Prof. Dr. Paul Van Cauwenberge

TGF-beta in chronic sinus diseases: From immuneregulation to remodeling

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This thesis is based on the following manuscripts published in an international peer reviewed journal.

1. Van Bruaene N, Perez Novo Claudina, Deruyck Natalie, Holtappels Gabriele, Van Cauwenberge Paul, Gevaert Philippe, Bachert Claus. Inflammation and remodeling patterns in early-stage chronic rhinosinusitis.
   Clin Exp Allergy. 2012 Jun;42(6):883-90.

2. Gevaert P*, Van Bruaene N*, Cattaert T, Van Steen K, van Zele T, Acke F, De Ruyck N, Blomme K, Sousa AR, Marshall RP, Bachert C. Mepolizumab, a humanized anti-IL-5 mAb, as a treatment option for severe nasal polyposis.
   J Allergy Clin Immunol. 2011 Nov;128(5):989-95.e1-8. Epub 2011 Sep 28.

3. Van Bruaene N, Bachert C. Tissue remodeling in chronic rhinosinusitis.
   Review in Curr Opin Allergy Clin Immunol. 2011 Feb ; 11(1) :8-11.

4. Van Bruaene N, L Derycke, Perez-Novo CA, Gevaert P, Holtappels G, De Ruyck N, C Cuvelier, Van Cauwenberge P, Bachert Claus. TGF-beta signaling and collagen deposition in chronic rhinosinusitis.
   J Allergy Clin Immunol 2009 Aug; 124(2):253-9, 259.e1-2 Epub 2009 Jun 4.

5. Van Bruaene N, Perez-Novo C, Basinski T, Van Zele T, Holtappels G, Schmidt-Weber C, Akdis C, Van Cauwenberge P, Bachert C, Gevaert P. T cell regulation in chronic paranasal sinus disease.
   J Allergy Clin Immunol 2008; 121:1435-1441. Epub April 16.

List of abbreviations

AC = available case analysis
Con = controls
CRSsNP = chronic rhinosinusitis without nasal polyps
COPD = Chronic obstrcutive pulmonary disease
CT = computed tomography
CRSwNP: chronic rhinosinusitis with nasal polyps
EAACI = European Academy of Allergy and Clinical Immunology
ECM = extracellular matrix
ECP = eosinophilic cationic protein
FESS = functional endoscopic sinus surgery
IFN = interferon
IgE = immunoglobulin E
IL = interleukin
IL-5Rα = interleukin 5 receptor, alpha subunit
IQR = interquartile range
IV = intravenous
LAP = latency associated protein
LOCF = last observation carried forward imputation
LTBP = latent TGF-beta binding protein
mAb = monoclonal antibody
MMP = matrix metalloproteinase
MPO = myeloperoxidase
mRNA = messenger ribonucleic acid
NP = nasal polyps
nPIF = nasal peak inspiratory flow
NREU = normalized relative expression units
PBMC's =Peripheral blood mononuclear cells
PCR = Polymerase Chain Reaction
PND = postnasal drip
PS = polyp score
pSmad 2 = phosphorylated smad 2
s.e. = standard error
SOL = soluble
T-bet = T-box transcription factor
TGF-beta = Transforming growth factor beta
TGF-beta R = Transforming growth factor beta receptor
TH = T helper cell
TIMP = tissue inhibitor of matrix metalloproteinase
TNF-a = Tumor necrosis factor alpha
TPS = Total polyp score
Treg = T regulatory cell

Summary

One of the critical factors involved in remodeling of upper airway disease is TGF-beta, acting as a master switch for the development of either chronic rhinosinusitis with or without polyp formation. TGF-beta impacts fibrosis formation through collagen production and its effect on the balance between MMPs and TIMP. Additionally, TGF-beta influences the differentiation of T cells towards Tregs, allowing different inflammatory patterns to establish in the case of deficiency.

We have shown that the regulation of TGF-beta, its receptors and down-stream signals (phosphosmad) and products (collagen) are differently regulated in CRSsNP and CRSwNP, which results in different clinical expression and remodeling patterns. CRSsNP represents fibrosis, whereas CRSwNP is characterized by oedema formation.

In line with a low TGF-beta expression, a significantly lower FOXP3 expression, but a significantly higher T-bet and GATA-3 expression in CRSwNP compared to controls was observed, suggesting a deficit in the T regulatory capacity, which leads to a strong increase in Th1 and Th2 effector cell signals. Eosinophils in Caucasian CRSwNP are activated and their survival is increased by IL-5, a Th2 cytokine suppressing the apoptosis of those granulocytes, and anti-IL5 has been identified as a therapeutic principle in eosinophilic CRSwNP. In CRSsNP, FOXP3, T-bet, GATA-3 and RORc expression were not significantly different from controls; the disease is characterized by a modest increase in IFN-gamma and TGF-beta1 mRNA and protein on the background of a functional T regulatory cell compartment in contrast to CRSwNP. Thus, T-cell mediated inflammation seems to play less of a role in CRSsNP compared to remodeling in this disease phenotype.

