propria EG2+ eosinophils (i.e. activated eosinophils) is significantly increased in the responders group (p = 0.005). This increase in the number of EG2+ eosinophils is present in both CRS patients without nasal polyposis and CRS patients with nasal polyposis. In addition, a trend towards significance isobserved for the number of lamina propria CD15+ neutrophils (p = 0.066) in the responders group. The number of epithelial CD14+ macrophages, on the other hand, is significantly increased in the non-responders group (p = 0.043). No differences were observed for all other studied parameters (tables 4 and 5). Non-responders and responders did not differ in the percentage CD34+ vessels positive for mAb MECA-79 (2.92% (IQ-range 0.73 – 9.91) and 1.62% (IQ-range 0.30 – 5.77) respectively) and mAb HECA-452 (0.33% (IQ-range 0.00 – 1.30) and 1.02% (IQ-range 0.33 – 1.48) respectively). No correlation was

TABLE 4. Number of positive cells per mm epithelium in inferior turbinate specimens at baseline (median – IQ range)

	Responders	Non-Responders
Eosinophils (MBP+)	1.00 (0.00 – 2.57)	0.05 (0.00 – 0.49)
Activated eosinophils (ECP/ EDN+)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)
Basophils (basogranulin+)	0.72 (0.00 – 2.50)	2.50 (0.00 – 5.90)
NK-cells (CD94+)	3.53 (1.54 – 7.26)	4.46 (0.78 – 11.27)
Neutrophils (CD15+)	2.67 (0.34 – 7.81)	1.30 (0.55 – 3.91)
Macrophages (CD14+)	0.79 (0.00 – 2.42) †	3.00 (0.70 – 8.57) †
IL-5	2.73 (0.59 – 5.54)	0.88 (0.00 – 2.94)

† $p < 0.05$ and ‡ $p < 0.01$

TABLE 5. Number of positive cells per mm^2 lamina propria in inferior turbinate specimens at baseline (median – IQ range)

	Responders	Non-Responders
Eosinophils (MBP+)	11.38 (0.96 – 24.55)	1.74 (0.33 – 11.94)
Activated eosinophils (ECP/ EDN+)	0.64 (0.11 – 1.39) ‡	0.00 (0.00 – 0.00) ‡
Basophils (basogranulin+)	9.40 (1.08 – 15.38)	9.06 (0.00 – 17.90)
NK-cells (CD94+)	14.29 (7.40 – 27.75)	11.18 (2.87 – 16.20)
Neutrophils (CD15+)	14.38 (5.85 – 24.33)	5.96 (1.51 – 41.05)
Macrophages (CD14+)	10.47 (1.09 – 25.80)	16.47 (4.14 – 25.78)
Endothelial cells (CD34+)	122.20 (90.50 – 145.52)	112.05 (89.60 – 142.00)
IL-5	16.47 (2.66 – 28.94)	11.23 (3.51 – 23.50)

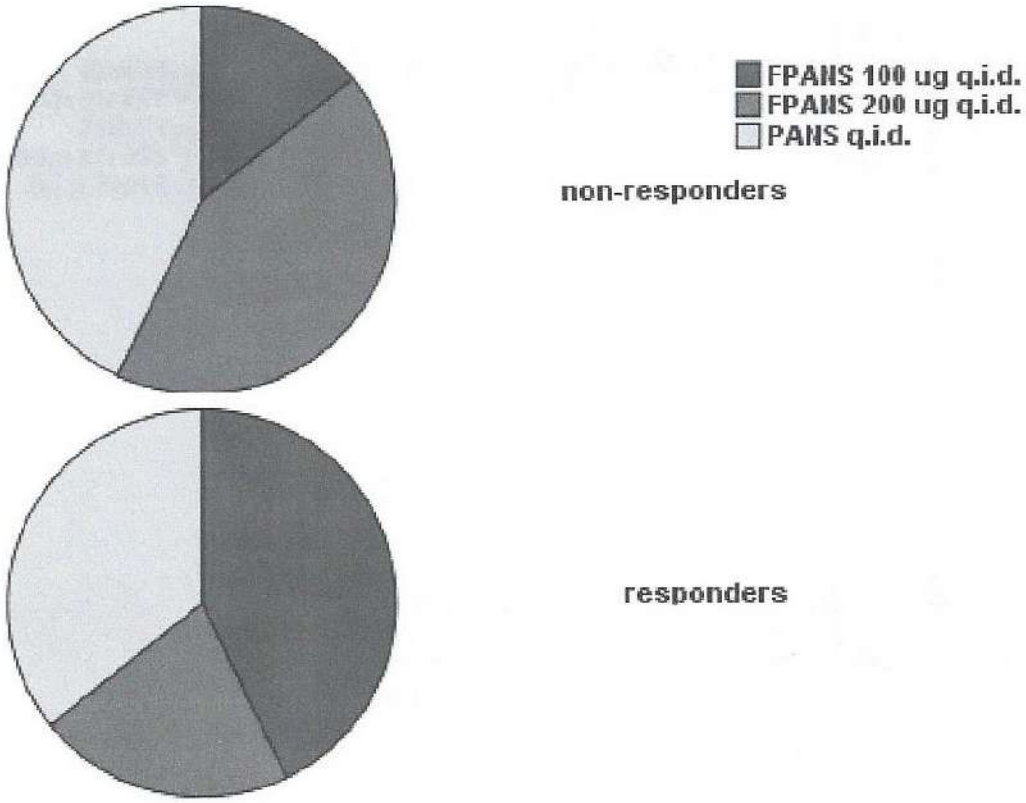
† $p < 0.05$ and ‡ $p < 0.01$

observed between the percentage of CD34+ vessels positive for mAb MECA and mAb HECA and all studied inflammatory cells.

Predictors of response

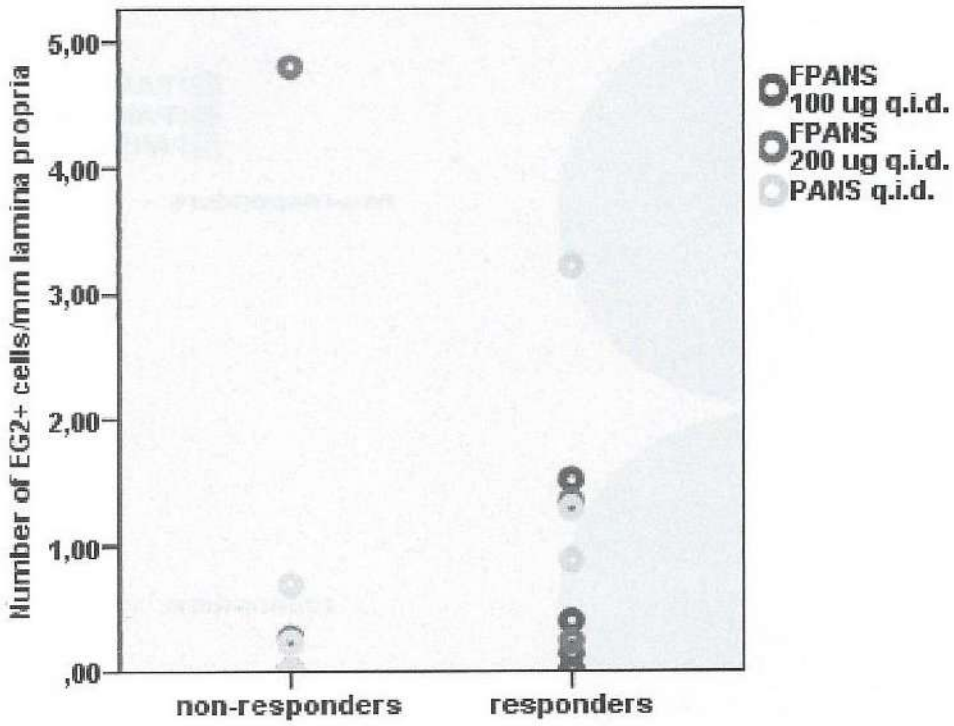
Main goal of this study was to identify one or more predictors of response to treatment within our data pool. A binary logistic regression analysis was performed to identify one or more of these predictors. Since only 21 patients were included in

FIGURE 2. Distribution of treatment scheme (%)



the non-responders group and 14 in the responders group, only 2 predictors were allowed. Both treatment and the number of lamina propria EG2+ eosinophils were identified as possible predictors of response. Only treatment was significantly (p = 0.048) associated with response (figure 2) in our model including treatment and the number of lamina propria EG2+ eosinophils. The impact of treatment was solely attributable to the FPANS 100 µg q.i.d. regimen compared to placebo (odds ratio 9.9; 95% CI 1.1 – 88.8). Although significantly higher baseline levels of lamina propria EG2+ eosinophils were observed in the responders group (figure 3), in our model this difference only showed a trend towards significance but did not reach significance yet (p = 0.091).

FIGURE 3. Predictors of response to treatment



DISCUSSION

Fluticasone propionate is a glucocorticoid with high topical activity and low systemic bioavailability. It is considered one of the most potent topical glucocorticoids currently available for the treatment of patients suffering from CRS with or without nasal polyposis. Various controlled trials have demonstrated that treatment with fluticasone propionate is well tolerated and effective in the majority of CRS patients with and without nasal polyposis[95;96;209-211]. Besides being effective in treating signs and symptoms of primary and recurrent CRS, the use of fluticasone propionate has been shown to effectively prevent post-surgical recurrence of disease[212]. Only two studies, one of which was recently published and describes the clinical results of this study[208], were unable to demonstrate a positive effect of fluticasone propionate on outcome[208;213].

It is generally accepted that topical glucocorticoids reduce the number of inflammatory cells in CRS mucosa, particularly the numbers of primed eosinophils. This may be achieved either directly via a reduction in cell viability and activation[102-104] or indirectly via a reduction in the secretion of chemotactic cytokines[105-108]. The exact mechanisms underlying the anti-inflammatory and immunoregulatory effects of topical glucocorticoids, however, remain to be fully explained.

Although most patients respond well, a substantial number of CRS patients with and without nasal polyposis is refractory to (topical) glucocorticoids and requires surgical intervention. Both increased disease severity and/or glucocorticoid resistance are likely involved. The exact cause of glucocorticoid resistance is unknown. Overexpression of glucocorticoid receptor β and/or a downregulation of glucocorticoid receptor α are two of the mechanisms that have been proposed[111;112;214]. Other mechanisms include immunomodulation, cigarette smoking, genetic predisposition, viral infection, allergen exposure, the presence of microbial superantigens, and tissue neutrophilia[100]. Ideally, those patients resistant to glucocorticoids are identified prior to starting topical glucocorticoid treatment. If identified early, those patients resistant to topical glucocorticoids can be treated alternatively with, for example, long-term macrolide antibiotics[58;115-118]. With this in mind, we aimed to identify a cellular marker predicting the response to surgery and/or post-operative fluticasone propionate aqueous nasal spray in a mixed group of CRS patients with and without nasal polyposis.

Inferior turbinates are easy accessible and present in all CRS patients with and without nasal polyposis. We chose to investigate inferior turbinate biopsies, as middle turbinate specimens can be absent in those CRS patients suffering from recalcitrant disease. Levels of various inflammatory mediators, including IL-5, were recently shown to be equally present in inferior turbinate specimens and nasal polyp tissue specimens[140]. Thus, inferior turbinate tissue specimens seem to be ideal for those doctors wanting to predict response to treatment in nasal biopsies.

Baseline inferior turbinate specimens of responders are characterized by high levels eosinophils, NK-cells, neutrophils, and an increase in the number of IL-5+ cells. Baseline inferior turbinate tissue specimens of non-responders, on the other

hand, are characterized by high levels of basophils, NK-cells and macrophages (table 4). Tissue specimens from both groups are characterized by high levels of CD34+ endothelial cells. Only a small percentage of these CD34+ endothelial cells do express functionally active L-selectin ligands (as detected by both mAb HECA-452 and mAb MECA-79). Although we have previously demonstrated that, in CRS patients without nasal polyposis, the extent of tissue eosinophilia correlates with the percentage of CD34+ endothelial cells expressing functionally active L-selectin ligands[181], no correlation was observed between the percentage of CD34+ endothelial cells expressing functionally active L-selectin ligands and any of the studied inflammatory cells in this study. Although a lack of power cannot be ruled out, results are in line with data obtained in a second study from our group investigating nasal polyps from CRS patients with nasal polyposis[207].

Both treatment with fluticasone propionate and the number of lamina propria EG2+ eosinophils were identified as possible predictors of response. Only treatment was significantly (p = 0.048) associated with response (figure 2) in our model including treatment and the number of lamina propria EG2+ eosinophils as variables. The impact of treatment was solely attributable to the FPANS 100 µg q.i.d. regimen compared to placebo. These results are in line with results published by Rowe-Jones et al who observed that CRS patients with or without nasal polyposis performed significantly better on all outcome measures when treated with fluticasone propionate 200 µg b.i.d. (a similar daily dose as our study) for 4 years following FESS in a randomized, stratified, prospective, double-blind, placebo controlled study [212], but do not confirm the overall results of our clinical data (in which responders and non-responders were grouped together)[208]. Our current division in responders and non-responders possibly explains the observed results, since the majority of patients included in our clinical study belonged to the non-responders group. A selection bias, however, cannot be excluded. Those patients treated with FPANS 200 µg q.i.d. and PANS q.i.d. post-operatively are more likely to suffer from recurrent disease (figure 2), results in line with our clinical data[208].

Recently, increased numbers of eosinophils were shown to be characteristic of both patients suffering from CRS with nasal polyposis and patients suffering from CRS without nasal polyposis. Levels, however, were shown to vary between individuals and within biopsies[122]. In this study, we show that those CRS patients

with increased numbers of lamina propria EG2+ eosinophils are more likely to respond to surgery, especially when treated post-operatively with FPANS 100 µg q.i.d., than those CRS patients with low numbers of lamina propria EG2+ eosinophils. Unfortunately, even though significantly higher baseline levels of lamina propria EG2+ eosinophils are observed in responding patients, it is only a trend towards association that is observed between the number of baseline lamina propria EG2+ eosinophils and response to treatment ($p = 0.091$). Most likely, the presence of one outlier in the non-responders group is explanatory (figure 3).

Similar to our data in CRS patients, low sputum eosinophils have been shown to predict the lack of response to beclomethasone treatment in symptomatic asthmatics[215]. Therefore, additional research in CRS patients with and without nasal polyposis studying the role of EG2+ eosinophils and response to FESS in combination with post-operative fluticasone propionate treatment is warranted.

CHAPTER 4

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CHAPTER 4.1

AMPHOTERICIN B NASAL LAVAGES: NOT A SOLUTION FOR PATIENTS WITH CHRONIC RHINOSINUSITIS

Ebbens FA, Scadding GK, Badia L, Hellings PW, Jorissen M, Mullol J, Cardesin A, Bachert C, van Zele TPJ, Dijkgraaf MGW, Lund V, and Fokkens WJ, *Journal of Allergy and Clinical Immunology 2006;118:1149-56.*

ABSTRACT

Background: Recently, it has been suggested that an exaggerated immune response to fungi is crucial in the pathogenesis of CRS. Based on this hypothesis, intranasal treatment with amphotericin B should benefit patients suffering from CRS. Data from 2 uncontrolled and 2 controlled trials are however conflicting.

Objective: In order to clarify the role of intranasal antifungal drugs in the treatment of CRS, we conducted a large double-blind placebo-controlled multicenter study, comparing the effectiveness of amphotericin B nasal lavages with placebo.

Methods: 116 randomly selected patients with CRS with or without nasal polyps were instructed to instill 25 mL amphotericin B (100 µg/mL) or placebo to each nostril twice daily for 3 months. Primary outcomes were a reduction in total VAS score and nasal endoscopy score. Secondary outcome measures included Peak Nasal Inspiratory Flow, polyp scores, quality of life (SF-36, RSOM-31) and patient symptom scores (individual VAS scores).

Results: Analysis was based on intention-to-treat and involved all patients randomly assigned. Mean VAS scores, SF-36 and RSOM-31 data, PNIF values, nasal endoscopy scores and polyp scores were similar in both treatment groups at the time of randomization and no significant differences were observed after 13 weeks of treatment.

Conclusion: Amphotericin B nasal lavages in the described dosing and time schedule are ineffective in the treatment of patients with CRS with or without nasal polyposis.

INTRODUCTION

Chronic rhinosinusitis (CRS) with or without nasal polyps (NP) is a multifactorial disease, associated with asthma, cystic fibrosis, primary ciliary dyskinesia, acetyl salicylic acid (ASA) intolerance and possibly allergy, affecting between 1 and 4% of the general population[15;25]. Although many hypotheses have been suggested, the pathogenesis of CRS is still largely unknown. The majority of patients with CRS suffer from nasal congestion, thick mucus production, reduced sense of smell, headache/facial pain, and reduced quality of life and usually require a combination of medical and surgical therapy to provide long-term symptom control[216].

A range of bacteria has been implicated as pathogens, though their exact role is unclear. It has long been recognized that, besides bacteria, fungi are responsible for some forms of CRS, although it can prove extremely difficult to confirm the diagnosis even with sophisticated culturing techniques. Recently, Ponikau et al showed by using novel collection and culturing methods, the presence of fungi in 202 of 210 (96%) consecutive patients with CRS, suggesting that fungi might be involved in more cases of CRS than hitherto suspected[122]. A similar high incidence of fungal colonization (91%) was subsequently reported from Europe[217]. It should, however, be noted that similar techniques demonstrate the presence of fungi in the nose of healthy controls as well. The presence of fungi alone therefore does not explain the chronicity of inflammation seen in CRS patients.

Recent observations suggest that peripheral blood mononuclear cells from the majority of CRS patients, but not from healthy controls, produce IL-5 and IL-13 when exposed to fungal extracts. On the basis of these and other observations, Ponikau et al have suggested that intranasal treatment with amphotericin B, an antifungal agent, is an appropriate treatment for patients suffering from CRS. In 2 uncontrolled trials, such therapy has been reported as safe and effective[123;124]. However, when subjected to a randomized, double-blind, placebo-controlled trial, involving 60 patients with CRS, no significant benefit from long term use (8 weeks) of an amphotericin B nasal spray[127] was observed and a recent double-blind placebo-controlled single-center study, including only 30 patients, failed to show major differences between amphotericin-treated subjects and placebo-treated subjects after 6 months although some differences were reported as significant[126].

In addition, treatment with high-dose oral terbinafine (6 weeks) failed to improve symptom scores and computed tomography (CT) scores in a double-blind placebo-controlled trial including 53 patients[131].

In order to clarify the role of intranasal antifungal drugs in the treatment of CRS, we conducted a large double-blind placebo-controlled multicenter study, comparing the effectiveness of amphotericin B nasal lavages with placebo in patients with CRS with or without NP delivered at the level of tertiary care Otorhinolaryngology clinics.