In fact, remodeling patterns are consistent in different ethnic groups, whereas inflammatory cell patterns vary, esp. the presence of eosinophil granulocytes in CRSwNP. These discrepancies may indicate that remodeling and inflammation may be dissociated processes, a hypothesis further supported by the finding of early signs of remodeling without inflammatory changes in early CRSsNP disease.

Samenvatting

De ontwikkeling van chronische rhinosinusitis met of zonder nasale polipose wordt in belangrijke mate beïnvloed door TGF-beta. TGF-beta zorgt voor de ontwikkeling van weefselfibrosering via collageenproductie en onrechtstreeks beïnvloedt TGF-beta ook het evenwicht tussen degraderende matrix metalloproteinases (MMPs) en het inhiberende TIMP (tissue inhibitor metalloproteinase). Naast het effect op weefselremodelering, heeft TGF-beta ook een effect op de differentiatie van T cellen naar T regulatoire cellen. Via dit mechanisme kunnen verschillende inflammatoire patronen tot stand komen in geval van TGF-beta deficiëntie.

We hebben aangetoond dat de regulatie van TGF-beta, de receptoren, intracellulaire signalen (phosphosmad) en producten (collageen) verschillend gereguleerd zijn in CRSsNP en CRSwNP. Dit resulteert in een unieke klinische expressie en een verschillend weefselremodelering patroon. CRSsNP wordt typisch gekenmerkt door fibrose, terwijl CRSwNP gekenmerkt wordt door oedeemvorming.

In overeenstemming met een lage expressie van TGF-beta in CRSwNP, werd een significant verlaagde expressie van FOXP3 (merker voor T-regulatoire cellen), maar een significant verhoogde expressie van T-bet en GATA-3 expressie in CRSwNP vastgesteld in vergelijking met controles. Deze bevinding suggereert een deficiëntie aan T-regulatoire capaciteit in CRSwNP, welke aanleiding kan geven tot een sterke toename van Th1 en Th2 effector celsignalen. Eosinofielen in Kaukasische CRSwNP zijn geactiveerd en de overleving is verlengd door IL-5, een Th2 cytokine welke de apoptose van deze granulocyten onderdrukt. Anti-IL5 is aldus een belangrijk therapeutisch target. In CRSsNP zijn FOXP3, T-bet, GATA-3 and RORc expressie niet significant verschillend van controles; deze ziekte is gekenmerkt door een beperkte toename in IFN-gamma en toename in TGF-beta mRNA en proteïne op een achtergrond van een functioneel T regulatoir cel repertoire. T cel gemedieerde inflammatie blijkt aldus een minder belangrijke rol te spelen in CRSsNP in vergelijking met weefselremodelering.

Remodeleringspatronen blijken uniform aanwezig te zijn in verschillende etnische groepen, terwijl de inflammatoire cellijnen zelf sterk kunnen uiteenlopen, zoals bijvoorbeeld de aanwezigheid van eosinofiele granulocyten in CRSwNP. Deze discrepanties kunnen indicatief

zijn voor een dissociatie tussen remodelering en inflammatie. Deze hypothese wordt ondersteund door de bevinding dat vroege tekenen van remodelering wel reeds aanwezig zijn in beginnende CRSsNP zonder dat er duidelijke inflammatoire veranderingen aan te tonen zijn in CRSsNP.

Chapter I

General introduction to chronic rhinosinusitis

1. Definition and subgroups

Chronic rhinosinusitis, by definition, is a disease of the paranasal sinuses that lasts longer than three months and is characterized by a chronic inflammation of the sinuses and the nose[1]. Symptoms of chronic sinusitis may include any combination of the following: nasal congestion, facial pain, headache, post nasal drip, loss of smell, an increase in previously minor or controlled asthma symptoms, aching teeth.

Chronic rhinosinusitis represents a significant health care problem with considerable medical costs and severe impact on lower airway disease and general health outcomes. In order to summarize the current knowledge on rhinosinusitis, the European Academy of Allergology and Clinical Immunology (EAACI) has developed the EP³OS (EAACI position paper on Rhinosinusitis and Nasal Polyps) document on what is currently known about pathophysiology, as well as guidelines for evidence based recommendations on diagnosis and treatment[1,2].

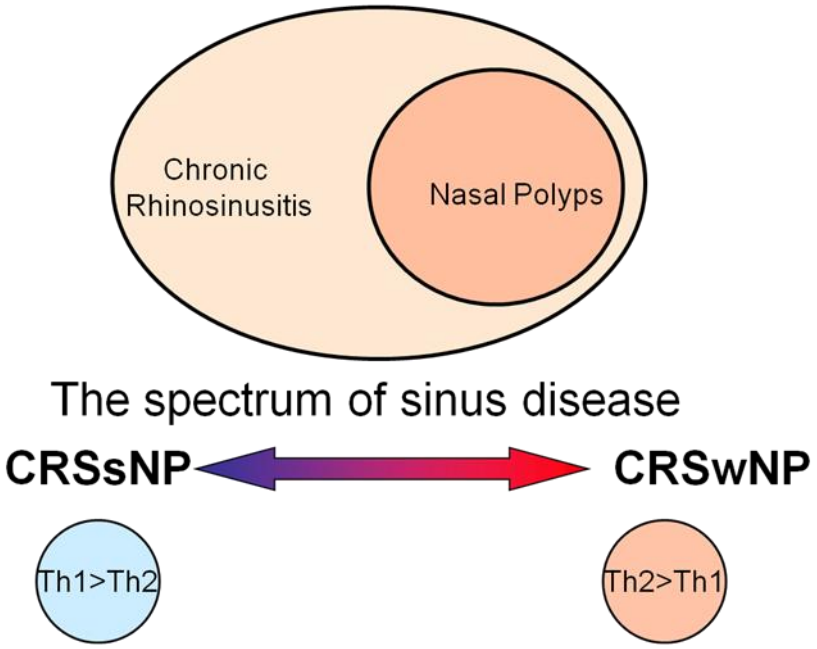
Chronic rhinosinusitis is defined as a group of disorders that is characterized by persistent inflammation of the nose and the paranasal sinuses, and can present with nasal polyp formation. Based on current consensus, the two major subgroups are chronic rhinosinusitis without (CRSsNP) and with nasal polyposis (CRSwNP). Besides these subgroups, nasal polyp formation also occurs in specific conditions such as cystic fibrosis (CF) and allergic fungal sinusitis (AFS), based on genetic defects in CF and a specific IgE-mediated immune response to fungi in AFS respectively.

The clinical diagnosis of chronic rhinosinusitis is currently based on symptoms and duration of symptoms, clinical examination, nasal endoscopy and CT-scan.
However, the clinical presentation is aspecific since the pattern of symptoms and signs is overlapping in patients with chronic sinus inflammation, whether there is formation of nasal polyps (CRSwNP) or not (CRSsNP). As a result, all chronic sinus disease is considered as one disease spectrum, “chronic rhinosinusitis”, which obstructs the development of pathophysiological knowledge and new therapeutic approaches.

However, when looking at inflammatory and remodeling patterns, chronic rhinosinusitis can be differentiated into distinct subgroups[3].

Based on these biological patterns CRS without and with NP represent distinct disease entities within the spectrum of chronic sinus disease. This initially gave rise to a T_H1/T_H2 dogma in chronic sinus disease, where CRSsNP could serve as a model for a Th1 biased disease, and CRSwNP as a model for Th2 driven eosinophilic disease.

CRSsNP is characterized by a predominant T_H1 milieu with high IFN-gamma and TGF-$beta_1$ concentrations, whereas CRSwNP typically show a T_H2 skewed eosinophilic inflammation with high levels of IL-5 and IgE[3].



Classification of nasal polyps and chronic rhinosinusitis.

Table: Clinical definition of chronic rhinosinusitis according to EP³OS guidelines[2]

<table>
<tr>
<td rowspan="2">Clinical definition of rhinosinusitis/nasal polyps

Rhinosinusitis (including nasal polyps) is defined as:

• Inflammation of the nose and the paranasal sinuses characterized by two or more symptoms:

- blockage/congestion;

- discharge: anterior/post nasal drip;

- facial pain/pressure;

- reduction or loss of smell;

and either

• Endoscopic signs:

- polyps;

- mucopurulent discharge from middle meatus;

- oedema/mucosal obstruction primarily in middle meatus;

and/or

• CT changes:

- mucosal changes within ostiomeatal complex and/or sinuses.</td>
<td>Severity of the disease

The disease can be divided into MILD and MODERATE/SEVERE based on total severity visual analogue scale (VAS)

score (0-10 cm):

MILD = VAS 0-4

MODERATE/SEVERE = VAS 5-10</td>
</tr>
<tr>
<td>Duration of the disease

Acute/Intermittent

< 12 weeks

Complete resolution of symptoms.

Chronic/Persistent

>12 weeks symptoms

No complete resolution of symptoms.</td>
</tr>
</table>

2. Epidemiology

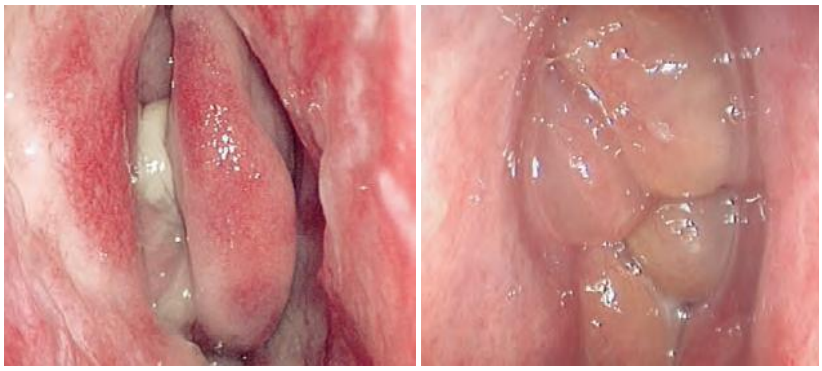
Chronic rhinosinusitis (CRSsNP) and nasal polyposis (CRSwNP) are diseases with high prevalence, estimated up to 15% and 4% respectively in industrialized countries. Chronic rhinosinusitis is one of the most common chronic illnesses in the United States with almost 31 million patients affected, and its prevalence is still increasing[4]. It occurs in both genders, and all ethnic groups. Prevalence appears to be increasing in women and individuals living in the southern US[5]. There is a considerable socio-economic burden, with loss of productivity and missed work/school. Quality-of-life scores are worse than those of other chronic diseases such as heart failure, asthma, and COPD[6].