METHODS

Participants

This double-blind, placebo-controlled multicenter trial, comprising 6 tertiary care Otorhinolaryngology clinics, investigated the effectiveness of intranasal amphotericin B (100 µg/mL) or placebo, used for 3 months in adult patients with CRS with or without NP and was prepared, conducted and reported in compliance with the EU Note for Guidance on Good Clinical Practice as laid down in the Dutch Medicines Law (Wet op de Geneesmiddelenvoorziening, art. 55). The trial has been approved by the medical ethical committee of each participating center. Prior to enrollment, each patient was required to read and sign an informed consent

TABLE 1. Inclusion criteria

Inclusion criteria
Clinical signs and symptoms related to CRS (nasal congestion, nasal discharge, headache and/or facial pain) which are present persistently or recurrently (i.e. intermittent or present > 6 weeks after the last surgical procedure) for a total period of at least 6 months
Endoscopic signs of CRS and/or nasal polyps
Sinus CT scan score of 5 according to the Lund & Mackay scoring system[51] performed within a period of 2 months before randomization
Older than 18 years of age

form. Patients presenting to the Otorhinolaryngology Department of the Academic Medical Center (Amsterdam, the Netherlands), Erasmus Medical Center (Rotterdam, the Netherlands), Royal National Hospital (London, UK), University Hospital Ghent, (Ghent, Belgium), University Hospital Leuven (Leuven, Belgium) or Hospital Clinic de Barcelona (Barcelona, Spain) from February 2002 to December 2004 were recruited. All patients aged 18 years or older with clinical signs and symptoms related to CRS, endoscopic signs of CRS and/or NP and sinus CT-scan score of at least 5 according to the Lund & Mackay scoring system[51] were eligible to enroll provided that none of the exclusion criteria was present (tables 1 and 2). To guarantee adequate access to sinonasal mucosa upon irrigation with intranasal amphotericin B, previous endoscopic sinus surgery (ESS) was obligatory for inclusion. The use of intranasal corticosteroids in a normal dosage was allowed when used consistently during the whole trial period. Antibiotics (either amoxicillin/clavulanic acid 500/125 mg three times daily or ciprofloxacin 750 mg twice daily combined with clindamycin 600 mg three times daily) were allowed at clinical exacerbation, but only after aerobic and anaerobic cultures were performed

TABLE 2. Exclusion criteria

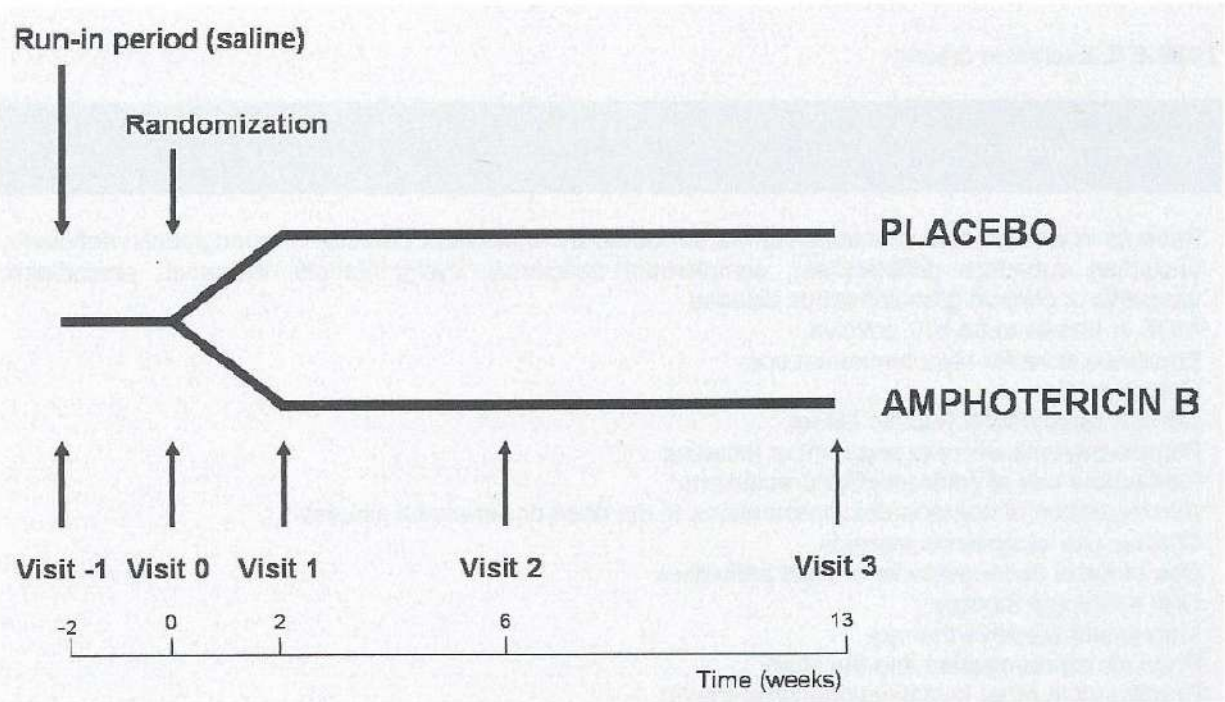
Exclusion criteria
Patients in whom nasal infections can be explained by anatomical defects, immunoglobulin deficiency (including sub-class deficiencies), complement deficiency, cystic fibrosis, Wegener, sarcoidosis, vasculitis or chronic granulomatous disease
AIDS or known to be HIV positive
Positive culture for Mycobacterium spp.
Osteoporosis
Chronic renal and/or hepatic failure
Female patients who are pregnant or lactating
Inadequate use of contraceptive precautions
Administration of homeopathic preparations to the nose or paranasal sinuses
Chronic use of systemic steroids
Use of nasal decongestants or local antibiotics
Oral antifungal therapy
Immunosuppressive therapy
Previous randomization into the study
Enrollment in other investigational drug-trial(s)
Psychiatric, addictive or any other disorder, compromising the ability to truly give informed consent
Concerns for compliance with the protocol procedures

by suction and injection in a port-a-cul (BD, Sparks, MD, USA). Systemic steroids were allowed for a maximum period of 14 days when prescribed for a disease other than upper airway pathology. All patient-related study data were recorded in Case Report Forms and internally monitored for accuracy and completeness.

Intervention

In this study, patients applied 25 mL amphotericin B solution (100 µg/mL) or placebo to each nostril twice daily using an Emcur (also named Rhinicur) nasal douching device (Emcur GmbH, Bad Ems, Germany). Amphotericin B is active against most moulds frequently identified within the paranasal sinuses and fractions involuntarily swallowed are not absorbed. The applied 100 µg/mL is approximately 30-100 times higher than the minimum inhibitory concentration for all relevant fungi[218].

FIGURE 1. Study design



Randomized allocation to one of the two treatment groups took place on visit 0. Prior to randomization, each patient was required to participate in a two week run-in period on placebo to get acquainted with the Emcur (also named Rhinicur) nasal douching device. Follow-up visits were scheduled at 2, 6 and 13 weeks after randomization.

Randomized allocation to one of the two treatment groups took place on visit 0. Prior to randomization, each patient was required to participate in a two week run-in period on placebo to get acquainted with the Emcur (Rhinicur) nasal douching device. Follow-up visits were scheduled at 2, 6 and 13 weeks after randomization (figure 1).

Upon randomization, the hospital pharmacist provided each participating patient with trial medication. Amphotericin B nasal lavage solution was prepared by dissolving amphotericin B for injection (Bristol-Meyer-Squib, New York, NY, USA) in sterile water containing 2.5% glucose, resulting in a clear yellow solution. Placebo nasal lavage solution was prepared by dissolving 3.4 mL/L Cernevit (Baxter, Deerfield, IL, USA) in sterile water containing 2.5% glucose, resulting in a similar clear yellow solution. Cernevit, a multivitamin preparation for use intravenously, was chosen as placebo for its color and absence of toxic effects on nasal mucosa. Upon preparation, solutions were kept in dark, light-rejecting bottles at 4°C and patients were instructed to keep trial medication refrigerated since amphotericin B has no antibacterial activity and contains no bacteriostatic agents. In those circumstances, the potency of the amphotericin B solution is retained for 1 month (data Bristol-Meyers Squibb[219]). Sterile water containing 2.5% glucose was used as a diluent since amphotericin B for injection forms precipitants when dissolved in saline. In contrast to saline, glucose is compatible with amphotericin B and has no effect on drug bioavailability[219]. The addition of glucose results in a reduction of nasal irritation due to low osmolarity and is advised as diluent by the manufacturer. No difference in appearance, taste or smell between placebo and amphotericin B solutions could be detected. Both groups continued current treatment regimens and were instructed to record and report any changes. Compliance was checked by looking at the amount of residual amphotericin B or placebo solution returned to the pharmacist at each visit.

Objective

In our present study, we compared the effectiveness of amphotericin B nasal lavages and placebo in the treatment of patients with CRS with or without NP delivered at the level of tertiary care Otorhinolaryngology clinics.

Outcome

We evaluated the effect of amphotericin B nasal lavages on quality of life and on the amount of nasal mucosal disease in patients with CRS with or without NP. Primary outcome measures included the change from baseline in patient symptoms by using total VAS score (sum of nasal blockage, rhinorrhoea, facial pain, postnasal drip and anosmia VAS score, see below) and the amount of mucosal disease as assessed by endoscopic examination in a standardized manner. Secondary outcomes included the change from baseline in disease specific patient symptoms by using the RSOM-31 (Rhinosinusitis Outcome Measure 31) questionnaire and individual VAS-scores, change in quality of life as assessed by the SF-36 (Short Form 36) questionnaire, change in nasal patency (PNIF) and change in polyp scores.

At each clinical visit, the amount of mucosal disease was assessed. The presence or absence of nasal secretions (0 = absent, 1 = clear to opaque, 2 = purulent), amount of crusting (0 = absent, 1 = mild, 2 = severe) and presence or absence of nasal polyps (0 = absent, 2 = present) was documented in predefined areas (e.g. middle meatus, ethmoid region). Sum scores were calculated by adding all independent values for both nostrils. The proportion of the total nasal cavity volume occupied by polyps was estimated as described by Johansson et al[220]. SF-36[56;57] health status questionnaires to assess physical (Physical Component Scale) and emotional (Mental Component Scale) effects, as well as RSOM-31 disease specific health questionnaires[60] were completed prior to randomization, at baseline and 3 months after randomization as described previously. An increase in SF-36 values implies improvement. An increase in the RSOM-31 total impact factor implies deterioration of disease. VAS scores (0-10 cm)[47] were completed at each visit, including visits 1 (2 weeks after randomization) and 2 (6 weeks after randomization). Symptoms scored by using VAS included nasal blockage, facial pain, rhinorrhoea, post nasal drip, anosmia, itching of the nose, itching of the throat, itching of the ears, nose bleeds, sneezing and headache. Lower VAS scores indicate less troublesome symptoms. PNIF was measured at each visit using a Youlten meter (Clement Clark International, Harlow, Essex, England), using the technique as described by Lund[221]. Measurements were repeated three times and highest measurements were recorded. An increase in PNIF value

implies improvement of nasal patency. Previous studies have shown good sensitivity and close correlation with nasal blockage symptoms and objective measures of nasal airway function (e.g. rhinomanometry)[222].

Sample size

Based on a prospective open-label pilot study conducted by the Mayo Clinic in 2000, in which improvement of symptoms and signs of CRS was observed in 38 (75%) of 51 patients upon treatment with amphotericin B nasal lavages[123], we hypothesized that amphotericin B nasal lavages are 25% better than placebo. Based on 0.8 power to detect a significant difference (P = 0.05, two-sided), 60 patients were required to enroll in each study group.

Randomization

Patients were randomly allocated at visit 0 to one of the two treatment groups (ratio 1:1) using a computer generated randomization schedule (block length of 4), provided by the Department of Biostatistics, Erasmus University Medical Center, Rotterdam, the Netherlands. Separate randomization lists were generated for each participating center and given to each pharmacy department. Patient numbers were sequentially assigned in time for each participating center. Numbered, light-rejecting bottles containing either amphotericin B or placebo were prepared and dispensed by an independent pharmacist in each participating center to each patient upon randomization. Patients were given a 1-month supply of medication each month (each month made freshly) to guarantee adequate potency of the medication. All study personnel and participants were blinded to the experimental conditions for the duration of the study. Randomization codes were revealed to the researchers only when recruitment and data collection were complete.

Data entry and monitoring

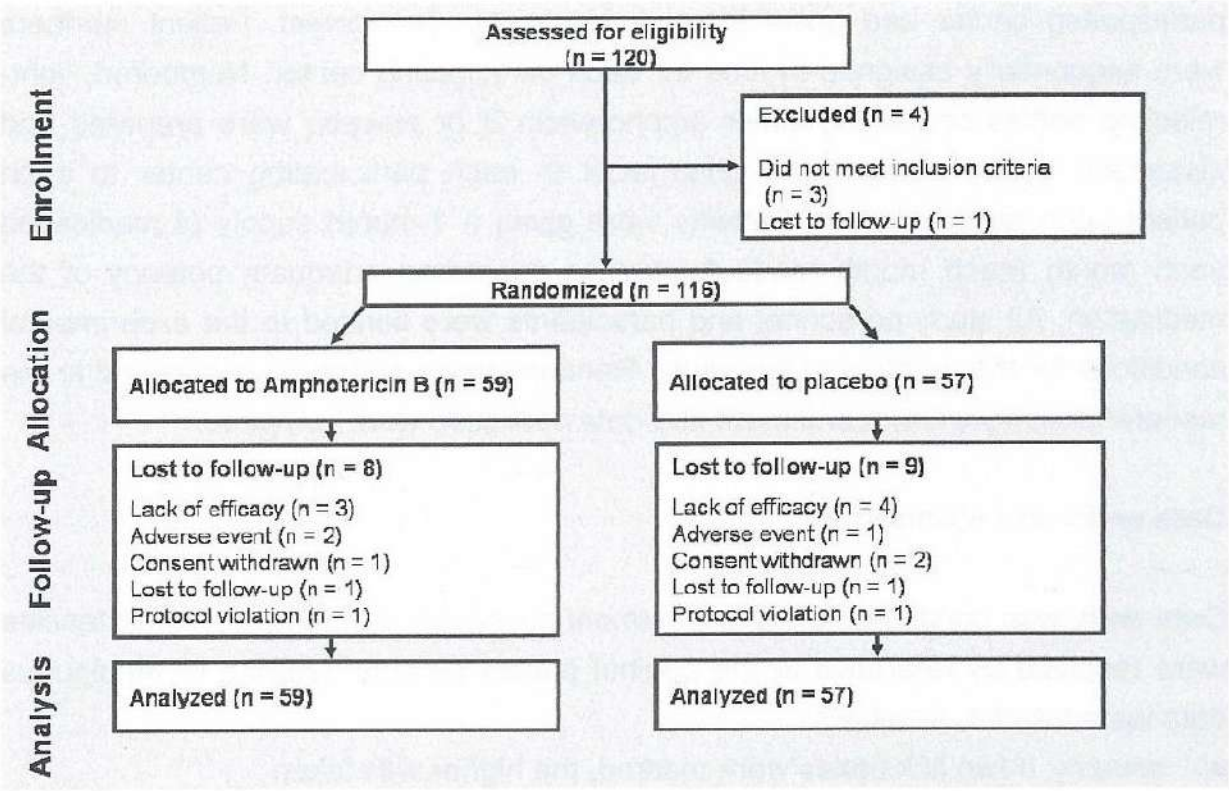
Data entry was conducted blind to treatment allocation. Errors and inconsistencies were resolved by reference to the original patient records. Missing or ambiguous data were treated as follows:

- severity: if two tick boxes were marked, the higher was taken

- missing data for VAS scores, nasal endoscopy scores, polyp scores and PNIF measurements: if scores on either side were present, the average of the two scores on either side was taken; missing values at visit -1, visit 3 and all other cases were treated as missing values
- missing data for SF-36 and RSOM-31: treated as missing data
- drop-outs (VAS scores, nasal endoscopy scores, polyp scores and PNIF measurements): last observation carried forward

No interim analyses of treatment outcomes were conducted. The occurrence of adverse events and the use of concurrent medication were monitored at each visit. When necessary, details as to the nature of the adverse event, date of occurrence, duration, intensity and severity, clinical course, necessary therapeutic measures and likely causality were recorded. In case of withdrawal, the reason of premature ending was recorded. Once 120 subjects were enrolled, inclusion of new patients in the trial was closed.

FIGURE 2. Flow of participants through the trial



Statistical analysis

Analyses were based on all patients randomly assigned to one of both treatment arms (intention-to-treat) and all patients with available follow-up (per-protocol). As specified in the trial protocol, outcome measures were evaluated by using change scores (3 months minus baseline). Two-sided *t*-test and chi-square analyses were performed to check for between-group demographic differences. Two-sided *t*-test and Mann-Whitney-U non-parametric tests were performed to check for between-group differences in clinical characteristics at baseline and for treatment effect. Paired *t*-test and Wilcoxon signed rank test were performed to check for significant changes from baseline within groups. Fisher's exact test was performed to check for significant differences in the occurrence of all adverse events. *P* values < 0.05 (1-sided) or equivalent for 2-sided tests were considered statistically significant. All statistical analyses were performed by using SPSS 12.01 (Chicago, IL, USA).

RESULTS

Participants flow

Recruitment took place between February 2002 and December 2004. Participants attended clinic visits at enrollment, at the time of randomization and two, six and thirteen weeks after randomization. Figure 2 shows the flow of participants through the trial. Table 3 summarizes baseline patient demographics and medical history. Table 4 summarizes baseline clinical characteristics. Overall, clinical characteristics were similar at baseline. Where in doubt (allergy, RSOM-31), *t*-test and chi-square analyses were performed. Although our study reached the desired enrollment of 120 patients, only 116 patients were randomized. Three patients did not fulfill the inclusion criteria at the time of randomization and one patient was lost to follow-up. 99 patients completed the trial, whereas 8 patients on amphotericin B and 9 patients on placebo did not. The dropout rate was approximately balanced between the two groups and no major differences in the reason of premature discontinuation were observed. Patients who dropped out were similar to those who completed in gender, endoscopic signs of disease and CT-scan scores. In general, amphotericin B and placebo nasal lavages were well tolerated by all patients and no serious side-effects were observed.

TABLE 3. Baseline characteristics of the study population (n = 116)

	Amphotericin B (n = 59)	Placebo (n = 57)
Age, years *(mean, sd)*	48.1 (11.1)	45.4 (12.7)
Male gender *(n, %)*	40 (68%)	37 (65%)
Asthma *(n, %)*	32 (54%)	30 (53%)
ASA intolerance *(n, %)*	17 (29%)	10 (18%)
Allergy (general) *(n, %)*	29 (49%)	37 (65%)
Allergy to fungi *(n, %)*	14 (24%)	9 (16%)
Smoking habits *(n, %)*		
current smoker	5 (9%)	7 (12%)
ex-smoker	19 (32%)	18 (32%)
non-smoker	35 (59%)	32 (56%)
Mean CT-scan score *(mean, sd)*	15.8 (6.3)	17.0 (5.2)
Presence of nasal polyps *(n, %)*	47 (80%)	48 (84%)
Number of surgical interventions *(mean, sd)*	3.3 (3.0)	3.2 (2.5)
Use of local steroids *(n, %)*	41 (70%)	38 (67%)

No statistical differences were observed between the placebo and active drug arm for all studied parameters

TABLE 4. Clinical characteristics of the study population (n = 116) at baseline

	Amphotericin B (n = 59)	Placebo (n = 57)
Total VAS score *(mean, sd)*	246 (98)	234 (108)
VAS nasal blockage *(mean, sd)*	51 (28)	45 (32)
VAS rhinorrhoea *(mean, sd)*	39 (28)	37 (32)
VAS post-nasal drip *(mean, sd)*	52 (28)	51 (31)
VAS reduced sense of smell *(mean, sd)*	75 (31)	69 (34)
VAS facial pain *(mean, sd)*	31 (32)	33 (32)
SF-36 health survey (PCS) *(mean, sd)*	42.4 (8.9)	43.6 (10.3)
SF-36 health survey (MCS) *(mean, sd)*	48.2 (10.3)	46.8 (11.4)
RSOM-31 total impact score *(mean, sd)*	150 (94)	176 (114)
PNIF *(mean, sd)*	139 (69)	145 (60)
Nasal endoscopy score *(mean, sd)*	7.7 (4.2)	7.2 (3.7)

No statistical differences were observed between the placebo and active drug arm for all studied parameters

Primary and secondary outcome measures

Analysis was based on intention-to-treat and involved all patients randomly assigned. Table 5 summarizes the result of all primary and secondary outcome measures (intention to treat). Mean VAS scores (total and individual), SF-36 and RSOM-31 data, PNIF values, nasal endoscopy scores and polyp scores were similar in both treatment groups at the time of randomization and no significant differences were observed for change scores at 13 weeks of treatment. Neither per-protocol analysis (data not shown), nor subgroup analyses (asthma, ASA intolerance, general allergy, fungal allergy, topical steroid treatment and presence or absence of nasal polyps) revealed significant differences between amphotericin B and placebo treated groups except for a significant improvement in change scores on total VAS, post-nasal drip VAS, and rhinorrhoea VAS in patients treated with placebo without asthma and/or topical steroid treatment. From baseline, both amphotericin B and placebo treated patients improved significantly on nasal endoscopy scores ($p = 0.017$ and $p = 0.004$ respectively), but no in-between group differences were observed. PNIF scores deteriorated significantly from baseline in the amphotericin B treated group ($p = 0.040$), but not in the placebo treated group ($p = 0.977$). Again, no significant in-between group differences were observed. For all other investigated variables, no significant changes from baseline were observed within treatment groups.

Adverse events

The proportion of patients experiencing an adverse event was similar between amphotericin B and placebo groups: 39 (66%) of 59 and 35 (61%) of 57 patients, respectively. On average, of the patients experiencing an adverse event, 2.0 (range 1-6) and 1.9 (range 1-6) incidents were reported in amphotericin B and placebo treated groups respectively. No significant difference in the occurrence of all types of adverse events was observed. The proportion of patients experiencing a serious adverse event, as judged by the investigators, was higher in the amphotericin B group than in the placebo group: 5 (9%) of 59 versus 0 (0%) of 57 patients, respectively. There was only one serious adverse event reported as drug-related (asthma attack). All adverse events reported are shown in table 6.

TABLE 5. Primary and secondary outcomes after 13 weeks of treatment

Change from baseline	Amphotericin B (n = 59)	Placebo (n = 57)	*t*- or z-value	*p* value
Total VAS score *(mean, sd)*	-3.1 (82.8)	-21.1 (101.2)	-1.356	0.31
VAS nasal blockage *(mean, sd)*	1.4 (31.3)	-3.4 (31.1)	-0.828	0.41
VAS rhinorrhoea *(mean, sd)*	-2.2 (23.0)	-2.8 (31.8)	-0.105	0.92
VAS post-nasal drip *(mean, sd)*	-4.9 (25.6)	-6.3 (30.4)	-0.272	0.79
VAS reduced sense of smell *(mean, sd)*	-0.4 (17.0)	-3.2 (22.6)	-0.742	0.46
VAS facial pain *(mean, sd)*	1.9 (21.9)	-4.9 (20.8)	-1.709	0.09
SF-36 health survey (PCS) *(mean, sd)*	0.6 (7.1)	1.4 (8.5)	0.474	0.64
SF-36 health survey (MCS) *(mean, sd)*	-0.3 (8.1)	1.9 (9.7)	1.153	0.25
RSOM-31 total impact score *(mean, sd)*	17.0 (86.4)	-3.6 (100.4)	-0.946	0.35
PNIF *(mean, sd)*	-11.9 (49.8)	-3.2 (53.5)	0.897	0.37
Nasal endoscopy score *(mean, sd)*	-1.1 (3.2)	-1.4 (3.7)	-0.471	0.64

A negative change indicates improvement, except for PNIF and SF-36 change scores. Analysis was based on intention-to-treat. *P* values of less than 0.05 were considered statistically significant. No significant differences were observed between placebo and amphotericin B treated patients for all investigated parameters.

Antibiotics were allowed at clinical exacerbation, but only after aerobic and anaerobic cultures were performed. Systemic steroids were allowed for a maximum period of 14 days when prescribed for a disease other than upper airway pathology. In total, 12 patients (20%) on amphotericin B and 10 patients (18%) on placebo needed a short course of antibiotics during the trial period. A course of systemic steroids was necessary in 1 patient (2%) in the amphotericin B treated group. Combined treatment with systemic steroids and antibiotics was necessary in 3 patients (5%) in the amphotericin B treated group and 2 patients (4%) in the placebo treated group.

DISCUSSION

Although it has long been recognized that fungi may cause severe acute and chronic rhinosinusitis in immunocompromised individuals[223], in immunocompetent

TABLE 6. Adverse effects

Adverse effects (n, %) *	Amphotericin B (n = 59)	Placebo (n = 57)
Acute exacerbation of CRS	9	7
Headache	13	10
Facial pain	1	1
Increased congestion and/or rhinorrhoea	6	8
Allergic rhinitis and/ or sneezing	3	0
Epistaxis	2	2
Common cold	1	3
Flu	3	3
Cacosmia	0	1
Mucocele	0	1
Otitis media	1	6
Otitis externa	2	0
Earache and/or fullness	6	5
Tinnitus	1	0
Nasal irritation due to douche	0	1
Toothache	0	3
Pharyngitis	1	0
Asthma exacerbation	5	1
Bronchitis	6	6
Pneumonia	1	1
Cough	3	1
Dyspnea	2	0
Chest pain	1	1
Dizziness	0	1
Itchy eyes	0	1
Eye infection	1	0
Fever	0	1
Reflux and/or gastric pain	2	1
Fungal and/or yeast infection skin	4	1
Psoriasis exacerbation	0	1
Skin rash	1	0
Surgery for stress incontinence	1	0
Cystitis	0	2
Cyst in groin	1	0
Backpain	2	2
Muscle ache	1	0
Gout	1	0
Insomnia	0	1

* Some patients reported multiple adverse effects.

hosts fungal elements are generally regarded innocent bystanders. Recently, Ponikau et al suggested that fungi play a more prominent role in the pathogenesis

of CRS in immunocompetent hosts than hitherto expected[122]. According to his concept, ubiquitous airborne fungi become entrapped in sinonasal mucus, are attacked by eosinophils and cause, via the release of toxic granules from eosinophils, secondary mucosal inflammation in susceptible individuals. If true, fungal eradication, by using intranasal antifungals, should improve the course of the disease. Indeed, in 2 pilot studies[123;124] such therapy was reported as successful. Both studies, however, were uncontrolled and thus susceptible to observer bias. When subjected to a double-blind placebo-controlled trial, no significant benefit from amphotericin B nasal spray or high-dose oral terbinafine was observed[127;131]. Although a second double-blind placebo-controlled study, showed some benefit from amphotericin B nasal lavages [9], this study included only 30 patients and outcome was mainly based on a reduction in inflammatory mucosal thickening of maxillary sinuses on CT of a standardized coronal cut, known to correlate poor with symptom scores[54;216].

We conclude, based on the results of our large double-blind, placebo-controlled multicenter study, that direct topical administration of intranasal amphotericin B is not a solution for patients suffering from CRS with or without NP, since neither major improvements nor significant differences between amphotericin B and placebo treated groups were observed. In addition, although not significant, placebo treated patients performed better on all posttreatment outcome measures (table 5), confirming the findings of Weschta et al, who showed that posttreatment symptom scores were distinctly better in the placebo treated arm[127].

Our sample primarily consisted of patients with CRS with a previous history of ESS referred to a tertiary otolaryngology center. This implies selection of patients with long-standing disease refractory to common medical therapy (e.g. local and systemic steroid treatment). Because of ethical reasons, the use of local corticosteroids was allowed when used consistently during the whole trial period. Although balanced between both treatment arms, an effect on treatment outcome cannot be excluded. Extrapolating outcomes to immunocompetent patients not treated with local corticosteroids, without previous ESS and seen in secondary otolaryngology centers is difficult. Comparison with results from previous published double-blind placebo controlled studies on intranasal application of amphotericin B[126;127], is however possible, especially since demographic details of patients in all

three studies are remarkably similar. The observed differences in treatment outcome, although marginal, may be explained by minor differences in patient demographic details. For instance, fungal allergy is slightly more common in the population studied by Ponikau et al and the presence of asthma and topical steroid treatment is less frequent in the population studied by Weschta et al.

In our study, subgroup analysis revealed no difference in outcome between patients with a proven allergy to fungi or not. Based on our results and the idea that a local immune response and not an allergy to fungi is responsible for the disease, it seems highly unlikely that treatment is only effective in patients for whom fungal sensitivity drives the expression of the disease.

Although previous pilot studies reported significant treatment effects after 4-12 weeks of treatment when using a 100 µg/mL amphotericin B solution twice daily (25 mL)[123;124], we observed no significant treatment effect upon 13 weeks of treatment. Therefore, we cannot exclude a possible treatment effect with longer treatment, but based on our results this seems very unlikely.

The injectable formulation of amphotericin B caries FDA-approved labeling solely for intravenous administration. Traditionally it is considered the most effective form of antifungal treatment. Several alternative routes of administration that use the injectable formulation have been reported, including administration of amphotericin B into the pleural cavity[224], bladder[225-228], synovial joints[229] and peritoneal space[230]. Although we assumed similar pharmacokinetics in our study, we did not study pharmacokinetics ourselves and results from previous studies have not been determined conclusively, except for bladder instillation[225-228].

Daily nasal irrigation is known to improve symptoms of CRS significantly[231;232]. Our study, however, showed only marginal effects of daily nasal irrigation. Reasons include the fact that most patients were on a daily nasal irrigation regimen before inclusion and all patients were required to participate in a two week run-in period on placebo.

Because we were interested in evaluating alleviation of symptoms and improvement of quality of life, we focused on patients' clinical responses as our

main outcome measure. Although previous studies showed a reduction in intranasal inflammatory mediators and fungal load upon treatment with amphotericin B[126], we believe the assessment of treatment effects should focus on clinical outcome measures. Since we did not study a reduction in fungal load, we cannot rule out the presence of persistent high fungal loads upon amphotericin B treatment in our study. This seems however, based on the results of Ponikau and others, highly unlikely.

In conclusion, our double-blind placebo-controlled multicenter trial shows no additional benefit of amphotericin B nasal lavages to intranasal steroids and irrigations in patients with CRS with or without NP with a previous history ESS. Our results suggest that extramucosal fungi are innocent bystanders in the upper respiratory tract and play no demonstrable role in the pathophysiology of CRS in immunocompetent patients.

CHAPTER 4.2

EFFECT OF TOPICAL AMPHOTERICIN B ON INFLAMMATORY MARKERS IN PATIENTS WITH CHRONIC RHINOSINUSITIS: A MULTICENTER RANDOMISED CONTROLLED STUDY

Ebbens FA, Georgalas C, Luiten S, van Drunen CM, Badia L, Scadding GK, Hellings PW, Jorissen M, Mullol J, Cardesin A, Bachert C, van Zele TPJ, Lund VJ, and Fokkens WJ, *The laryngoscope 2009;119:401-408*

ABSTRACT

Background: Recently, it has been suggested that an exaggerated immune response to fungi is crucial in the pathogenesis of chronic rhinosinusitis (CRS). Based on this rationale, the use of topical antifungals (amphotericin B) has been advocated. Studies on its clinical effectiveness are however contradictory.

Objective: To examine the effect of nasal antifungal treatment on secreted mediators in samples of nasal lavage fluid from patients with CRS with or without nasal polyps (NP).

Methods: Part two of a prospective double-blind, placebo-controlled multicenter clinical trial investigating the effect of 13 weeks of treatment with amphotericin B or placebo on the levels of pro-inflammatory cytokines, chemokines and growth factors (i.e. IL-1β, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin, RANTES, MCP-1, MCP-2, MCP-3, VEGF, EGF, FGF-basic, HGF, Gro-α) and albumin via a fluorescent enzyme immunoassay in nasal lavage specimens of CRS patients with or without NP.

Results: Topical amphotericin B had no significant effect on the level of any of the tested pro-inflammatory cytokines, chemokines and growth factors in CRS nasal lavage samples. Treatment with placebo, however, increased the level of MIP-1α and MIP-1β which, mediators involved in wound healing.

Conclusion: Topical amphotericin B has no effect on activation markers of nasal inflammatory cells in nasal lavage specimens of patients suffering from CRS with or without nasal polyposis.

INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nose and paranasal sinuses that is present for at least 12 weeks without complete resolution and is characterized by distinctive symptoms (e.g. nasal blockage, nasal discharge, facial pain and/or reduced sense of smell) and either endoscopic signs or computed tomography (CT) changes characteristic of the disease[1]. The etiology of CRS with or without nasal polyps (NP) is debated and its pathophysiology remains controversial. Recently, a fungal etiology has been proposed[122].

Various studies have shown that under optimal conditions, fungi can be identified within the nose and paranasal sinuses of nearly every individual[233;234]. While the presence of these fungi may not cause the disease CRS in itself, it has been suggested that fungi may trigger a cascade of events, ultimately resulting in the accumulation and degranulation of eosinophils in susceptible individuals[122]. Based on this rationale, the use of topical antifungals (amphotericin B) has been advocated[123]. Studies on its clinical effectiveness are however contradictory[123;124;126-128].

Cytokines, chemokines, and growth factors are potent biologic factors involved in the regulation of inflammation, immune defense and wound healing. Although we have not yet achieved a full understanding of the precise mechanisms underlying the pathogenesis of CRS, a variety of these mediators is suggested to be involved[1]. Interleukin (IL)-5, eotaxin, and transforming growth factor-beta (TGF-β) seem to be crucial players in the regulation of eosinophilic inflammation and extracellular matrix breakdown and/or deposition in CRS patients with concurrent NP. In addition, a variety of other inflammatory mediators including IL-1, IL-3, IL-4, IL-6, IL-8, IL-13, tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) and Growth related oncogene-α (Gro-α) may be increased in CRS tissue specimens with or without NP[1;183]. Importantly, as is the case for at least some of these mediators, cytokine levels have been shown to correlate with clinical signs and symptoms of CRS and effective anti-inflammatory treatment (a.o. macrolides) has been shown to significantly reduce cytokine levels in some individuals[235].

In this study we aimed to examine the effect of topical amphotericin B on various pro-inflammatory mediators. Using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA), we assessed nasal lavage specimens of 39 CRS patients for a variety of inflammatory markers before and following 13 weeks of nasal lavage with topical amphotericin B. This study was performed as part of a double-blind randomized controlled multicenter clinical study in which we observed no effect on clinical outcome of topical amphotericin B treatment in patients suffering from CRS with or without NP[128].

METHODS

Participants

This study was based on a double-blind, placebo-controlled, multicenter trial assessing the effectiveness of intranasal amphotericin B (100 mg/mL) when used for 3 months in adult patients with CRS with or without NP[128]. It included patients presenting to the Otorhinolaryngology Department of the Academic Medical Center (Amsterdam, The Netherlands), Erasmus Medical Center (Rotterdam, The Netherlands), Royal National Hospital (London, United Kingdom), University Hospital Ghent (Ghent, Belgium), University Hospital Leuven (Leuven, Belgium), or Hospital Clinic de Barcelona (Barcelona, Spain) between February 2002 and December 2004. All adult patients with clinical symptoms of CRS, endoscopic signs of CRS with or without NP and sinus CT scan score of at least 5 according to the Lund and Mackay scoring system[51], who had undergone previous endoscopic sinus surgery were eligible to enroll. To guarantee adequate access to sinonasal mucosa on irrigation with intranasal amphotericin B and to improve the representativeness of the lavage material, previous endoscopic sinus surgery (ESS) was obligatory for inclusion. Exclusion criteria included immunodeficiency (AIDS, chronic systemic steroid use, immunosuppressive treatment, immunoglobulin deficiency, complement deficiency), inability to provide consent or concerns regarding compliance, actual or suspected pregnancy, use of oral antifungals, use of topical decongestants or antihistamines, Mycobacterium infection, osteoporosis and chronic renal or liver failure.

Intervention

In this study, patients applied 25 mL of a 100 mg/mL amphotericin B solution or placebo to each nostril twice daily using an Emcur (also named Rhinicur) nasal douching device (Emcur GmbH, Bad Ems, Germany). Amphotericin B is active against most moulds frequently identified within the paranasal sinuses while fractions involuntarily ingested are not systemically absorbed. The applied concentration (100 mg/mL) is approximately 30 to 100 times higher than the minimum inhibitory concentration for all relevant fungi[218]. The study protocol was approved by the medical ethical committee of each participating center and all participating patients read and signed an informed consent form before enrolment.

Study design

Randomized allocation to one of two treatment groups took place on visit 0. Before randomization, each patient was required to participate in a 2-week run-in period on saline in order to get acquainted with the Emcur nasal douching device. Follow-up visits were scheduled at 2, 6, and 13 weeks after randomization. On randomization, the hospital pharmacist provided each participating patient with trial medication. Amphotericin B nasal lavage solution was prepared by dissolving amphotericin B for injection (Bristol-Myers-Squibb, New York, NY, USA), resulting in a clear yellow solution. Placebo nasal lavage solution was prepared by dissolving 3.4 mL/L Cernevit (Baxter, Deerfield, IL, USA) in sterile water containing 2.5% (w/v) glucose, resulting in a solution identical in color and smell to the amphotericin B solution. At visit 0 and visit 3 (13 weeks of treatment) mucus samples were collected from all patients by flushing each nostril with 20 mL of sterile saline using a sterile syringe with a blunt curved needle. At the start of this procedure, patients were asked to take a deep inspiratory breath and hold it until one of the investigators injected sterile saline into one of the nostrils. Upon injection, patients were asked to forcefully exhale through the nose during flushing. The return was collected in a sterile pan and stored at -80°C until analysis.

Detection of pro-inflammatory mediators in nasal lavage specimens

Upon analysis, a random sample of nasal lavages obtained from 39 of 116 patients (19 treated with placebo and 20 treated with amphotericin B) was defrosted to room temperature, vortexed and centrifuged at 1400 rpm. Supernatants were collected for subsequent analysis. All study personnel was blinded to the treatment allocation for the duration of the investigations. Randomization codes were revealed to the researchers once data collection was complete.

Determination of IL-5 and eotaxin concentrations was performed using a sandwich immunoassay containing 30 analytes (i.e. IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-2 receptor (IL-2R), IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, interferon α (IFN-α), IFN-γ, granulocyte colony-stimulating factor (G-CSF), GM-CSF, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, interferon-inducible protein of 10 kDa (IP-10), monokine induced by IFN-γ (MIG), eotaxin, RANTES, monocyte chemoattractant protein 1 (MCP-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF-basic) and hepatocyte growth factor (HGF) (Biosource™, Invitrogen, Breda, the Netherlands). Gro-α concentrations were determined using a custom-made sandwich immunoassay containing 3 analytes (i.e. MCP-2, MCP-3 and Gro-α, Biosource™, Invitrogen, Breda, the Netherlands). IL-3 and albumin concentrations were determined by performing separate sandwich immunoassays (Biosource™, Invitrogen, Breda, the Netherlands and Bethyl Laboratories INC, Montgomery, TX, USA respectively). All assays were performed as described by the manufacturer (Biosource™, Invitrogen, Breda, the Netherlands (30-plex, 3-plex and IL-3 assay) and Bethyl Laboratories INC, Montgomery, TX, USA (albumin assay)). Plates were analyzed using a Luminex 100™ instrument (Luminex BV, Oosterhout, the Netherlands, 30-plex, 3-plex and IL-3 assay) or using a Versamax microtiter plate reader (Versamax, Molecular Devices Ltd, Sunnyvale, CA, USA, albumin) at a wavelength of 450 nm.

Statistical analysis

Statistical analysis was carried out using SPSS 14.0 (Chicago, IL, USA). All variables were tested for normality both graphically and by using the Kolmogorov-Smirnov test. Cytokine, chemokine, and growth factor values reported as below threshold were recoded to zero. Adjusted values were computed by dividing the observed mediator concentration by the corresponding albumin concentration of the same lavage specimen. Both unadjusted and adjusted values were analyzed. Change values (3 months minus baseline) were computed to evaluate treatment effect. Demographic and clinical characteristics in both groups at baseline were compared using χ^2 and Fisher's Exact tests (proportions) and two-sided *t* tests and Mann-Whitney *U* tests (continuous variables) as required. Changes in mediator concentrations *within* each group were assessed using Wilcoxon signed-rank tests. Differences in mediator changes *between* both groups were assessed using Mann-Whitney U tests. Using the Bonferroni adjustment for multiple comparisons, the level of statistical significance was set to 0.0015 (2-sided).