However, estimating the prevalence of CRS is difficult due to shortcomings in current epidemiological methodology, and the heterogeneity of the disease. Recently, the GA^{2}LEN network of excellence, funded by the European Union, conducted a large pan European study to evaluate the prevalence of CRS in Europe. A postal questionnaire was sent to a random sample of adults aged 15-75 in 19 centres in Europe. Participants reported symptoms of chronic rhinosinusitis, age, gender, and smoking history. Definition of chronic rhinosinusitis was based on the current EP^{3}OS definition. Information was obtained from 57128 responders living in 19 centers in 12 countries. The overall prevalence of chronic rhinosinusitis by EP^{3}OS criteria was 10.8%. Chronic rhinosinusitis was more common in smokers than non-smokers[7]. Co-morbidities such as asthma and aspirin hypersensitivity are frequent in nasal polyposis. This is not the case in chronic rhinosinusitis without polyp formation.

3. Clinical aspects of chronic rhinosinusitis

3.1 Nasal endoscopy

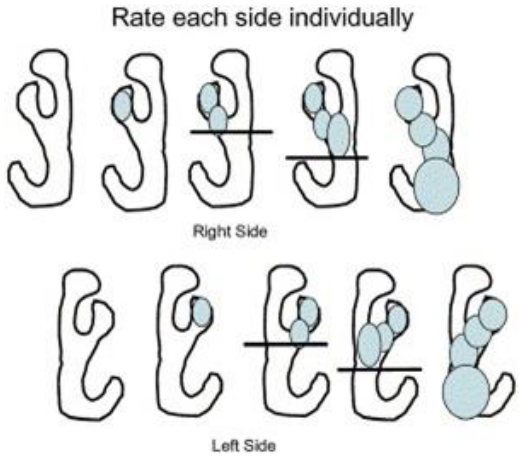
Clinical examination of chronic rhinosinusitis patients is based on nasal endoscopy. Using a nasal endoscope, endoscopy provides a detailed examination of both the nasal cavity and sinuses.



Typical nasal endoscopic view of chronic rhinosinusitis without nasal polyps (left). Mucopurulent discharge from middle meatus can be seen. Right image showing nasal polyps.

We used the following four category endoscopic staging system:

Score 0: no polyps visible
Score 1: small polyps visible in the middle meatus, not reaching below the inferior border of the middle meatus
Score 2: polyps reaching the lower border of the middle turbinate
Score 3: large polyps reaching the lower border of the inferior turbinate or polyps medial to the middle meatus
Score 4:large polyps causing complete obstruction of the inferior meatus



3.2 Computed tomography

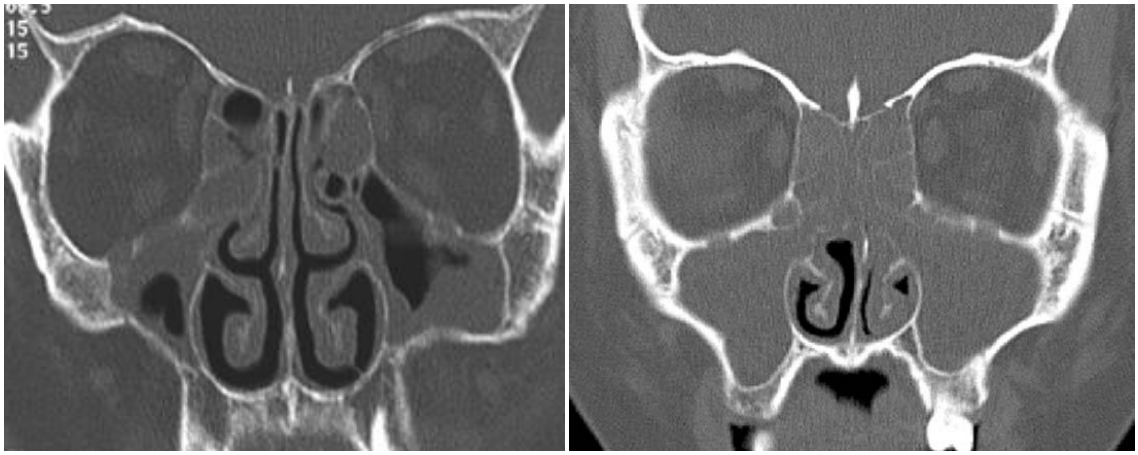
CT scan imaging is the technique of choice for chronic rhinosinusitis, showing extent of the disease and anatomy. Plain sinus X-rays are insensitive and nowadays obsolete.