TABLE 1. Baseline demographic and clinical characteristics

	Placebo (n = 19)	Amphotericin B (n = 20)	*p* value
Age (y), mean (SD)	41.7 (15.3)	46.8 (8.11)	0.21
Male gender, n (%)	11 (58%)	16 (80%)	0.14
ASA intolerance, n (%)	4 (21.1%)	7 (35.0%)	0.33
Asthma, n (5%)	11 (58%)	9 (45%)	0.42
Allergy (general), n (%)	12 (63%)	8 (40%)	0.30
Fungal allergy, n (%)	1 (7%)	3 (16%)	0.61
Smoking habits, n (%)			
Current smoker	3 (15.8%)	3 (15.0%)	0.95
Ex-smoker	9 (47.4%)	9 (45.0%)	0.88
Non-smoker	7 (36.8%)	8 (40.0%)	0.84
Mean CT score, mean (SD)	18.1 (3.40)	18.1 (4.25)	1.00
Presence of nasal polyps, n (%)	17 (89.5%)	18 (90%)	1.00
Number of surgical interventions, median (IQ range)	3 (1.0-5.0)	2.50 (2.0-4.75)	0.93
Use of local steroids, n (%)	12 (63%)	14 (72%)	0.56

RESULTS

Participants

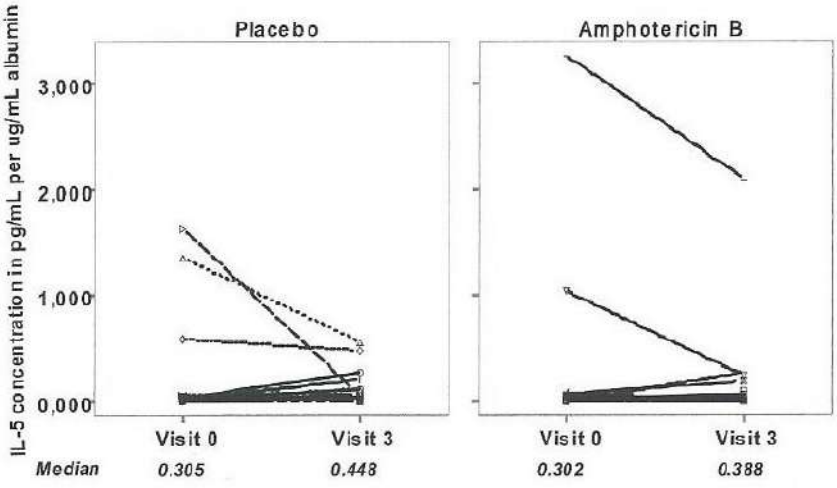
Of the 116 included patients included in this double-blind placebo-controlled multicenter trial, 99 patients completed the study per protocol. From these 99 patients who completed the trial, paired nasal lavage samples of thirty-nine patients (20 from the amphotericin B treated group and 19 from the placebo treated group) were randomly selected. No significant differences in demographic and clinical characteristics were observed at baseline between both groups (table 1).

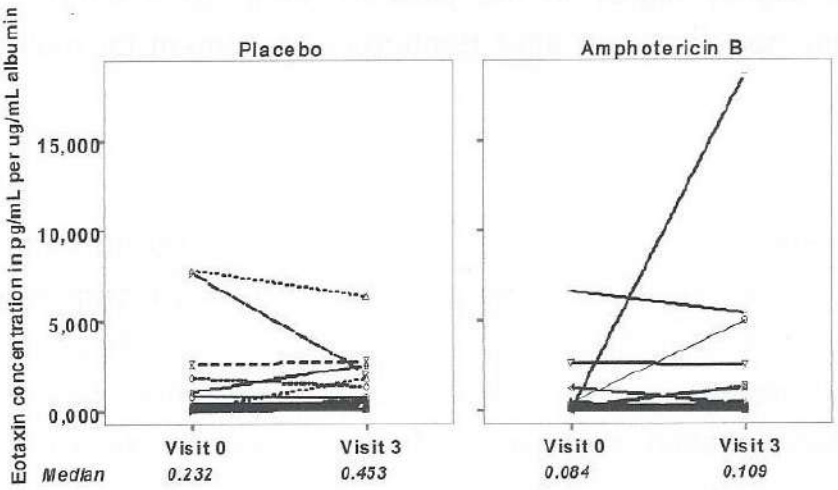
Albumin and cytokine concentrations

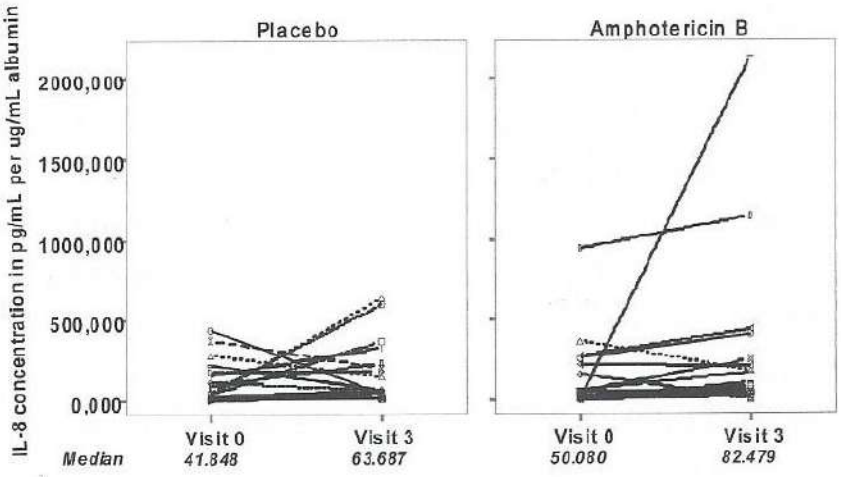
Baseline

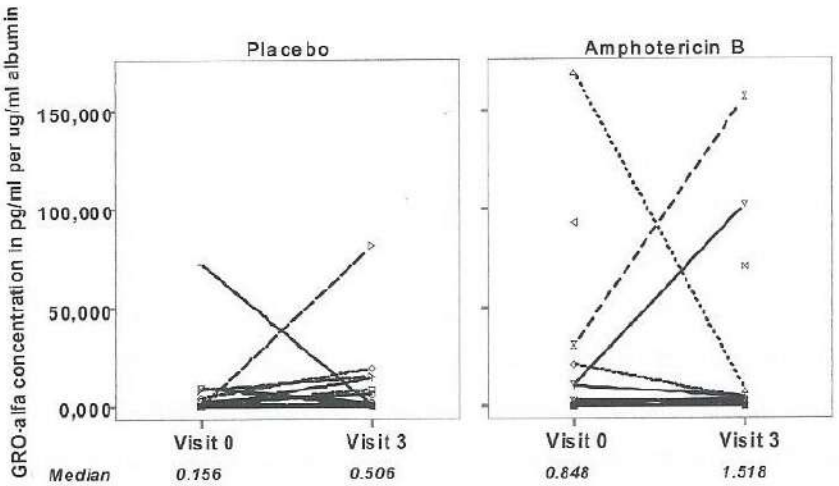
The median albumin concentration in nasal lavage fluid at baseline was 109.6 mg/mL in the amphotericin B group and 96.8 mg/mL in the placebo group (p = 0.29). No significant differences were observed for all cytokine, chemokine, and growth factor concentrations, either before or after adjustment for the observed albumin concentration, between both groups. Although the albumin-adjusted G-

FIGURE 1. Change in albumin-adjusted IL-5, IL-8, eotaxin and GRO-α concentrations between both groups




Placebo
Amphotericin B
Eotaxin concentration in pg/mL per ug/mL albumin
15,000
10,000
5,000
0,000
Visit 0
Visit 3
Median
0.232
0.453
Visit 0
Visit 3
0.084
0.109


Placebo
Amphotericin B
IL-8 concentration in pg/mL per ug/mL albumin
2000,000
1500,000
1000,000
500,000
0,000
Visit 0
Visit 3
Median
41.848
63.687
Visit 0
Visit 3
50.080
82.479


Placebo
Amphotericin B
GRO-alfa concentration in pg/ml per ug/ml albumin
150,000
100,000
50,000
0,000
Visit 0
Visit 3
Median
0.156
0.506
Visit 0
Visit 3
0.848
1.518

GSF concentration was slightly higher in the placebo group (p = 0.03), this difference was statistically not significant after Bonferroni adjustment for multiple comparisons.

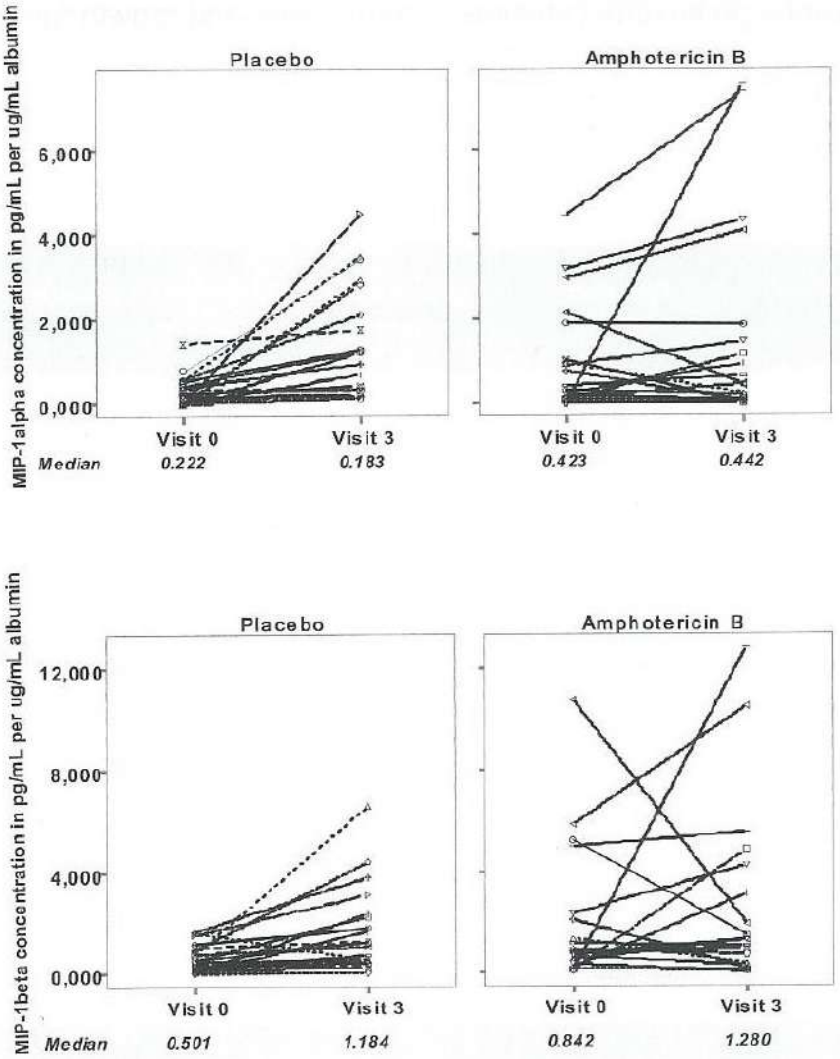
Three months of treatment

Albumin concentrations after 3 months of treatment were essentially unchanged (median 88.8 mg/mL in the amphotericin B treated group and 36.3 mg/ml in the placebo treated group, p = 0.76 and p = 0.1 respectively). The levels of most mediators at baseline correlated closely with levels at 3 months from baseline. Although the adjusted concentration of 26 out of 34 mediators increased in the amphotericin group, none of these changes was statistically significant. More importantly, in the placebo group the adjusted concentration of most tested mediators (20 of 34) increased as well (figure 1). Although this increase in the placebo group was more pronounced for IL-15 and HGF (p = 0.025 and p = 0.015 respectively), it was not significant after Bonferroni adjustment for multiple comparisons. The observed increase in MIP-1α and MIP-1β in the placebo group, however, was statistically significant ($p < 0.0001$ and p = 0.001 respectively) (figure 2). When comparing change values for the adjusted mediator levels between both groups, no significant differences were observed (table 2). Although repeat analysis for most unadjusted cytokine concentrations revealed similar results as described above for the adjusted cytokine concentrations (table 3), now we did no longer observe a significant change from baseline in the concentration of both MIP-1α and MIP-1β within each group.

DISCUSSION

If the inflammation observed in CRS patients is the result of an immune reaction to fungi, reducing the presence of this inflammatory trigger may improve the course of the disease[122]. Ideally, treatment should eliminate the fungus without causing harm to the host. Topical treatment, thus, seems most attractive. Although the injectable formulation of amphotericin B carries US Food and Drugs Administration-approved labeling solely for intravenous administration, several alternative routes of administration that use the injectable formulation have been reported including the administration of amphotericin B into the pleural cavity[224] and bladder[228].

FIGURE 2. Change in albumin-adjusted MIP-1α and MIP- 1β concentrations between both groups



Recently, amphotericin B nasal lavages have been advocated in the treatment of CRS. Studies on its clinical effectiveness, however, have yielded conflicting results[123;124;126-128].

In recent years, several hypotheses have been put forward regarding topical amphotericin's mechanism of action. Besides possessing an antifungal effect, it has been suggested that amphotericin B may reduce inflammation via a direct cytotoxic effect on nasal polyp epithelial cells[236;237] or that it may have anti-

inflammatory properties[238;239]. In this study we aimed to examine the effect of amphotericin B nasal lavages on several cytokines, chemokines and growth factors

TABLE 2. Median change from baseline in albumin-adjusted mediator concentrations

Concentration in pg/mL per µg/mL albumin	Placebo (n = 19)	Amphotericin B (n = 20)	*p* value
IL-1β	0	0	0.41
IL-3	1.33	-0.64	0.52
IL-4	0.008	0	0.85
IL-5	0.01	0.004	0.80
IL-6	0.033	0.186	0.60
IL-8	10.53	36.29	0.33
IL-13	0	0.01	0.79
TNF-α	0.015	0.01	0.71
Eotaxin	0.088	0.0059	0.78
IFN-γ	0.023	0.0087	0.56
MIP-1α	0.696	0	0.06
MIP-1β	0.617	0.04	0.17
GM-CSF	0	0	0.11
GRO-α	0.09	0	0.60
RANTES	0	-0.02	0.97

TABLE 3. Median change from baseline in raw mediator concentrations

Concentration in pg/mL per µg/mL albumin	Placebo (n = 19)	Amphotericin B (n = 20)	*p* value
IL-1b	0	0	0.28
IL-3	75.34	-63.04	0.27
IL-4	0	0	0.89
IL-5	0	-0.48	0.73
IL-6	-3.40	-0.27	0.46
IL-8	358.58	3870.19	0.18
IL-13	0	0	0.96
TNF-α	0.12	1.33	0.46
Eotaxin	6.72	1.90	0.82
IFN-γ	1.50	1.68	0.33
MIP-1α	8.06	-12.50	0.19
MIP-1β	9.64	-7.01	0.46
GM-CSF	0	0	0.11
GRO-α	0	0	0.40
RANTES	0	0	0.91

that are known to be involved in leukocyte migration to sites of inflammation and other stages in the inflammatory cascade.

We used nasal lavages to collect mucus, a technique that has been used frequently by others to collect and subsequently study the presence or absence of pro-inflammatory mediators. This method has been shown to have relatively low within-subject variability[240]. However, since unknown fractions of lavage fluid may be swallowed or absorbed, this technique can be associated with potentially unpredictable dilutions of nasal secretions. This could pose a problem when interpreting the observed cytokine, chemokine, and growth factor concentrations. It has been shown that the use of albumin as a marker of dilution improves the accuracy of quantifying endogenous substances in nasal secretions[241]. Although we did not observe major differences in outcome when comparing the unadjusted and adjusted mediator concentrations, to our opinion adjusted mediator concentrations provide a more accurate estimate of true mediator concentrations.

If effective, one would expect that any clinical effect of topical amphotericin B is associated with concurrent demonstrable effects on CRS mediators. However, in agreement with our clinical data[128], we demonstrate that 13 weeks of treatment with topical amphotericin B does not result in a statistically significant reduction in the level of any of the tested cytokines, chemokines or growth factors. Our results are in line with recent data by Shin et al, who showed that topical amphotericin B treatment does not result in a significant reduction in the level of IL-5, IL-8, IFN-γ, and RANTES and Weschta et al, who showed that topical amphotericin B treatment does not result in a significant reduction in eosinophilic cationic protein (ECP) and tryptase levels[238;239]. Thus, any direct or indirect anti-inflammatory effect of topical amphotericin B in patients with CRS appears highly unlikely. Although Weschta et al observed that neither topical amphotericin B treatment nor fungal state before and after treatment significantly influenced the level of any of the tested inflammatory activation markers[238;239], we cannot exclude the possibility that treatment with topical amphotericin B may have an effect on the inflammatory markers in those patients who are fungus positive at inclusion. However, since fungi have been shown to be omnipresent[233;234], it seems highly unlikely that only fungus negative patients were included in our study.

Although no significant differences were observed when comparing both treatment groups, within the placebo group a clear increase in both MIP-1α and MIP-1β (adjusted concentrations) is observed. Although these results could be a chance occurrence, MIP-1α and MIP-1β belong to the CC family of chemokines and are crucial for T-cell chemotaxis to inflamed tissue. They both play an important role in the regulation of transendothelial migration of monocytes, dendritic cells, and NK cells. Thus, it is not surprising that MIP-1α and MIP-1β are key players in the pathogenesis of many inflammatory conditions including asthma, granuloma formation, arthritis, multiple sclerosis, pneumonia and psoriasis[242]. Moreover, MIP-1α is also critical for macrophage chemotaxis to sites of cutaneous wound repair and may promote healing by inducing inflammatory responses against various pathogens such as viruses[243] and parasites[244;245]. Since most patients who received placebo performed better on all clinical outcome measures when compared to those who were treated with amphotericin B[128], we suggest that the increase in MIP-α and MIP-1β may reflect enhanced tissue repair in those patients treated with placebo, a result that is in accordance with clinical data demonstrating a positive effect of saline douching in patients suffering from CRS[246-248]. However, further studies that could characterize the role of MIP-1α and MIP-1β in the pathogenesis of CRS are needed. In the meanwhile, our study adds to the body of evidence that suggests a limited (if any) role for topical amphotericin B in the treatment of patients suffering from CRS.

CHAPTER 4.3

THE MOLD CONUNDRUM IN CHRONIC RHINOSINUSITIS RHINOSINUSITIS

Based on a publication by Ebbens FA, Georgalas C, and Fokkens WJ, *Current Allergy and Asthma Reports (in press)*

INTRODUCTION

Although bacteria have long been implicated in the pathogenesis of most forms of CRS, it has been recognized that fungi are implicated in some forms. Fungal spores, due to their ubiquitous nature, are continuously inhaled and deposited on the airway mucosa. Although they rarely behave as pathogens in the airways of healthy individuals, they may occasionally be the cause of disease.

In 1983, Katzenstein et al identified non-invasive *Aspergillus* species in thick, tenacious, dark-colored eosinophilic mucus (so called eosinophilic or allergic mucin) obtained from the nose and paranasal sinuses of patients suffering from CRS with nasal polyposis and introduced the term "allergic *Aspergillus* sinusitis" because of histopathological similarities with allergic bronchopulmonary aspergillosis[249]. Later the term "allergic fungal sinusitis" (AFS) was coined after other non-invasive fungi were shown to produce similar symptoms[250]. As clinical evidence of AFS accumulated, controversy regarding its definition (including the presence of allergy to fungi as a diagnostic criterion), its prevalence and pathogenesis emerged[251;252]. Ponikau et al demonstrated the presence of both fungi and eosinophils in the nose and paranasal sinuses of nearly all CRS patients by using novel collection, culturing and histology techniques, thus suggesting that the majority of CRS patients actually suffers from AFS[122]. Their findings stimulated new discussions about the definition, prevalence and disease mechanisms of AFS.

PREVALENCE AND MICROBIOLOGY OF FUNGI

An obvious diagnostic criterion for AFS is the presence of non-invasive fungi in the nose and paranasal sinuses. However, contradictory results have been published with prevalence rates ranging from 0% to 100% in both CRS patients and healthy controls (table 1)[122;126;127;129;131;217;249;253-268]. As was suggested by Ponikau et al[122], differences in collection and detection techniques may explain the observed differences in fungal yield. Of all collection techniques, the nasal lavage technique is considered the most accurate collection technique[253;260;261].

Although Ponikau et al describe a prevalence rate of 100% upon culture in their study using novel collection and detection techniques[122], most authors agree that

polymerase chain reaction (PCR) is superior to both culture and Grocott methanamine silver stains (GMS) in detecting fungal elements[254;255;257;260;264]. A recent development is the publication of the detection of one or more fungal hyphae in 100% of CRS mucus specimens using the fluorescein-labeled chitinase stain[259]. Although no other studies using this technique have been published to date, this finding is interesting and warrants future research.

The ubiquitous presence of fungi in the nose and paranasal sinuses of both CRS patients and healthy controls could lead to the argument that it is not the presence or absence of fungi, but rather a specific fungal species or fungal load that is relevant for disease development. Cultures collected via the novel technique described by Ponikau et al[122] grew 37-40 different genera with 2.7-3.2 species per CRS patient and 2.3-3.1 per healthy control[122;257;258;260]. *Aspergillus, Penicillium, Cladosporium, Candida, Aureobasidium* and *Alternaria* appeared most frequently with no significant differences between the two groups. A different study, assessing the amount of fungal DNA present in tissue specimens obtained from CRS patients and healthy controls, demonstrated no differences in fungal load between the two groups[256], rendering it unlikely that fungal species and fungal load play a role in disease development. Whether an increase in fungal *allergen* content is involved in CRS pathogenesis remains unclear.

HYPERSENSITIVITY TO FUNGI

A generally accepted concept has been that immunoglobulin E (IgE)-mediated systemic fungal allergy drives the pathologic process behind AFS. Indeed, various authors have studied sensitization rates to fungi in CRS patients, demonstrating values ranging from 18% to 75%[122;123;128;259]. However, there were no significant differences between those patients with fungi and those patients without fungi in their nose and paranasal sinuses[263]. Importantly, Ponikau et al reported sensitization rates in CRS patients that are no different from those in healthy controls[122], although others report higher levels of fungus specific IgE in those CRS patients with eosinophilic mucin (with or without fungi)[269]. Although higher in CRS patients with eosinophilic mucin, no significant differences were observed between this group of patients and group of patients suffering from allergic rhinitis

TABLE 1. Prevalence of fungi (adapted from Ebbens et al[233])

Author	Year	Collection technique	Site of collection	Detection technique	Fungi in CRS patients (%)	Fungi in controls (%)
Katzenstein et al[249]	1983	Surgically excised mucosa	Paranasal sinuses	Histology	6.2	
Ponikau et al[122]	1999	Nasal lavage	Nasal cavity	Culture	96	100
		ESS guided sampling*	Paranasal sinuses	Histology	81	
Catten et al[253]	2001	Cytology brush	Nasal septum, inferior turbinate	PCR	40	
		Cotton swab	Nasal septum inferior turbinate	PCR	0	
Taylor et al[259]	2002	ESS guided sampling	Paranasal sinuses	GMS stain	76	
				Fluorescein-labeled chitinase stain	100	
Buzina et al[260]	2003	Nasal lavage	Nasal cavity	Culture	91.3	91.3
		ESS guided sampling	Paranasal sinuses	Culture	84.0	
				GMS stain	70.2	
Braun et al[217]	2003	Nasal lavage	Nasal cavity	Culture	91.3	91.3
		ESS guided sampling	Paranasal sinuses	Histology	75.5	
Scheuller et al[256]	2004	ESS guided sampling	Middle meatus	PCR	21.1	36.8
Kostamo et al[265]	2004	Nasal lavage	Nasal cavity	Culture	16.7	0
		ESS guided sampling	Paranasal sinuses	PAS* & GMS stain	16.7	
Granville et al[266]	2004	Surgically excised mucosa	Paranasal sinuses	GMS stain	11.7	
Weschta et al[127]	2004	Nasal lavage	Nasal cavity	Culture, fluorescence microscopy & PCR	63.3	
Jiang et al[261]	2005	Cotton swab	Middle meatus	Culture	11.8	
		Nasal lavage	Nasal cavity	Culture	49	
Kim et al[257]	2005	Nasal lavage	Nasal cavity	PCR	92.5	
				Culture	23.2	
Polzelhl et al[255]	2005	Nasal lavage	Nasal cavity	Culture	25	
				PCR	44	
Kennedy et al[131]	2005	Nasal lavage, mucus sampling	Nasal cavity	Histology, culture	77.4	
Rao et al[254]	2006	ESS guided sampling	Ethmoid bulla or ethmoid sinus	PCR	6.5	0
				Culture	0	0
Murr et al[258]	2006	ESS guided sampling	Middle meatus	PCR	45.9	45.9

TABLE 1. Prevalence of fungi (continued)

Author	Year	Collection technique	Site of collection	Detection technique	Fungi in CRS patients (%)	Fungi in controls (%)
Corradini et al[268]	2006	Nasal lavage	Nasal cavity	Culture	77	
Tosun et al[263]	2007	Nasal lavage	Nasal cavity	PCR	43.9	
Hafidh et al[262]	2007	Nasal lavage, mucus sampling	Nasal cavity	Histology, culture	46.6	70
Aydil et al[264]	2007	ESS guided sampling	Nasal cavity and paranasal sinuses	Microscopy Culture PCR	46.7 26.7 60.0	5.6 22.2 33.3
Liang et al[129]	2008	Nasal lavage	Nasal cavity	Culture	54.7-65.6	

* ESS: Endoscopic Sinus Surgery
* PAS: periodic acid Schiff stain

with proven allergy to fungi but without sinus involvement[269]. In addition, Shin et al recently showed that IgE levels to various common airborne fungi (*Alternaria, Aspergillus, Cladosporium* and *Penicillium)* are similar in 18 CRS patients and 15 healthy controls, rendering it unlikely that an allergy to a specific fungus is involved[270]. Interestingly, a recent study demonstrated that patients with CRS who do not have an allergy to the fungus that is present in their eosinophilic mucin may have elevated IgE levels to other fungi[269]. This leads to the obvious question whether the presence of type I hypersensitivity to fungi is relevant for disease development. Most likely, the presence of type I hypersensitivity to fungi represents concurrent fungal allergy in the majority of CRS patients.

LOCAL HOST DEFENSE AGAINST FUNGI

Inflammatory cells

Cellular immune responses vary according to the fungal species, the morphotype encountered and the anatomical site of interaction. Whereas yeasts and spores are often effectively phagocytosed, the larger size of hyphae precludes effective ingestion and requires interaction with different inflammatory cells. Although

eosinophils, neutrophils, macrophages and monocytes are all important antifungal effector cells, most research in CRS has focused on the role of eosinophils in antifungal immune defense.

The concurrent presence of fungi and eosinophils in nearly all CRS tissue specimens has lead to the suggestion of a cause and effect relationship[122;271;272]. Indeed, Wei et al recently demonstrated a concentration-dependent increase in (CRS) eosinophil migration towards both CRS nasal mucin and CRS nasal tissue extracts[273], suggesting that fungi may trigger inflammatory cells to initiate a complex localized eosinophilic reaction. However, one should note that most CRS patients in this study suffered from asthma (9/10 patients) and/or atopy (4/10 patients). Since eosinophils from subjects with asthma (both allergic and non-allergic asthma) are known to exhibit a primed phenotype that is likely the consequence of eosinophil interaction with cytokines in the peripheral blood, resulting in increased eosinophil migration-, adhesion-, and degranulation capacities, it may well be that the presence of asthma and/or atopy rather than that of fungi explains the observed concentration-dependent increase in eosinophil migration[274;275]. This hypothesis is supported by recent data in sheep, in which primed eosinophils were shown to be more effective in immobilizing and killing gastrointestinal parasites in the presence of specific anti-parasite antibodies in comparison to unprimed eosinophils[276].

Cytokines and chemokines

Various cytokines and chemokines have been implicated in CRS pathogenesis[1;2]. Cytokines and chemokines are low molecular weight proteins with growth, differentiation, and activation functions that regulate and determine the nature of both innate and adaptive immune responses[277]. Recently, striking differences were observed between CRS and healthy control peripheral blood mononuclear cell (PBMC) cytokine responses when cultured with extracts from 4 common airborne fungi (*Alternaria, Aspergilllus, Cladosporium* and *Penicillium*). When cultured with *Alternaria* extract, PBMCs of CRS patients produced significantly more IL-5 and IFN-γ in comparison to healthy control PBMCs. In addition, some PBMCs of CRS patients produced more IL-5 in response to *Aspergillus* and *Cladosporium* extracts. PBMCs from all CRS patients produced IL-13 upon culture

with all tested fungal extracts[270]. Together, these data suggest that fungi may induce PBMC cytokine production (mainly T_H2 cytokines). Unfortunately, 61% of the patients demonstrated increased IgE levels to common aeroallergens and 78% suffered from bronchial asthma. Since PBMCs from asthmatic subjects (both allergic and non-allergic) are known to produce more IL-5 in response to allergen in comparison to both allergic rhinitic subjects and healthy controls[278] and since a second study (in which the frequency of atopy was equally distributed) showed only minimal changes in IL-5 and IFN-γ expression upon culture with *Aspergillus* and *Alternaria* extracts[279], the role of fungi in inducing a T_H2 cytokine response remains to be determined.

Antimicrobial peptides

Cathelicidins and defensins are two major families of cationic antimicrobial peptides involved in innate immunity at mucosal surfaces. Recently, Ooi et al demonstrated that LL-37, the free C-terminal peptide of human cathelicidin hCAP18 (human cationic antimicrobial peptide 18kDa), is significantly upregulated in a dose-response effect at the mRNA and protein level in CRS patients without eosinophilic mucin in response to *Aspergillus fumigatus* and *Alternaria tenuis*. However, in CRS patients with eosinophilic mucin (but without fungal presence) no significant increase in LL-37 was observed at either the mRNA or the protein level in response to *Aspergillus* challenge. No increase in expression in both tissue and secreted LL-37 was observed upon *Alternaria* challenge[280]. Although the idea is interesting, since neither CRS patients with eosinophilic mucin and fungal presence nor a control group were included in this study, the exact role of LL-37 in the CRS pathogenesis remains to be determined.

Besides cathelicidins and defensins, various other antimicrobial peptides, including lactoferrin, lysozyme and secretory leukoprotease inhibitor, have been identified in nasal secretions[281] and sinus mucosa[282]. Lactoferrin possesses a variety of functions, including antibacterial, antifungal, and antiviral activities[283]. More recently, it has also been shown to possess antibiofilm properties[284]. Bacterial biofilms are present in the majority of CRS patients and may contain large amounts of fungal elements[285]. Downregulation of lactoferrin was recently observed in those CRS patients with nasal polyps[286] and/or those with biofilms[287]. However,

no difference was observed between CRS patients with or without eosinophilic mucin, those with or without fungal allergy and those with or without fungi present[286;287].

Surfactant proteins

Pulmonary surfactant is a mixture of phospholipids and proteins. Four different surfactant proteins (SPs) are known to exist: SP-A, SP-B, SP-C and SP-D[288]. SP-D binds and agglutinates micro-organisms and enhances phagocytosis, chemotaxis, and cytokine production. SP-D has been shown to play an important role in the immune response to *Aspergillus fumigatus* in the lung and is present in submucosal glands of CRS patients without eosinophilic mucin, CRS patients with eosinophilic mucin but without fungal allergy and healthy controls. Highest levels are detected in healthy controls. In CRS patients with eosinophilic mucin and fungal allergy, however, SP-D protein levels are below the detection level. *In vitro* studies demonstrate that *Alternaria tenuis* upregulates SP-D mRNA in those CRS patients with and those without eosinophilic mucin. *Aspergillus fumigatus*, on the other hand, increases SP-D mRNA expression in CRS patients without eosinophilic mucin only[289]. Absence of SP-D protein may result in failure to clear fungi from the nose and paranasal sinuses and, as a result, disease development.

FUNGUS ANTI-HOST EFFECTS

Besides innate and adaptive antifungal immune responses that may contribute to disease development, fungus anti-host effects may be involved in CRS pathogenesis. Ubiquitous airborne fungi (especially *Alternaria* and *Aspergillus*) are known to produce proteases that bind to protease-activated receptors (PARs) expressed on epithelial cells, airway cells, leukocytes and blood vessels, thereby activating intracellular signaling pathways that give rise to multiple responses, including the production and release of mediators involved in tissue damage[290-292]. In addition to an indirect effect, recent studies indicate that *Alternaria alternata* may activate eosinophils directly. *Alternaria alternata,* but not IL-5, has been shown to induce eosinophil IL-8 synthesis and eosinophil surface expression of CD11b (a β_2-integrin that is used by eosinophils to adhere to β-glucan, a major fungal cell wall component[293]) and CD63 (a component of eosinophil granule membranes) in

healthy volunteers, patients with allergic rhinitis and patients with bronchial asthma. Upon recognition of *Alternaria alternata*, eosinophils released eosinophil derived neurotoxin (EDN)[294] and this response may play a pivotal role in CRS pathogenesis.

THERAPY

Antifungals

If CRS inflammation is caused by an immune reaction to fungi, reducing the presence of this inflammatory trigger could potentially improve the course of the disease[122]. Ideally, treatment should eliminate the fungus without causing harm to the host. In 1996, 22 fungal cultures grown from 15 AFS patients were studied by Bent and Kuhn for *in vitro* susceptibility to five common antifungal drugs. Ketoconazole and amphotericin B were shown to be most effective, independent of fungal organism tested[295]. Despite the potential for clinical effectiveness, the use of systemic antifungals is limited by adverse systemic reactions. Topical treatment may have the advantage that high concentrations may be achieved locally without causing major systemic side effects. Although the injectable formulation of amphotericin B carries US Food and Drug Administration-approved labeling solely for intravenous administration, several alternative routes of administration that use the injectable formulation have been reported, including the administration of amphotericin B into the pleural cavity[224] and bladder[228]. Recently, amphotericin B nasal lavages have been advocated in the treatment of CRS. Although safe to use and despite initial evidence of benefit in two uncontrolled trials[123;124], one subsequent uncontrolled prospective trial[125] and four subsequent double-blind placebo-controlled studies (one of which is presented in chapters 4.1 and 4.2) investigating the effect of topical amphotericin B nasal lavages[126;128;129] and nasal sprays[127] either failed to show benefit[127-129] or showed, at best, only modest radiological benefit without symptomatic improvement[126].

Although several uncontrolled reports have suggested that oral antifungal agents are effective in the treatment of CRS[130], a recent double-blind placebo-controlled study by Kennedy et al treated 53 CRS patients with high-dose oral terbinafine for a period of 6 weeks and demonstrated no improvement in subjective and objective

outcome measures[131]. Thus, the use of both topical and oral antifungals in the treatment of patients with CRS is not substantiated by the majority of publications.

Antifungal immunotherapy

If CRS stems from a hypersensitivity to retained fungal elements (a conclusion that should be questioned as mentioned previously) the removal of fungal elements may minimize ongoing stimulation. However, when the underlying hypersensitivity remains untreated, the disease is expected to recur. From 1994 onwards, Mabry et al prospectively treated 23 patients with AFS with antifungal immunotherapy following thorough exenteration of the involved sinuses. A decreased need for both systemic and topical corticosteroids, a marked decrease in polyp recurrence and a lessening of long-term nasal and sinus crusting was observed over a treatment period of 1-3 years in 11 patients[296]. Cessation of immunotherapy after 3 years did not result in recurrence of symptoms in the 7 to17 months follow-up[297]. When interpreting these data, one should note that no placebo group was included, that controls included those patients that dropped out from the immunotherapy group, that several patients were lost to follow-up, that most patients were treated with immunotherapy to both fungal and non-fungal antigens and that all patients were treated with nasal irrigations and topical steroids for a variable period of time post-operatively. But, even though many confounders are present, the results of this study are intriguing. Even if one assumes that fungal allergy is not causative of CRS, one may conclude that antifungal immunotherapy is effective in reducing signs and symptoms in those CRS patients with concurrent fungal allergy. Future placebo-controlled studies are necessary to reveal the true role of antifungal immunotherapy in the treatment of CRS patients.

CONCLUSIONS AND FUTURE DIRECTIONS

The role of fungi in CRS remains unclear. Initial enthusiasm from studies showing that fungi can be detected in the nose and paranasal sinuses of nearly all CRS patients, were tempered when it was demonstrated that they are present in healthy controls as well. Currently, there are more questions than answers concerning the role of fungi in the pathophysiology of CRS. Further studies are necessary to clarify the role of fungi in CRS, assess which fungal organisms, if any, are pathogenic,

and what exactly characterizes the immunologic response to fungi that may potentially result in the development of disease. Presently, in the absence of convincing microbiological and immunological data or evidence of effectiveness of both topical and oral antifungals, the case against the fungus remains unproven.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

INTRODUCTION

CRS constitutes a heterogeneous group of diseases with potentially different underlying etiologies. So far, CRS is clinically divided in CRS with nasal polyposis and CRS without nasal polyposis based on the presence of nasal polyps upon nasal endoscopy. Despite many studies, the exact pathophysiology of CRS is largely unknown. Although many treatment strategies exist, CRS has a strong tendency to recur and many patients do not respond to current treatment strategies at all. Based on this knowledge, this thesis is focused around three themes:

1) CRS is a heterogeneous group of diseases characterized by an inflammation of the nose and paranasal sinuses. A better understanding as to the exact nature of the observed inflammation is necessary to design optimal treatment regimens for all CRS patients. Although most authors agree that eosinophils and T_H2 cytokines are characteristic of most CRS tissue specimens (especially those tissue specimens obtained from CRS patients with nasal polyposis), their presence does not exclude the involvement of other leukocytes and mediators.

2) Topical glucocorticoids are the treatment modality of first choice in patients suffering from CRS with and without nasal polyposis. Efficacy of topical glucocorticoids is, however, variable and depends on long-term treatment regimens. To date, the potential mechanisms predicting topical glucocorticoid responsiveness remain largely unknown.

3) Recently, a fungal etiology has been proposed to play a prominent role in the pathogenesis of CRS with or without nasal polyposis, an idea based on the premise that an altered local immune response to sinonasal fungi results in the generation of disease in susceptible individuals. If true, fungal eradication, by using intranasal antifungals, should improve the course of the disease.

INVOLVEMENT OF LEUKOCYTES AND MEDIATORS OTHER THAN EOSINOPHILS AND T_H2 CYTOKINES IN THE PATHOGENESIS OF CRS

Are eosinophils present in all CRS tissue specimens?

Classically, CRS without nasal polyposis tissue specimens are said to be neutrophilic[65] and CRS with nasal polyposis tissue specimens are said to be eosinophilic[65;75;76]. Recent data, however, suggest that increased numbers of eosinophils are characteristic of both patients suffering from CRS with nasal polyposis and patients suffering from CRS without nasal polyposis[122]. Levels, however, may vary between individuals and within biopsies[271].

If we assume that eosinophils are characteristic of CRS with nasal polyposis tissue specimens, do eosinophils play an exclusive role in its pathogenesis?

Confirming results by other groups[156;157;187], in chapter 3.1 we show that, compared to control inferior turbinate, the number of eosinophils is increased significantly in CRS patients with nasal polyposis with or without concurrent atopy, asthma, and/or ASA intolerance. As patients with concurrent atopy, asthma and/or ASA intolerance were included, the presence of these co-morbid disease, themselves linked to an increase in (tissue) eosinophilia (chapter 2.2), may be (partly) explanatory for the observed link between tissue eosinophilia and CRS. In chapter 2.3 we demonstrate that in tissue specimens of CRS patients with nasal polyposis without atopy, asthma, and ASA intolerance, the number of tissue eosinophils is increased only slightly with no difference between nasal polyps and healthy control inferior turbinates. Based on these results, one should question the exclusive role for eosinophils in the pathogenesis of CRS with nasal polyposis.

Eosinophils are frequently present in CRS with nasal polyposis tissue specimens, especially in those patients with concurrent atopy, asthma and/or ASA intolerance. Is selective recruitment of eosinophils involved in these cases?