The Lund-Mackay system is a validated staging system for assessing extent of the pathology. The system relies on a scoring system ranging from 0 to 2 as description of the extent of opacification of each sinus system and of the ostiomeatal complex: 0 in the case of absent opacification, 1 partial opacification and 2 in the case of complete opacification, deriving a maximum score of 12 per side.

	Left	**Right**
Maxillary Sinus (0,1,2)		
Anterior Ethmoid (0,1,2)		
Posterior Ethmoid (0,1,2)		
Sphenoid (0,1,2)		
Frontal Sinus (0,1,2)		
Ostiomeatal complex (0,2)*		
Total score		

0: no abnormalities 1: partial opacification 2: total opacification

0: not occluded* 2. occluded*



CT scan of paranasal sinuses, coronal view showing typical changes with obstruction of the ostiomeatal complex in chronic rhinosinusitis without polyps (left). Right image showing massive nasal polyposis with complete opacification of the maxillary and ethmoidal sinuses.

3.3 Management of chronic rhinosinusitis

Several therapies are used in the treatment of chronic rhinosinusitis, however, corticosteroids and antibiotics remain the cornerstones of the current medical treatment. When medical treatment fails, functional endoscopic sinus surgery (FESS) is indicated in order to restore physiologic aeration and drainage of the sinuses, which can facilitate the resolution of mucosal disease. However, FESS does not directly treat the underlying inflammatory disorder, therefore intensive post-operative medical management is mandatory.

3.3.1 Chronic rhinosinusitis with nasal polyps

Patients suffering from nasal polyposis complain mostly of nasal congestion, hyposmia or anosmia, anterior rhinorrhoea or postnasal drip. Medical treatment is intended to reduce the size and extent of the nasal polyps and control mucosal inflammation. Corticosteroids remain the cornerstone of treatment, they can be administered topically by either sprays or drops (instillation) or systemically.

3.3.1.1 Topical glucocorticosteroids

Topical intranasal corticosteroids have shown to be safe and effective in reducing polyp size, nasal obstruction, rhinorrhoea and sneezing in people with nasal polyposis[8-10]. Their anti-inflammatory effect is localized and their systemic absorption has been shown to be negligible[11-13]. However, due to the mechanical obstruction of the sinuses by the nasal polyps, it is often impossible for sprays to reach within the sinuses. Therefore, nasal drops are likely to be more effective, because these solutions can reach further within the sinuses[14]. The patient is asked to assume a series of positions: first the head down forward position in order to reach the frontal and ethmoid sinuses, then right lateral supine position for the maxillary sinuses, and finally in the supine position to reach the sphenoid sinuses, each for one to two minutes.

Topical glucocorticoids are also helpful in preventing the regrowth of nasal polyps following sinus surgery[15].

3.3.1.2 Systemic glucocorticosteroids

The use of systemic corticosteroids has been widely used in treatment of nasal polyposis, however systemic side effects limit its usefulness.

In a double blind placebo controlled trial methylprednisolone shows a fast and significant effect of oral methylprednisolone on nasal polyp size, nasal symptoms and nPIF, with however a relapse as soon as 4 weeks and a total recurrence as early as 3 months after start of treatment[16].

Adverse effect of systemic steroid use include diabetes, peptic ulcer disease, glaucoma, severe hypertension, and advanced osteoporosis. Even a short course of steroids can significantly increase the blood pressure and glucose levels in patients with predisposition to hypertension and diabetes.

3.3.1.3 Doxycyline

A chronic microbial trigger is currently suggested to play an important role in the pathogenesis of chronic rhinosinusitis with nasal polyposis. Colonization with Staphylococcus aureus is present in 64 percent of patients with chronic rhinosinusitis with nasal polyposis, compared with approximately 30 percent in healthy controls or patients with chronic rhinosinusitis without nasal polyps. In addition, IgE antibodies directed against Staphylococcal superantigens have been found in the tissues of a high percentage of colonized polyposis patients. A randomized, double-blind, placebo-controlled trial was conducted to assess whether doxycycline could reduce nasal polyp size and provide anti-inflammatory effects. Doxycycline (200 mg on the first day followed by 100 mg once daily for 20 days) caused a statistically significant reduction in polyp size beginning at week 2 and this effect was sustained for 12 weeks. A significant reduction in nasal secretion eosinophil cationic protein (ECP) was also found after 20 days of doxycycline treatment[17].

3.3.1.4 Other treatment options

3.3.1.4.1 Anti-IL-5

Nasal polyposis is characterized by abundant tissue eosinophilia in more than 80% of the Caucasian patients, and is frequently associated with asthma. IL-5 is essential for the differentiation of eosinophils, but it also activates and prolongs survival of the mature cells in the tissue. Hence, IL-5 represents a specific therapeutic target.

TGF-beta not only has pro-fibrotic and immunomodulatory properties, it is also known for its ability to counter effects on IL-5: TGF-beta counteracts the survival-prolonging effects of IL-5 on eosinophils[18]. TGF-beta inhibits the release of eosinophil peroxidase. Thus, TGF-beta seems to inhibit eosinophil survival and function[18].