L-selectins on eosinophils and their glycosylated ligands on endothelial cells have been shown to be involved in the recruitment of eosinophils to diseased sinus mucosa in CRS patients without nasal polyposis[181]. In CRS patients with nasal polyposis, however, no correlation was observed (chapter 2.2). Although both eosinophils and glycosylated L-selectin ligands are increased in tissue specimens

of patients suffering from CRS with nasal polyposis, especially in those patients without asthma and/or ASA intolerance, the percentage of glycosylated L-selectin ligands is decreased in those nasal polyp patients with associated asthma and ASA intolerance whereas the number of eosinophils clearly increases. Thus, glycosylated L-selectin ligands on endothelial cells are unlikely to be a major determinant of tissue eosinophilia in patients suffering from CRS with nasal polyposis. Altogether, these data confirm our notion that the mild eosinophilia related to CRS with nasal polyposis without asthma and ASA intolerance is different from the marked eosinophilia related to CRS with nasal polyposis with asthma and/or ASA intolerance.

Should the eosinophil be abandoned as causative effector cell?

Lower levels of eosinophils do not exclude a causative role for eosinophils in the pathogenesis of CRS with nasal polyposis. As was shown by Koller et al, although eosinophils are less abundant in CF, the few eosinophils that are present are able to release high amounts of ECP[191]. In chapter 3.2 we show that activated (EG2+) eosinophils are present in tissue specimens of patients suffering from CRS with or without nasal polyposis and that successful post-operative treatment with topical glucocorticoids is related to their presence. Thus, activated eosinophils are likely to be involved in the pathogenesis of CRS in some individuals. In individuals lacking activated eosinophils, however, other mechanisms may be involved.

Although eosinophils should not be abandoned as causative effector cells, other effector cells may be involved in CRS with nasal polyposis pathogenesis

Besides eosinophils, nasal polyps are characterized by elevated levels of neutrophils (chapter 3.1). Recent data from Asia suggest that non-Caucasian nasal polyps, nasal polyps macroscopically remarkably similar to Caucasian nasal polyps, are characterized by abundant tissue neutrophilis and not abundant tissue eosinophils[66;77;78]. Similar results are observed in nasal polyps obtained from patients suffering from CF (chapters 2.2 and 2.3)[66;77]. Thus, neutrophils could also be involved in CRS with nasal polyposis pathogenesis.

Is there any evidence that neutrophils are involved in airway inflammation in general and in CRS with nasal polyposis in specific?

In asthma, in about 50% of asthmatic patients, evidence has accumulated that neutrophil mediated inflammatory mechanisms are involved in producing enhanced bronchial reactivity and reversible airflow obstruction [144]. Given the increased prevalence of nasal polyposis in asthmatic individuals, our observation that neutrophils are as abundant as eosinophils in nasal polyps of patients with or without concurrent atopy, asthma and/or ASA intolerance (chapter 3.1), the predominance of neutrophils in non-Caucasian nasal polyps and CF nasal polyps, and the role of neutrophils in asthma, neutrophils should be considered as potential players in the pathogenesis of CRS with nasal polyposis.

Are two independent pathways involved in CRS with nasal polyposis pathogenesis?

Following the data on the distribution of specific cell types in CRS with and without nasal polyposis in relationship to potential co-morbidities and treatment outcome, we are forced to consider that cell types other than eosinophils are important in the pathogenesis of CRS. This other cell type could well be the neutrophil. Given the more neutrophilic polyps seen in Asia, a provocative idea emerges that in the Caucasian CRS population, two (independent) pathophysiological mechanisms exist that centre on eosinophils and neutrophils respectively. Possibly, we can substantiate this idea by having a critical look at cytokines and chemokines involved in eosinophil- and neutrophil-mediated disease mechanisms.

The level of eosinophil-related mediators and most T_H2 cytokines is reduced in CRS with nasal polyposis tissue specimens

In chapter 2.1 we demonstrate that protein levels of the eosinophil-related mediators GM-CSF, eotaxin, and RANTES are slightly decreased in CRS patients with nasal polyposis. Of the T_H2 cytokines, IL-5 protein levels are increased but not significantly (chapter 2.1). Similar results are observed using immunohistochemistry in CRS patients with nasal polyposis but without any co-morbid disease (chapter 2.3). In CRS with nasal polyposis tissue specimens, the levels of the T_H2 cytokines IL-4 and IL-13 are decreased (chapter 2.1). A 4-fold decrease in the level of IL-4 is observed in all nasal polyps. This decrease was shown to be highly significant. Since high levels of IL-4 and IL-13 are linked to

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allergic inflammation, based on our results, one may conclude that atopy is not involved in the pathogenesis of nasal polyposis, confirming results obtained from population studies[18].

What is the role of increased IL-5 levels in CRS with nasal polyposis?

Confirming previous results[66;139-141], we show in chapter 3.1 that nasal polyps (with or without atopy, asthma, and/or ASA intolerance) are characterized by a significant increase in the number of IL-5+ cells. IL-5 is key cytokine for eosinophil maturation, differentiation, and survival. Although increased in a mixed group of nasal polyp patients (with or without atopy, asthma and/or ASA intolerance), the number of IL-5+ cells in nasal polyps of patients without atopy, asthma, and ASA intolerance is similar to the number of IL-5+ cells in control inferior turbinate specimens (chapter 2.3), suggesting that increased numbers of IL-5+ cells are linked to the presence of atopy, asthma, and/or ASA intolerance. Although the number of IL-5+ cells is significantly increased in a mixed group of nasal polyp patients using immunohistochemistry, using ELISA, we show that the actual increase in IL-5 protein is only marginal in both a mixed group of nasal polyp patients and patients without atopy, asthma and ASA intolerance (chapter 2.1). As protein levels in tissue as assessed with ELISA do not necessarily correlate with the number of positive cells as assessed with immunohistochemistry (i.e. the total number of cells producing IL-5 is increased despite decreased IL-5 expression levels per cell, resulting in lower IL-5 protein levels), additional studies are necessary to further delineate the true role of atopy, asthma, and/or ASA intolerance in IL-5 upregulation and, indirectly, tissue eosinophilia.

Do increased IL-5 levels and tissue eosinophilia always coincide?

In chapter 2.3, we show that, in CF nasal polyps, the number of IL-5+ cells is increased significantly without a significant increase in tissue eosinophilia. An increase in the number of neutrophils is, however, characteristic of CF nasal polyps. Today, there is some evidence that suggests that, besides being involved in eosinophil recruitment, maturation and activation, IL-5 is also involved in neutrophil recruitment[192]. Although IL-5 is unable to directly act on neutrophils, anti-IL-5 treatment has been shown to block neutrophil accumulation *in vivo*[192]. Thus, together with our observation that not only eosinophils, but also neutrophils are important effector cells in nasal polyposis and our observation that increased

numbers of IL-5+ are present in CF nasal polyps without marked tissue eosinophilia, the idea that high levels of IL-5 are involved in tissue eosinophilia only should be challenged.

How about other neutrophil mediators?

In chapter 2.1 we show that IL-8 is tremendously increased in all nasal polyps. IL-8 is a potent neutrophil recruiting and activating factor, produced by macrophages, epithelial cells and endothelial cells. Unpublished data by our group demonstrate that, in nasal polyps, it is the epithelium that produces the enormous amounts of IL-8. Besides being involved in neutrophil recruitment, the presence of elevated levels of IL-8 has been related to collagen breakdown and tissue remodeling via activation of matrix metalloproteinase 8 (MMP-8) in nasal polyp tissue specimens[87]. In patients with blood eosinophilia, but not in patient with normal levels of blood eosinophils, IL-8 has been shown to be chemotactic for eosinophils[143] and treatment with anti-IL-8 antibodies has been shown to (partially) block eosinophil survival in cultured nasal polyp epithelial cells[298]. Thus, although high levels of IL-8 do not exclude a role for eosinophils in the pathogenesis of CRS with nasal polyposis, based on many results presented in this thesis, we conclude that neutrophils and neutrophil mediators (IL-8 and possibly IL-5) are likely to play an important role in the pathogenesis of (at least a subgroup of patients suffering from) CRS with nasal polyposis.

POTENTIAL MECHANISMS PREDICTING TOPICAL GLUCOCORTICOID RESPONSIVENESS IN CRS PATIENTS

How do topical glucocorticoids exert their effect?

Although we know that the biological action of glucocorticoids is mediated through activation of intracellular glucocorticoid receptors[100], to date, the exact mechanisms underlying the anti-inflammatory and immunoregulatory effect of glucocorticoids remain to be fully explained. Clinical efficacy of topical glucocorticoids in CRS patients depends in part on the ability of glucocorticoids to reduce airway eosinophil infiltration (either directly via a reduction in eosinophil viability and activation[102-104] or indirectly via a reduction in the secretion of cytokines[105-108]) and in part on the ability of glucocorticoids to interfere with prostanoid synthesis[109].

What mechanisms are involved in glucocorticoid resistance?

Although most CRS patients respond to glucocorticoid therapy, a small subset of patients demonstrates persistent tissue inflammation despite treatment with high doses of glucocorticoids. The exact cause of this so called glucocorticoid resistance is unknown. Overexpression of glucocorticoid receptor β and/or a downregulation of glucocorticoid receptor α are two of the mechanisms that have been proposed[111;112]. Other mechanisms include immunomodulation, cigarette smoking, genetic predisposition, viral infection, allergen exposure, the presence of microbial superantigens, and tissue neutrophilia[100].

Do topical glucocorticoids reduce the number of tissue eosinophils?

In the past, treatment with oral glucocorticoids has been shown to result in a significant reduction in the number of tissue eosinophils in patients suffering from CRS with nasal polyposis[141]. In our hands, treatment with topical glucocorticoids did not result in a significant reduction in the number of tissue eosinophils (chapter 3.1), although a slight reduction is observed. Explanatory for these contradictory results may be the inclusion of patients refractory to topical glucocorticoids.

Do topical glucocorticoid reduce the number of IL-5+ cells?

In chapter 3.1 we show that treatment with topical glucocorticoids results in a slight decrease in the number of tissue eosinophils in a mixed group of CRS patients with nasal polyposis (with or without asthma and/or ASA intolerance). The number of IL-5+ cells, however, is increased significantly. As the percentage of cells producing IL-5 has been shown to be increased in fluticasone insensitive nasal polyp patients upon fluticasone treatment[111], the limited reduction of tissue eosinophils and the increase in IL-5 may be explained by the inclusion of glucocorticoid resistant patients.

Does the level of tissue eosinophils predict glucocorticoid resistance or responsiveness in CRS patients?

In chapter 3.2 we show that both treatment with fluticasone propionate 100 µg q.i.d. and high levels of inferior turbinate EG2+ (activated) eosinophils are linked to a favorable outcome one year after FESS. As increased numbers of eosinophils are characteristic of both patients suffering from CRS with nasal polyposis and patients suffering from CRS without nasal polyposis[122], the role of EG2+

eosinophils as a predictor of response to post-operative glucocorticoid treatment is promising. Additional research in CRS patients with and without nasal polyposis studying the role of EG2+ eosinophils and response to FESS in combination with post-operative fluticasone propionate treatment is, however, warranted. As tissue neutrophilia is associated with an increased prevalence of glucocorticoid resistance[100], those patients not responding to topical glucocorticoids and lacking activated tissue eosinophils may exhibit a neutrophil predominance. Although speculative, the idea is interesting in the light of many observations presented in this thesis.

Do topical glucocorticoids interfere with prostanoid synthesis?

Part of the clinical efficacy of topical glucocorticoids is based on the ability of glucocorticoids to interfere with prostanoid synthesis[109]. Cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2) are key enzymes in the generation of prostanoids from arachidonic acid. COX-1 is considered to be expressed constitutively and COX-2 is considered to be induced under pro-inflammatory conditions. Both isoforms are expressed in nasal polyps. Most studies published to date focus on the role of COX-2 in nasal polyp pathogenesis and suggest that glucocorticoids downregulate COX-2 mRNA and protein levels *in vitro*[194;197;198]. In chapter 3.1 we show that treatment with topical glucocorticoids does not reduce, but instead increases (although not significantly), the number of COX-2+ cells in nasal polyp epithelium. In contrast, treatment with topical glucocorticoids significantly decreases the number of COX-1+ cells in nasal polyp epithelium. As recent data suggest that COX-2 may have anti-inflammatory properties[195], treatment with topical glucocorticoids may skew nasal polyp prostaglandin production in an anti-inflammatory direction. Together with the observed downregulation in the number of COX-1+ cells, treatment with topical glucocorticoids may result in reduced levels of pro-inflammatory prostanoids and, as a consequence, disease resolution.

EFFECT OF INTRANASAL ANTIFUNGALS IN THE TREATMENT OF CRS WITH AND WITHOUT NASAL POLYPOSIS

Are topical treatment modalities other than topical glucocorticoids available to treat patients suffering from CRS with or without nasal polyposis?

TABLE 1. Studies on topical amphotericin B in CRS patients

Author	Active drug (n)	Placebo (n)	Dose	Duration	Method	Study design	Outcome
Ponikau[123]	51	0	100 µg/mL (20 mL) twice daily in each nostril	3-17 months	Nasal lavage	Non-placebo controlled single center study	Positive
Ricchetti[124]	74	0	1:1000 (20 mL) twice daily in each nostril	4 weeks	Nasal lavage	Non-placebo controlled single center study	Positive
Weschta[127]	28	32	3 mg/mL (200µL) four times daily in each nostril	8 weeks	Nasal spray	Randomized placebo-controlled double-blind single center study	Negative
Ponikau[126]	10	14	250 µg/mL (20 mL) twice daily in each nostril	6 months	Nasal lavage	Randomized placebo-controlled double-blind single center study	Positive (CT) & negative (symptoms)
Helbling[125]	21	0	1% (0.1 mL) three times daily in each nostril	3 months	Nasal spray	Non-placebo controlled single center study	Negative
Ebbens[128]	59	57	100 µg/mL (20 mL) twice daily in each nostril	13 weeks	Nasal lavage	Randomized placebo-controlled double-blind multicenter study	Negative
Liang[129]	32	32	4 µg/mL (250 mL) once daily in each nostril	4 weeks	Nasal lavage	Randomized placebo-controlled study	Negative

Recently, a fungal etiology has been proposed to play a prominent role in the pathogenesis of CRS with or without nasal polyposis (chapter 4.3), an idea based on the premise that an altered local immune response to sinonasal fungi results in the generation of disease in susceptible individuals[122]. Based on this premise, nasal lavages with topical amphotericin B have been advocated in the treatment of CRS with or without nasal polyposis.

Can we think of a mechanism that explains a positive effect of topical antifungals in the treatment of patients suffering from CRS with or without nasal polyposis?

If we assume that topical antifungals (a.o. amphotericin B) are effective in the treatment of CRS patients by reducing a local inflammatory response, various mechanisms may be involved. First and most likely, amphotericin B may reduce fungal load and, as a consequence, reduce the inflammatory response in the nose and paranasal sinuses. Second, amphotericin B may have a direct (cytotoxic) effect on nasal polyp epithelial cells. Although amphotericin B is a sterol-binding agent with high affinity for ergosterol (the dominant fungal sterol) and low affinity for cholesterol (the mammalian sterol), recent evidence suggests that topical amphotericin B is able to modify cell membrane structures of nasal polyp epithelial cells resulting in increased membrane permeability and disruption of cells[236]. These cytotoxic effects are, however, observed at dosages of 50 µM of amphotericin B, dosages much higher than those used in any clinical study. Third, amphotericin B may exhibit anti-inflammatory properties. Although interesting, the three studies (including our study that is presented in chapter 4.2) published to date centered around this theme show no difference between placebo and amphotericin B treated patients in the concentrations of various pro-inflammatory cytokines, chemokines and growth factors[238;239;299].

If we assume that topical antifungals reduce fungal load and, as a consequence, the inflammatory response in the nose and paranasal sinuses, treatment should lead to disease resolution. Is this true?

Despite initial evidence of benefit in two uncontrolled trials[123;124], one subsequent uncontrolled prospective trial[125] and four subsequent double-blind placebo-controlled studies (one of which is presented in chapter 4.1) investigating the effect of topical amphotericin B nasal lavages[126;128;129] and nasal sprays[127] in CRS patients with and without nasal polyposis, either failed to show benefit[127-129] or showed, at best, only modest non-relevant radiological benefit without symptomatic improvement (table 1)[126].

Do the observed results exclude an effect of topical antifungals in the treatment of CRS with or without nasal polyposis?

Although all randomized controlled trials do not support the use of topical antifungals in the treatment of CRS with or without nasal polyposis, dosage, treatment time, and route of administration may have influenced treatment outcomes. As recent *in vitro* data suggest that amphotericin B nasal lavages are

ineffective in killing fungi at concentrations of 100 µg/mL when used for 6 consecutive weeks[300] (dosages used by both Ponikau et al[123] and our group[128]), the lack of effect may be explained by inadequate dosing. Although inadequate dosing of topical amphotericin B may explain the observed results, treatment with topical amphotericin B in a concentration of 250 µg/mL, a dosage that was demonstrated to be effective in killing fungi within 6 weeks, was recently shown to be ineffective as well[126]. Thus, together with the observation by Weschta et al that fungal eradication does not alleviate CRS signs and symptoms[127], the role of fungal eradication in disease resolution should be questioned.

CONCLUSIONS

Based on results presented in this thesis, the role of eosinophils as unique effector cell in the pathogenesis of CRS with nasal polyposis should be challenged. We postulated that two (independent) pathophysiological mechanisms exist that centre on eosinophils and neutrophils respectively. Those patients suffering from eosinophil-mediated disease are likely to respond to topical glucocorticoids, those not suffering from eosinophil-mediated disease are less likely to respond to topical glucocorticoids. Future studies are necessary to elucidate the role (activated) eosinophils in glucocorticoid responsiveness and to elucidate the role of neutrophils and the neutrophil chemoattractant IL-8 in the pathogenesis of CRS with nasal polyposis. As multiple randomized controlled trials have demonstrated that topical antifungals are ineffective in the treatment of patients suffering from CRS, treatment of CRS patients with topical antifungals should be abandoned.

APPENDICES

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SUMMARY

Chapter 1

In this chapter normal paranasal sinus anatomy and (patho)physiology, disease definition, disease classification and epidemiology of CRS are reviewed. In addition, the association between asthma, acetyl salicylic acid (ASA) intolerance, atopy, ciliary impairment, cystic fibrosis (CF) and CRS is discussed. One paragraph focuses on essential steps necessary to diagnose and stage CRS with and without nasal polyposis. Another paragraph discusses the role of inflammatory cells and mediators in CRS inflammation, focusing on differences between CRS with and CRS without nasal polyposis. In the final paragraph evidence-based treatment modalities are reviewed.

Chapter 2.1

Most data on Caucasian nasal polyps are based on tissue specimens obtained from patients suffering from atopy, asthma and/or ASA intolerance. In this chapter we studied the effect of atopy, asthma and ASA intolerance on the presence of various cytokines, chemokines and growth factors (i.e. IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin, RANTES, MCP-1, VEGF, EGF, FGF-basic, and HGF) in tissue specimens of patients suffering from CRS with nasal polyposis using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA). Compared to control nasal mucosa, the concentration of most mediators (including IL-4, IL-13 and GM-CSF) is lower in all nasal polyps. The concentrations of some mediators (including IL-5, IL-6, IL-7, IL-8 and IL-10), however, was shown to be increased in nasal polyps of patients with or without concurrent atopy, asthma and/or ASA intolerance. Only the increase in IL-8 was shown to be statistically significant. Atopy, asthma and/or ASA intolerance were shown not to influence outcome.

Chapter 2.2

L-selectins on leukocytes and their counterreceptors (glycosylated ligands) on endothelial cells have been shown to be involved in leukocyte recruitment in

chronic rhinosinusitis without nasal polyps. In this chapter we studied the expression level of glycosylated L-selectin ligands in nasal polyps obtained from patients with and without CF and compared their levels to the presence of various leukocyte subsets. All nasal polyps are characterized by a decrease in the number of CD34+ vessels. Although both eosinophils and the percentage glycosylated L-selectin ligands are increased in tissue specimens of patients suffering from non-CF nasal polyposis and CF-nasal polyposis, no correlation is observed between the percentage of glycosylated L-selectin ligands and the number of tissue eosinophils. As similar results are observed for neutrophils, macrophages, basophils and mast cells, it is highly unlikely that glycosylated L-selectin ligands are key players in guiding a single leukocyte subset to diseased sinus mucosa in CRS patients with nasal polyposis.

Chapter 2.3

Nasal polyps frequently occur in patients with CF. Although macroscopically remarkably similar to non-CF nasal polyps, controversy exists as to whether these polyps should be considered distinct histopathological entities. In this chapter the number of various leukocytes (eosinophils, neutrophils, basophils, mastcells, and macrophages), mediators (IL-4, IL-5, IL-6, and eotaxin) and the adhesion molecule VCAM-1 was studied in both non-CF and CF nasal polyps using immunohistochemistry. Increased numbers of macrophages and neutrophils and an increase in the number of IL-6 expressing cells are striking features of CF nasal polyps. Although eosinophils are more abundant in non-CF nasal polyps, differences between both groups are statistically not significant. In contrast to what was expected, results from this study suggest that an upregulation of IL-5 expressing cells is characteristic of CF nasal polyps but not of non-CF nasal polyps of patients without atopy, asthma and/or ASA intolerance.

Chapter 3.1

Influx of inflammatory cells is one of the hallmarks of nasal polyposis. Since glucocorticoids are known to exhibit strong anti-inflammatory effects, glucocorticoids are frequently used in the treatment of CRS. Anti-inflammatory effects of glucocorticoids are, in part, attributed to their interference with prostanoid synthesis. Cyclo-oxygenases (COX) are key enzymes in the synthesis of both pro- (COX-1, COX-2) and anti-inflammatory prostanoids (COX-2) and their role in CRS

pathogenesis has long been studied. In this chapter, the role of topical glucocorticoids on COX-1, COX-2 and other inflammatory markers was studied in nasal polyps of patients with and without concurrent atopy, asthma and/or ASA intolerance. Inflammation in nasal polyps was shown to be characterized by an influx of eosinophils, increased numbers of IL-5+ cells and an increase in the number of IgE+ cells. In addition, an increase in the number of COX-1+ cells was observed. Topical glucocorticoids significantly downregulate the number of COX-1+ cells in nasal polyp epithelium. Upon topical glucocorticoid therapy, the number of COX-2+ cells is upregulated slightly and the number of IL-5+ cells is upregulated significantly.

Chapter 3.2

In the majority of CRS patients suffering from primary or recurrent CRS, topical glucocorticoids are highly effective. A subset of CRS patients, however, does not respond to (topical) glucocorticoids and requires surgical intervention. In this chapter we show, using a binary logistic regression model with potential relevant parameters, that post-operative treatment with fluticasone propionate aqueous nasal spray (FPANS) 100 µg q.i.d. (and not treatment with FPANS 200 µg q.i.d. or placebo) is significantly associated with response to treatment. In addition, a trend towards association between increased numbers of activated (EG2+) eosinophils at baseline and response to treatment was demonstrated. Together, our data suggest that those CRS patients with higher levels of activated eosinophils treated post-operatively with FPANS 100 µg q.i.d. are less likely to suffer from post-operative recurrent sinonasal disease.

Chapter 4.1

Recently, it has been suggested that an exaggerated immune response to fungi is crucial in the pathogenesis of CRS. If true, treatment with (topical) antifungals should benefit CRS patients. In this chapter we describe the results of a large double-blind placebo-controlled multicenter study, comparing the effectiveness of amphotericin B nasal lavages with placebo. Mean VAS scores, SF-36 and RSOM-31 data, PNIF values, nasal endoscopy scores and polyp scores were shown to be similar in both treatment groups at the time of randomization and no significant differences were observed after 13 weeks of treatment, suggesting that amphotericin B nasal lavages in the described dosing and time schedule are

ineffective in the treatment of patients suffering from CRS with or without nasal polyposis.

Chapter 4.2

In this chapter we studied the effect of 13 weeks of treatment with amphotericin B or placebo on the levels of pro-inflammatory cytokines, chemokines and growth factors (i.e. IL-1β, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxin, RANTES, MCP-1, MCP-2, MCP-3, VEGF, EGF, FGF-basic, HGF and Gro-α) in nasal lavage specimens of CRS patients that participated in our double-blind placebo-controlled multicenter study using a recently developed technology for concurrent testing of specimens for a variety of proteins (Multiplex ELISA). Topical amphotericin B was shown not to have a significant effect on the level of any of the tested pro-inflammatory cytokines, chemokines and growth factors. Treatment with placebo, however, increased the level of MIP-1α and MIP-1β, mediators involved in wound healing.

Chapter 4.3

Recently, it has been suggested that an exaggerated immune response to fungi is crucial in the pathogenesis of CRS. In this chapter the prevalence of fungi in CRS patients, the role of fungal hypersensitivity in the disease process and defense mechanisms against fungi are reviewed. This chapter concludes with a discussion on the role of antifungal drug therapy and antifungal immunotherapy in the treatment of patients suffering from CRS with or without nasal polyposis.

Chapter 5

In this chapter the results obtained in this thesis are discussed in general.

SAMENVATTING

Hoofdstuk 1

In dit hoofdstuk worden de normale anatomie van het neusbijholte complex en de (patho)fysiologie, definitie, classificatie en epidemiologie van chronische sinusitis (CRS) besproken. Daarnaast gaat dit hoofdstuk in op de associatie tussen astma, aspirine (acetylsalicylzuur) intolerantie, allergie, trilhaardysfunctie, cystic fibrosis (CF) en CRS. In het tweede deel van dit hoofdstuk worden een aantal stappen in het diagnostisch proces, nodig om de diagnose CRS (met of zonder neuspoliepen) te kunnen stellen, besproken. CRS is een ontsteking van de neus en neusbijholten, gekenmerkt door de aanwezigheid van verschillende cytokines, chemokines en groeifactoren. In het derde deel van dit hoofdstuk wordt het verschil in voorkomen van bepaalde cytokines, chemokines en groeifactoren tussen patiënten die lijden aan CRS zonder neuspoliepen en patiënten die lijden aan CRS met neuspoliepen besproken. In het laatste deel van dit hoofdstuk wordt ingegaan op de verschillende behandelmogelijkheden van CRS.

Hoofdstuk 2.1

De meeste studies op het gebied van Westerse neuspoliepen zijn gebaseerd op studies in weefsels van patiënten die naast neuspoliepen ook last hebben van allergie, astma en/of aspirine intolerantie. In dit hoofdstuk hebben we gekeken naar het effect van allergie, astma en/of aspirine intolerantie op het voorkomen van verschillende cytokines, chemokines en groeifactoren (o.a. IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxine, RANTES, MCP-1, VEGF, EGF, FGF-basic, en HGF) in neuspoliepen van patiënten die lijden aan CRS met neuspoliepen. Hiervoor hebben we gebruik gemaakt van een recent ontwikkelde techniek waarbij tegelijkertijd naar meerdere cytokines, chemokines en groeifactoren gekeken kan worden (Multiplex ELISA). In vergelijking tot gezond neusslijmvlies zijn de concentraties van de meeste cytokines, chemokines en groeifactoren (waaronder IL-4, IL-13 en GM-CSF) in alle neuspoliepen verlaagd. De concentraties van enkele cytokines (waaronder IL-5, IL-6, IL-7, IL-8, en IL-10) zijn echter verhoogd. Statistisch onderzoek laat zien dat de toename in IL-8 statistisch significant is. Er werden geen verschillen gezien tussen

neuspoliepen van patiënten met of zonder allergie, met of zonder astma en/of met of zonder aspirine intolerantie.

Hoofdstuk 2.2

Bepaalde eiwitten op leukocyten (L-selectins) en hun met suikers gedecoreerde receptoren op bloedvaten spelen een belangrijke rol bij het aantrekken van leukocyten naar chronisch ontstoken neus- en neusbijholte slijmvlies van patiënten die lijden aan CRS zonder neuspoliepen. In dit hoofdstuk hebben we gekeken naar het voorkomen van met suikers gedecoreerde receptoren voor L-selectins op bloedvaten van neuspoliepen van patiënten met en zonder CF en of het voorkomen van deze met suikers gedecoreerde receptoren voor L-selectins samenhangt met het voorkomen van bepaalde leukocyten in neuspoliepen. Het aantal CD34+ bloedvaten is verlaagd in neuspoliepen van patiënten met en zonder CF. Het aantal eosinofielen en het percentage met suikers gedecoreerde receptoren voor L-selectins is echter toegenomen in neuspoliepen van patiënten met en zonder CF. Er werd geen verband gezien tussen het aantal eosinofielen en het percentage met suikers gedecoreerde receptoren voor L-selectins. Eveneens werd geen verband gezien tussen het aantal neutrofielen, macrofagen, basofielen en mestcellen en het percentage met suikers gedecoreerde receptoren voor L-selectins op bloedvaten van neuspoliepen van patiënten met en zonder CF. Op grond van deze bevindingen lijkt het onwaarschijnlijk dat met suikers gedecoreerde receptoren voor L-selectins een cruciale rol spelen bij het aantrekken van één bepaald soort leukocyt naar ontstoken neus- en neusbijholte slijmvlies van patiënten met CRS met neuspoliepen.

Hoofdstuk 2.3

Neuspoliepen komen frequent voor bij patiënten met CF. Hoewel deze neuspoliepen met het blote oog erg lijken op neuspoliepen van patiënten zonder CF, is het onduidelijk of deze twee soorten neuspoliepen als een of als twee ziektebeelden gezien moeten worden. In dit hoofdstuk hebben we met behulp van immunohistochemie gekeken naar het voorkomen van verschillende soorten leukocyten (eosinofielen, neutrofielen, basofielen, mest cellen en macrofagen), verschillende soorten cytokines (IL-4, IL-5, IL-6), het chemokine eotaxine en de receptor VCAM-1 in neuspoliepen van patiënten met en zonder CF. CF neuspoliepen worden gekenmerkt door een toename van het aantal macrofagen

en neutrofielen. Ook het aantal IL-6+ cellen is duidelijk toegenomen. Hoewel eosinofielen iets vaker lijken voor te komen in neuspoliepen van patiënten zonder CF, zagen wij geen significant verschil in het aantal eosinofielen in neuspoliepen van patiënten met en zonder CF. Bovendien werd geen verband gezien tussen het aantal eosinofielen en het aantal IL-5+ cellen. Het aantal IL-5+ cellen is namelijk significant verhoogd in CF neuspoliepen, maar niet in neuspoliepen van patiënten zonder CF.

Hoofdstuk 3.1

Neuspoliepen worden gekenmerkt door de aanwezigheid van grote hoeveelheden ontstekingscellen. De aanwezigheid hiervan kan worden geremd door corticosteroïden. Daarom worden deze geneesmiddelen vaak voorgeschreven aan patiënten die lijden aan CRS. Corticosteroïden hebben een krachtig ontstekingsremmend effect, een effect dat voor een groot deel bepaald wordt door de remming van de productie van prostaglandinen. Cyclo-oxygenases (COX) zijn sleutel enzymen betrokken bij de aanmaak van ontstekingsbevorderende (COX-1, COX-2) en ontstekingsremmende (COX-2) prostaglandinen. In dit hoofdstuk hebben we gekeken naar het effect van lokale corticosteroïden op de aanwezigheid van COX-1, COX-2, verschillende leukocyten, het cytokine IL-5 en het antilichaam IgE in neuspoliepen van patiënten die lijden aan CRS met neuspoliepen (met of zonder allergie, astma en/of aspirine intolerantie). Het ontstekingsbeeld in deze neuspoliepen wordt gekenmerkt door de aanwezigheid van grote hoeveelheden eosinofielen, een toegenomen aantal IL-5+ cellen en een toegenomen aantal IgE+ cellen. Daarnaast is het aantal COX-1+ cellen toegenomen. Behandeling met lokale corticosteroïden zorgt ervoor dat het aantal COX-1+ cellen significant afneemt in het epitheel van neuspoliepen. Alhoewel het aantal eosinofielen licht afneemt onder invloed van lokale corticosteroïden, is de afname in het aantal eosinofielen niet significant. Het aantal COX-2+ cellen neemt licht toe onder invloed van lokale corticosteroïden. Behandeling met lokale corticosteroïden zorgt voor een significante stijging in het aantal IL-5+ cellen.

Hoofdstuk 3.2

Het merendeel van de patiënten met CRS (ook wanneer sprake is van recidief ziekte) reageert goed op behandeling met lokale corticosteroïden. Een klein deel van de patiënten met CRS reageert echter niet op behandeling met lokale

corticosteroïden. Chirurgie is dan vaak de enige andere oplossing. In dit hoofdstuk tonen we met behulp van een binair logistisch regressie model aan dat postoperatieve behandeling met fluticason 4 maal daags 100 µg (en niet behandeling met fluticason 4 maal daags 200 µg of placebo) ervoor zorgt dat patiënten een lagere kans op recidief ziekte hebben. Daarnaast lijkt het erop dat patiënten die een groter aantal geactiveerde (EG2+) eosinofielen in hun neusslijmvlies hebben, beter reageren op behandeling (p = 0.091). Het lijkt er dus op dat CRS patiënten met een toegenomen aantal geactiveerde (EG2+) eosinofielen die postoperatief behandeld worden met fluticason 4 maal daags 100 µg een betere prognose hebben.

Hoofdstuk 4.1

Onlangs is er gesuggereerd dat een overmatige afweerreactie tegen schimmels in de neus en neusbijholten cruciaal is voor het ontstaan van CRS. Als dit inderdaad het geval is, dan zou behandeling met (lokale) antimycotica effectief moeten zijn. In dit hoofdstuk beschrijven we de resultaten van een grote dubbelblinde placebogecontroleerde multicenter studie waarin het effect van neusspoelingen met amfotericine B (een antimycoticum) en placebo met elkaar vergeleken wordt. Er werden geen verschillen gezien in de gemiddelde VAS-score, SF-36 score, RSOM-31 score, PNIF score, neusendoscopie score en poliep score op het moment van randomisatie en 13 weken na behandeling tussen de met amfotericine B behandelde groep en de met placebo behandelde groep. Op grond van deze resultaten concluderen wij dat amfotericine B neusspoelingen niet effectief zijn en om die reden niet toegepast moeten worden als behandeling voor patiënten met CRS met en zonder neuspoliepen.

Hoofdstuk 4.2

Onlangs is er gesuggereerd dat een overmatige afweerreactie tegen schimmels in de neus en neusbijholten cruciaal is voor het ontstaan van CRS. Als dit inderdaad het geval is, dan zou behandeling met (lokale) antimycotica effectief moeten zijn. In dit hoofdstuk hebben we het effect van amfotericine B neusspoelingen vergeleken met het effect van placebo neusspoelingen op het voorkomen van bepaalde cytokines, chemokines en groeifactoren (o.a. IL-1β, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70 subunits), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, G-CSF, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, eotaxine, RANTES,

MCP-1, MCP-2, MCP-3, VEGF, EGF, FGF-basic, HGF and Gro-α) in neuslavaten van CRS patiënten die hebben deelgenomen aan de hierboven genoemde dubbelblinde placebogecontroleerde multicenter studie. Hiervoor hebben we gebruik gemaakt van een recent ontwikkelde techniek waarbij tegelijkertijd naar meerdere cytokines, chemokines en groeifactoren gekeken kan worden (Multiplex ELISA). Het niveau van alle geteste cytokines, chemokines en groeifactoren was voor en na behandeling met amfotericine B neusspoelingen gelijk. Behandeling met placebo resulteerde in een significante toename van MIP-1α en MIP-1β, twee chemokines die betrokken zijn bij de wondgenezing.

Hoofdstuk 4.3

Onlangs is er gesuggereerd dat een overmatige afweerreactie tegen schimmels in de neus en neusbijholten cruciaal is voor het ontstaan van CRS. In dit hoofdstuk worden het voorkomen van schimmels in de neus en neusbijholten, de rol van een overgevoeligheid voor schimmels bij het onstaan van CRS en een aantal mogelijke afweermechanismen tegen schimmels besproken. Het hoofdstuk sluit af met een paragraaf over de rol van antimycotica en anti-schimmel immunotherapie in de behandeling van patiënten die lijden aan CRS met of zonder neuspoliepen.

Hoofdstuk 5

In dit hoofdstuk worden de resultaten die in dit proefschrift zijn beschreven samengevat en in een bredere context geplaatst.

ABBREVIATIONS

ACP	antrochoanal polyp
AFS	allergic fungal sinusitis
AP	alkaline phosphatase
APES	aminophosphate ethylsilane
ASA	acetyl salicylic acid
CD	cluster of differentiation
CF	cystic fibrosis
COX	cyclooxygenase
CRS	chronic rhinosinusitis
CT	computed tomography
ECP	eosinophilic cationic protein
EDN	eosinophil derived neurotoxin
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ENT	ear, nose, and throat
ESS	endoscopic sinus surgery
FESS	functional endoscopic sinus surgery
FGFbasic	basic fibroblast growth factor
FPANS	fluticasone propionate aqueous nasal spray
GC	glucocorticoids
GM-CSF	granulocyte macrophage colony stimulating factor
G-CSF	granulocyte colony stimulating factor
GMS	Grocott methanamine silver stain
GR	glucocorticoid receptor
Gro-α	growth related oncogene α
hCAP18	human cationic antimicrobial peptide 18 kDa
HGF	hepatocyte growth factor
ICAM	intercellular adhesion molecule
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
IP-10	inteferon inducible protein of 10 kDa

LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MBP	major basic protein
MCP	monocyte chemoattractant protein
MIG	monokine induced by IFN-γ
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NM	(control) nasal mucosa
NP	nasal polyp
NSAID	non-steroidal anti-inflammatory drug
PANS	placebo aqueous nasal spray
PAR	protease activated receptor
PAS	periodic acid Schiff stain
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNIF	peak nasal inspiratory flow
RANTES	regulated upon activation normal T cell expressed and secreted
RSDI	rhinosinusitis disability index
RSOM-31	rhinosinusitis outcome measure 31
RT	room temperature
SF-36	medical outcomes study short form 36
SNOT-20	sinonasal outcome test 20
SP	surfactant protein
SPT	skin prick test
TIMP	tissue inhibitor of metalloproteinase
TSA	tyramide signal amplification
TGF	transforming growth factor
TNF	tumor necrosis factor
TRIS	tris(hydroxymethyl)aminomethane
VAS	visual analogue scale
VCAM	vascular adhesion molecule
VEGF	vascular endothelial growth factor

CONTRIBUTING AUTHORS

G.F.J.P.M Adriaensen MD MSc
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

I. Alobid MD, PhD
Department of Otorhinolaryngology, Hospital Clinic & Institut d'Investigacions Biomediques August Pi y Sunyer, Barcelona, Spain

Prof. C. Bachert MD, PhD
Department of Otorhinolaryngology, University Hospital Ghent, Ghent, Belgium

L. Badia MB BS, FRCS
Department of Rhinology, Royal National Throat Nose and Ear Hospital, London, United Kingdom

A. Cardesin MD
Department of Otorhinolaryngology, Hospital Clinic & Institut d'Investigacions Biomediques August Pi y Sunyer, Barcelona, Spain

E.J.J. de Groot BSc
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

M.G.W. Dijkgraaf PhD
Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center Amsterdam

Prof. W.J. Fokkens MD, PhD
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

C. Georgalas MD, PhD
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

Prof. P.W. Hellings MD, PhD
Department of Otorhinolaryngology, University Hospital St. Rafael, Leuven, Belgium

Prof. M. Jorissen MD, PhD
Department of Otorhinolaryngology, University Hospital St. Rafael, Leuven, Belgium

S. Luiten BSc
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

Prof. V.J. Lund MD, FRCS, FRCSEd
Department of Rhinology, Royal National Throat Nose and Ear Hospital, London, United Kingdom

M. Maldonado MD, Phd
Department of Otorhinolaryngology, Hospital Clinic & Institut d'Investigacions Biomediques August Pi y Sunyer, Barcelona, Spain

Prof. J. Mullol MD, PhD
Department of Otorhinolaryngology, Hospital Clinic & Institut d'Investigacions Biomediques August Pi y Sunyer, Barcelona, Spain