In vitro studies have shown that anti-IL-5 treatment resulted in eosinophil apoptosis and decreased tissue eosinophilia[19].

A first double-blind placebo-controlled studies has been performed with a monoclonal anti-IL5 antibody (reslizumab) in nasal polyp patients[20]. This study showed that one single administration of 3 mg/kg and 1mg/kg of a humanized anti-IL-5 is safe and well-tolerated therapy however only 50% of the patients showed a clinical response with reduction of polyp size. Subgroup analysis showed that high local IL-5 concentrations in nasal secretions predicted a positive response.

We performed a phase 2 study to determine the efficacy of two injections of a monoclonal anti-IL5 antibody (mepolizumab) on nasal polyp volume in subjects with severe nasal polyposis. The efficacy was studied by nasal endoscopy and CT-scan imaging[21]. In addition, markers of biological activity such as IL-5 and nasal eosinophilia were assessed over a period of eleven months post last dose. Two injections of mepolizumab were safe and well tolerated and significantly reduced the size of nasal polyps for at least 2 months post dosing based on endoscopic scoring and blinded CT scan assesment.

3.3.1.4.2 Anti-IgE

In patients with nasal polyps, a local massive multiclonal IgE response has recently been described[22]. Evidence accumulates that S. aureus derived enterotoxins act as superantigens

resulting in a multiclonal T- and B-cell activation with massive IgE formation within the airways. Therefore, IgE could be an interesting therapeutic target. In lower airway disease, omalizumab, a humanized monoclonal anti-IgE antibody was used in severe asthmatics. Treatment with omalizumab resulted in marked reduction of serum IgE and a reduction of IgE+ cells in the airway mucosa.

A double blind randomized placebo controlled study is currently conducted in our department with omalizumab in patients with severe nasal polyposis.

3.3.2 Chronic rhinosinusitis without nasal polyps

Patients with chronic rhinosinusitis without nasal polyps typically complain for longer than 12 weeks about nasal obstruction, together with one or more of the following symptoms: discolored nasal discharge, headache with frontal pain and sometimes smell disturbances.

In case of mild symptoms, treatment with topical steroids and nasal irrigations with saline is appropriate. In the case of failure after three months, or in the case of moderate to severe symptoms, a long term antibiotic treatment is suggested by the current E³POS guidelines.

3.3.2.1 Topical glucocorticosteroids

The efficacy of glucocorticoid nasal sprays was evaluated in a trial of 167 patients with CRS and persistent symptoms despite two weeks of oral antibiotics, in which subjects were randomized to budesonide nasal spray (128 micrograms twice daily) or placebo for 20 weeks[13]. The active therapy significantly reduced both morning -1.40 (95% CI, -2.18 to -0.62) and evening -1.37 (95% CI, -2.15 to -0.58) symptom scores from baseline, compared to placebo, with the greatest impact in patients with underlying allergic rhinosinusitis.

For patients who have persistent symptoms despite consistent use of glucocorticoid nasal sprays, we advise them to change to nasal glucocorticoid instillations, as described previously.

3.3.2.2 Low-dose macrolides

Several reports have concluded that long-term administration of low-dose macrolide antibiotics is helpful in chronic rhinosinusitis[23-25]. It is unclear if this is due to anti-

inflammatory or antimicrobial effects. One placebo-controlled trial in 64 patients with chronic rhinosinusitis evaluated monotherapy with the macrolide roxithromycin[25]. Patients were treated with 150 mg roxithromycin daily for 3 months. After 12 weeks of therapy, patients reported small but statistically significant benefits compared with placebo on the primary outcome measure of symptom score as well as several objective measures. However, long term treatment and routinely use of antibiotics still remains matter of debate due to the risk of development of multiresistant bacterial species.

3.3.3 Surgery

Functional endoscopic sinus surgery (FESS) has revolutionalized the surgical treatment of chronic rhinosinusitis, first introduced by Messenklinger and Stammberger. It has become the standard surgical intervention for patients with chronic rhinosinusitis (CRS) refractory to medical therapy. Performed through the nasal cavity using endoscopes and inciting no external scars, these advantages have renewed an interest in the surgical intervention of chronic sinus diseases.

4. T cell immunology in chronic sinus diseases

Chronic rhinosinusitis with and without nasal polyposis are chronic sinus diseases, both characterized by persistent inflammation of the nasal and paranasal mucosa. Recent research has demonstrated that these pathologies can be differentiated into distinct subgroups, based on the expression of inflammatory mediators[3], giving rise to a Th1/Th2 dogma in chronic sinus disease.

Chronic rhinosinusitis without polyps demonstrates a Th 1 typed inflammation, with high levels of IFN-gamma and TGF-beta.

Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a Th2 skewed eosinophilic inflammation, with high levels of IL-5 and total IgE, and low TGF-beta concentrations[26].

Because the intracellular mechanisms behind this initial T cell polarization remain largely unclear, this was subject for further research.