Prof. C. Picado MD PhD
Department of Otorhinolaryngology, Hospital Clinic & Institut d'Investigacions Biomediques August Pi y Sunyer, Barcelona, Spain

J. Renkonen DDS PhD
Transplantation laboratory & Infection Biology Research Program, Haartman Institute, University of Helsinki, Helsinki
HUSLAB, Helsinki University Central Hospital, Helsinki, Finland

Prof. R. Renkonen MD PhD
Transplantation laboratory & Infection Biology Research Program, Haartman Institute, University of Helsinki, Helsinki
HUSLAB, Helsinki University Central Hospital, Helsinki, Finland

A.B. Rinia MD MSc
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

G.K. Scadding MA, MD, FRCP
Department of Rhinology, Royal National Throat Nose and Ear Hospital, London, United Kingdom

S. Toppila-Salmi MD PhD
Department of Eye, Ear and Oral Diseases, Tampere University Hospital and University of Tampere, Tampere, Finland

C.M. van Drunen PhD
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

D. van Egmond BSc
Department of Otorhinolaryngology, Academic Medical Center Amsterdam, Amsterdam, the Netherlands

T. P.J. van Zele MD, PhD
Department of Otorhinolaryngology, University Hospital Ghent, Ghent, Belgium

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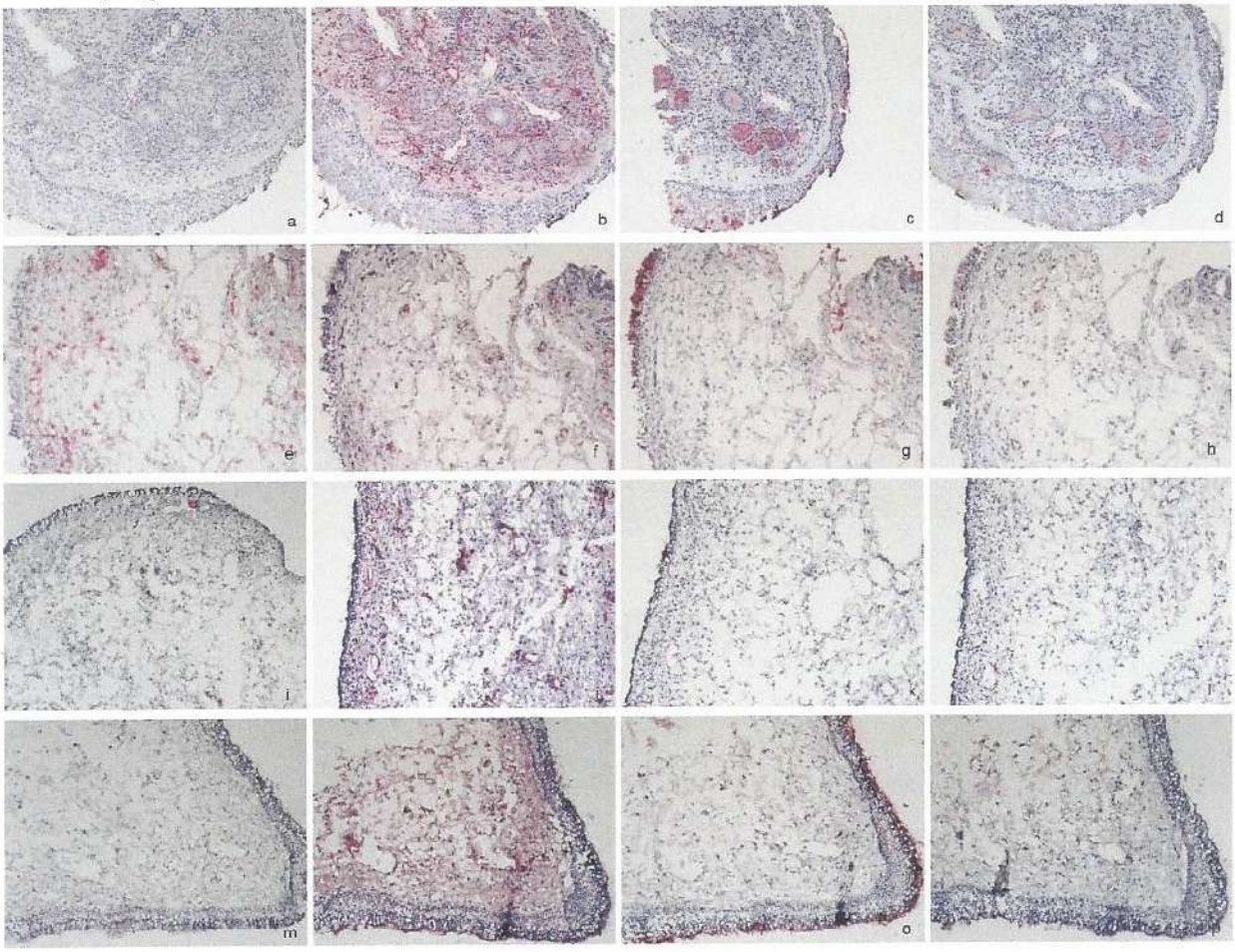
ABOUT THE AUTHOR

Fenna Afien Ebbens was born in Leidschendam, the Netherlands on May 24th 1977. She received her primary education at the Openbare Basischool 'De Boekhorst' in Voorhout and her secondary education (gymnasium) at the Rijnlands Lyceum Oegstgeest from which she graduated in 1995. From 1995 until 1996 she studied Biology and Art History at the University of Oregon (Eugene, OR, USA). In 1996, she started her medical training at the Erasmus University Rotterdam. In 2002 she received her medical degree (cum laude). Following this degree, she continued her education at the University of Amsterdam starting the PhD project on chronic rhinosinusitis which is described in this thesis (promotor: Prof. dr. W.J. Fokkens). In 2005 she started her specialist training in Otorhinolaryngology at the Academic Medical Center. In February 2006, she won the 1st Prize for Research of of the European Academy of Allergy and Clinical Immunology, Ear Nose and Throat section, presenting work described in this thesis. In June 2008, she won the Prize for Clinical Research 2008 of the European Rhinologic Society, presenting work described in this thesis. Currently, she is continuing her specialist training in Otorhinolaryngology (supervisor: Prof. dr. W.J. Fokkens). She is married to Victor Groothengel.

APPENDIX 1 : CHAPTER 2.2

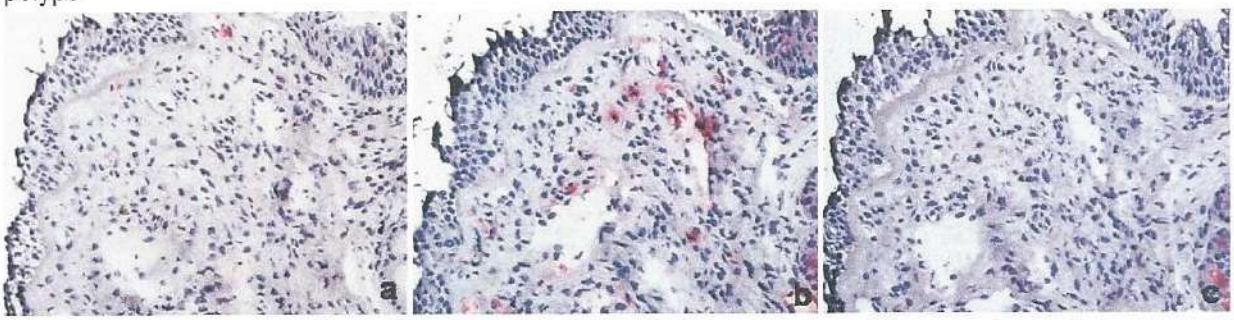
FIGURE 4. Presence of eosinophils, CD34+ vessels, mAb HECA-452+ vessels and mAb MECA-79+ vessels (magnification 10x)



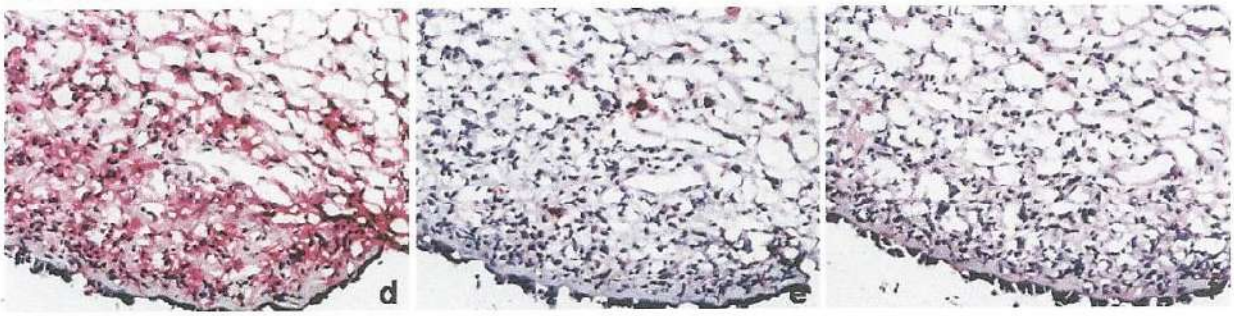
Control inferior turbinate (a-d), simple nasal polyp (e-h), CF nasal polyp (i-l) and antrochoanal nasal polyp (m-p) stained for eosinophils (MBP+ cells: a, e, i, and m), endothelial cells (CD34+ cells: b, f, j, and n), and functionally active L-selectin ligands (mAb HECA-452+ cells: c, g, k, and o; mAb MECA-452+ cells: d, h, l, and p). Note high numbers of eosinophils in simple nasal polyps, abundant CD34+ endothelial cells in control inferior turbinate and relative absence of CD34+ endothelial cells in simple nasal polyps, CF nasal polyps and antrochoanal nasal polyps. In addition, note the relative abundancy of mAb MECA-79+ CD34+ endothelial cells in simple nasal polyps.

APPENDIX 2 : CHAPTER 2.3

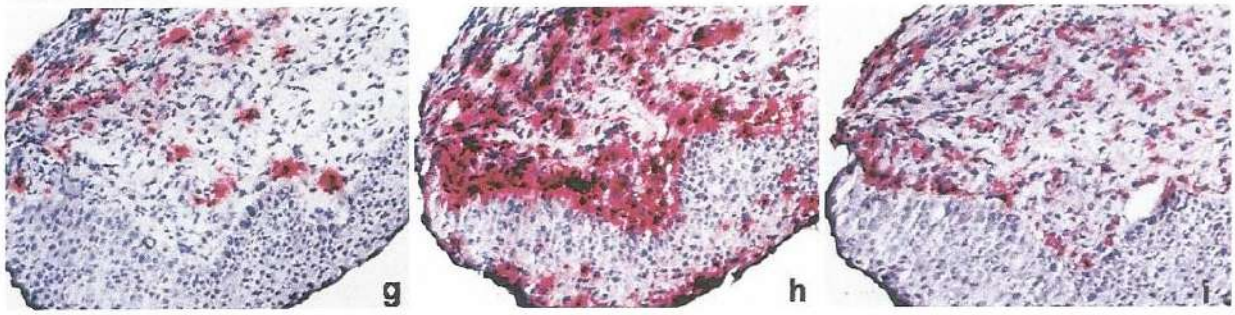
FIGURE 1. Immunohistochemistry of control inferior turbinate, non-CF nasal polyps and CF nasal polyps



Control inferior turbinate (magnification 20x). Note a) the presence of low numbers of eosinophils (MBP+ cells), b) low numbers of neutrophils (elastase+ cells) and c) the presence of low numbers of IL5+ cells.



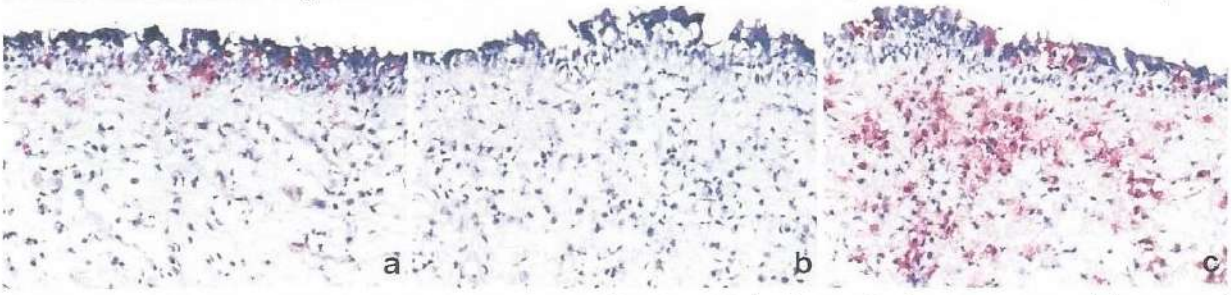
Non-CF nasal polyp (maginification 20x). Note d) the presence of increased numbers of eosinophils (MBP+ cells), e) relatively low numbers of neutrophils (elastase+ cells) and f) the presence of low numbers of IL-5+ cells.



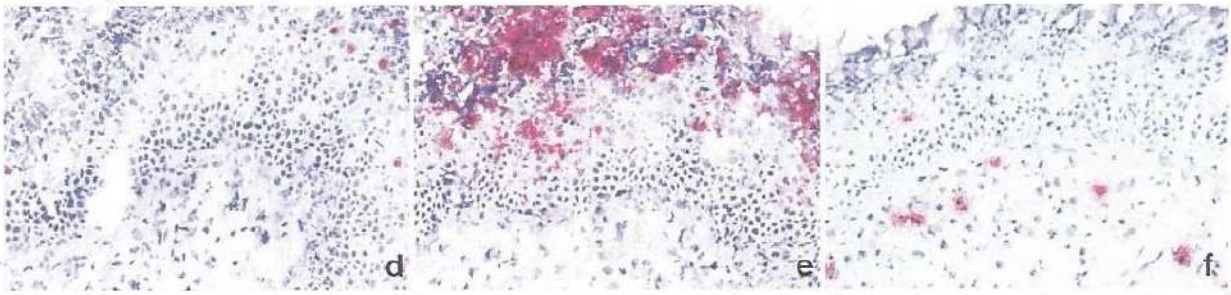
CF nasal polyp (magnification 20x). Note g) the presence of slightly increased numbers of eosinophils (MBP+ cells), h) high numbers of neutrophils (elastase+ cells) and i) the presence of high numbers of IL-5+ cells.

APPENDIX 3 : CHAPTER 3.1

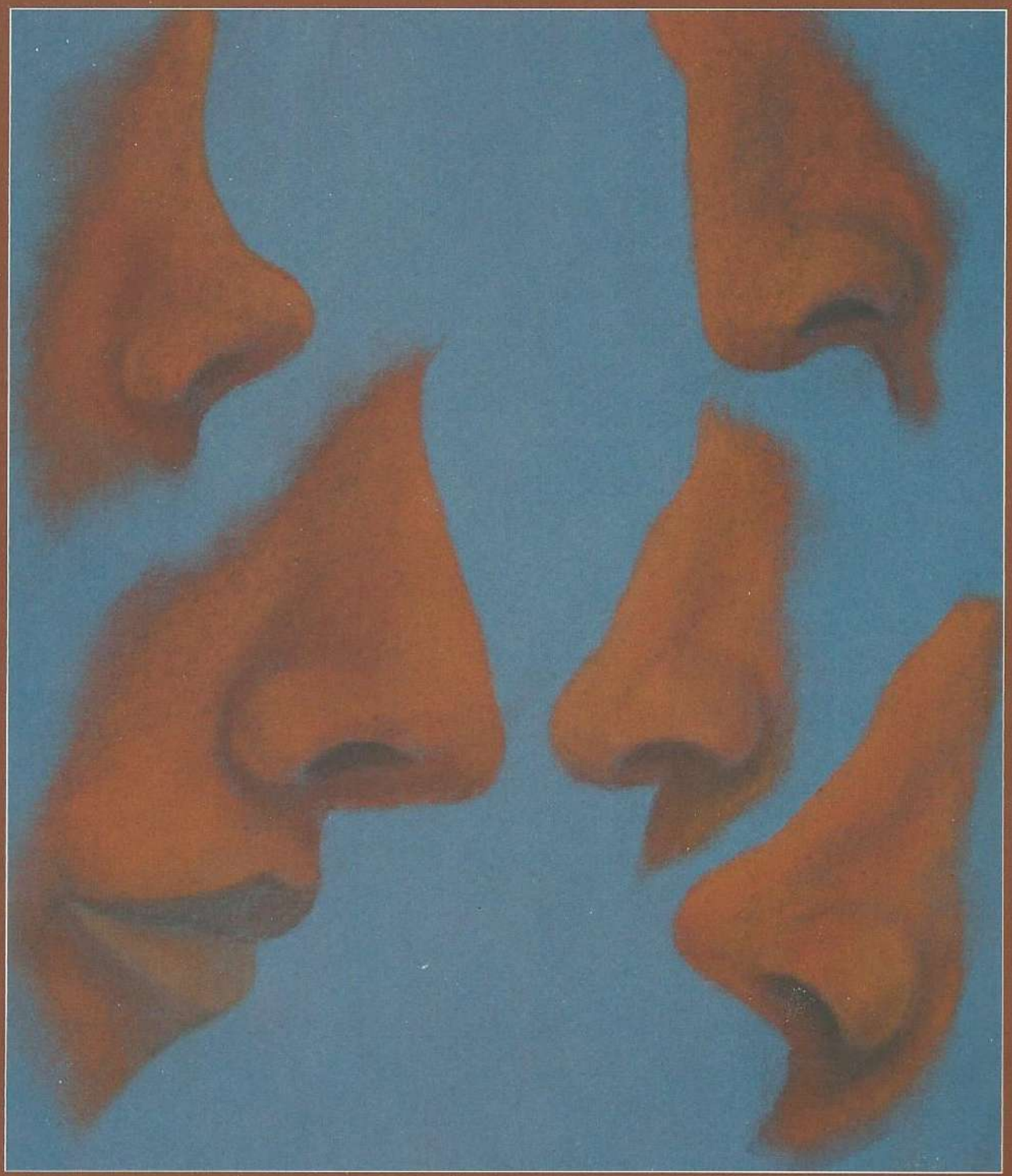
FIGURE 2b. Influence of glucocorticoids on the number of COX-1+ cells, COX-2+ cells and eosinophils



A non-glucocorticoid treated nasal polyp (magnification 20x). Note a) the presence of increased numbers of epithelial COX-1+ cells, b) the presence of low numbers of epithelial COX-2+ cells and c) the presence of increased numbers of eosinophils (MBP+ cells) in both nasal polyp epithelium and nasal polyp lamina propria.



A topical glucocorticoid treated nasal polyp (magnification 20x). Note d) the presence of low numbers of epithelial COX-1+ cells, e) the presence of increased numbers of epithelial COX-2+ cells and f) the presence of decreased numbers of eosinophils (MBP+ cells) in both nasal polyp epithelium and nasal polyp lamina propria.



STELLINGEN

behorend bij het proefschrift

CONTROVERSIES IN CHRONIC RHINOSINUSITIS

1. An exclusive role for eosinophils in the pathogenesis of CRS with nasal polyposis should be questioned (this thesis)

2. The role of neutrophils in the pathogenesis of CRS with nasal polyposis is underestimated (this thesis)

3. A significant increase in interleukin-8 is characteristic of all CRS with nasal polyposis tissue specimens (this thesis)

4. Glycosylated L-selectin ligands on endothelial cells are unlikely to be a major determinant of tissue eosinophilia or tissue neutrophilia in patients suffering from CRS with nasal polyposis (this thesis)

5. Topical glucocorticoids downregulate the expression of cyclo-oxygenase 1 in nasal polyp epithelium (this thesis)

6. Amphotericin B nasal lavages are not the solution for patients with chronic rhinosinusitis (this thesis)

7. Als je je neus volgt, zal er niets aan je neus voorbijgaan

8. ENTER: ear nose and throat (research): evidence rules! (abbreviations.com)

9. De toekomst is zwanger en niemand weet wat zij gaat baren (Afrikaans spreekwoord)

10. Een stelling is gemakkelijker te weerleggen dan op te stellen (Aristoteles)