4.1 T cell subsets

T cells play a central role in cell mediated immunity. Progenitor T cells migrate from the bone marrow to the thymus were they are selected by positive selection (recognition of MHC) and negative selection (recognition of self antigens). Once CD4+ cells have survived the selection procedure in the thymus, they move to the periphery where antigen encounter occurs. Antigen presenting cells (APCs) such as macrophages, dendritic cells and B cells present the antigen to the T cell receptor in the form of a peptide-MHC II complex.

Importantly, in the case of superantigens this presentation in the MHC II peptide binding groove does not occur, superantigens are able to cross link the MHC molecule and the T-cell receptor directly. It has been demonstrated that significantly more nasal polyp patients are colonized with Staphylococcus aureus. An increased response to S. aureus enterotoxins was observed in nasal polyps, reflected by a severe eosinophilic inflammation and higher total IgE production[22].

Upon antigen recognition, differential maturation towards a Th1 or Th2 phenotype will occur. Factors that can influence this decisive step are the cytokine milieu and the phenotype of the APC.

Different T cell subsets have been described, each with distinct functions. Initially, only two subsets of T helper (h) cells were described: Th1 and Th2 cell types.

Based on knowledge acquired from allergic rhinitis and asthma, it is established that Th1 cells characteristically interact with external pathogens (e.g. bacteria, viruses) and secrete interleukin 2 (IL-2), IL-3, tumor necrosis factor alpha, and interferon gamma.

Th2 cells adapt B-cell production of immunoglobulins and humoral immunity. Th2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13. IL-4 and IL-13 facilitate B-cell heavy-chain isotype switching from immunoglobulin G (IgG) to immunoglobulin E (IgE) production, with subsequent release of IL-5. IL-5 encourages eosinophilic inflammation, facilitating the allergic response and airway hyperresponsiveness. The IgE produced in response to allergens populates mast cells and basophils. Later exposure to the same allergen permits release of proinflammatory molecules, such as histamine. The IL-5-facilitated eosinophilic inflammation creates the nasal symptoms and airway inflammation characteristic of allergic rhinitis and asthma[27,28], which are both associated with an abundance of Th2 cell response[29,30].

The existence of a dedicated population of suppressive T cells was subject of controversy for many years. Recent advances in characterization of this T cell population, called regulatory T cells have firmly established their existence and their critical role in the immune system. Regulatory T cells are a specialized subpopulation of T cells that actively suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens[31]. It is assumed that regulatory T cells play a key role in diseases characterized by dysregulated peripheral tolerance such as asthma, atopic dermatitis, allergic rhinitis and autoimmune diseases[9-11]. The activity of T-regulatory cells can also suppress the response of T cells to exogeneous antigens such as Staphylococcal enterotoxin B[32].

Recently, a subset of highly proinflammatory T cells that produce interleukin 17 (Th 17 cells) has been identified, these could play an important role in immunity and disease. The role of TH17 cells in allergy is still largely unclear, but experimental models suggest that TH17 cells

may be important for neutrophilic inflammation in acute airway inflammation. Many functions that were initially attributed to Th1 cells are being shown to be part of Th17 responses[33].

4.2 Transcription factors

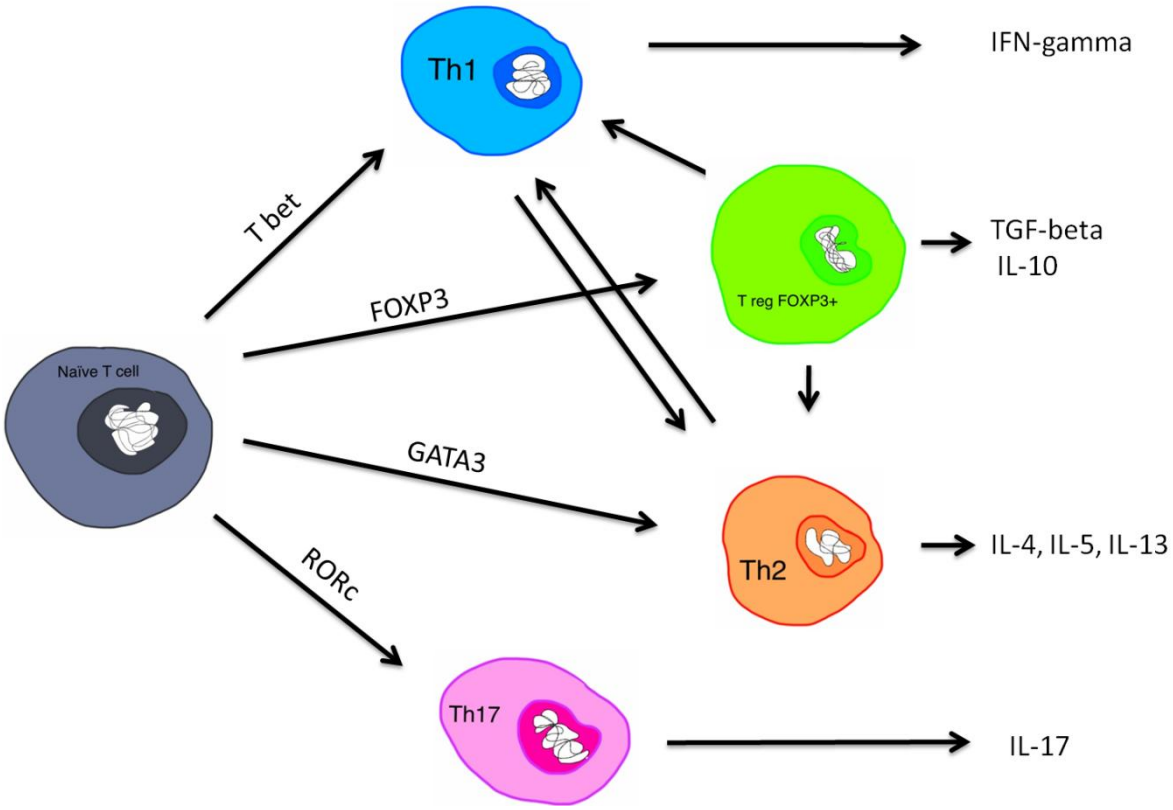
Naïve T-cells differentiate towards different T cell subtypes based on the differential expression of transcription factors. T-bet (T-box transcription factor) expressed by Th1 cells expressing IFN-γ involves commitment towards Th1 cells, absence of T-bet results in elimination of IFN-γ production by Th1 cells[34].

T-bet is restricted to the Th1 subset, and Tbet transactivates the IFN gamma gene, induces IFN-gamma production in retrovirally transduced primary T-cells, and redirects polarized Th2 cells towards the Th1 direction[35].

GATA-3 (gata binding protein 3) is critical for commitment towards Th2 cells and controls the expression of interleukin (IL)-4 and IL-5[36,37].

FOXP3 is a novel member of the forkhead transcription factors, and is recognized to be essential for the development and function of T-reg cells. FOXP3 acts as a master regulator for the development and function of T-reg cells. Genetic mutations in the gene encoding FOXP3 have been identified in both humans and mice. Humans with mutations in FOXP3 suffer from a severe and rapidly fatal autoimmune disorder known as Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome[38].

The transcription factor involved in Th17 differentiation is called RORγt, equivalent to RORc in humans[39].



Naïve T-cells differentiate towards different T cell subtypes based on the differential expression of certain transcription factors. T-bet (T-box transcription factor) involves commitment towards Th1 cells[40]; GATA-3 (gata binding protein 3) is critical for commitment towards Th2 cells, and controls the expression of interleukin (IL)-4, IL-5 and IL13[41,42]. Moreover, the balance between Th1 and Th2 is controlled by an intriguing subset of T cells, called T-regulatory cells (Tregs). The differentiation towards Treg cells is controlled by the transcription factor FOXP3. TGF-beta and IL-10 are indirect markers for induced Treg cell types, Tr1 and Th3 respectively[43]. RORc controls the differentiation towards Th17 cells.

4.3 Regulatory T cell subsets

Various populations of T cells have been described over the past years. T regulatory cells can be divided in two categories: natural and induced populations of regulatory T cells have been described, and they probably have overlapping functions in the control of the immune response[44].

4.4 Naturally occurring T regulatory cells

Naturally occurring regulatory T (nTreg) cells functionally mature in the thymus, and exert their suppressive effect via cell-cell contact or via soluble mediators. The development of nTreg cells occurs under the control of the transcription factor FOXP3, and they are

characterized by CD4(+)CD25(+) phenotype. They exert their suppressive effect via cell-cell contact. $CD4^+CD25^+$ regulatory T cells constitute 7 to 10 % of the total $CD4^+$ T cell population[45]. It is hypothesized that nTreg cells can migrate to sites of inflammation at mucosal surfaces and inhibit Th2 and Th1 cells via cell-cell contact [11].

4.5 Induced type T regulatory cells: Tr1 and Th3 cells

Inducible Tregs (iTregs) are generated from naïve T-cells in the periphery. The most important subsets of iTregs are Tr1 and Th3 cells. They suppress immune function by secretion of predominantly IL-10 and TGF-beta, respectively[46]. It is probable that both natural and inducible populations have complementary and overlapping functions.

FOXP3 was initially thought to be a specific marker for nTregs that could not be activated in peripheral Tcells[47-49]. However, recent studies could demonstrate the induction of FOXP3 in iTregs both in vivo and in vitro [50-52], thus being a marker for both nTregs and iTregs.

5. TGF-beta

Transforming growth factor beta (TGF-beta) is a multifunctional and pleiotropic growth factor involved in many processes, affecting processes ranging from regulation of cellular differentiation and growth to inflammation, wound healing, bone formation, and contributing to the pathogenesis of diseases as diverse as autoimmune disease and carcinogenesis.

It's main activities are extensive. It has a growth inhibitory action on epithelial cells, endothelial cells and hematopoietic cells. TGF-beta also regulates the function of immune cells, for which it is a strong suppressor of activation of T cells and of antibody secretion by B cells.

TGF-beta has an effect on chemotaxis, cellular differentiation, apoptosis and extracellular matrix production. It's effect on extracellular matrix production is manifested by enhanced expression of extracellular matrix proteins and suppression of expression of matrix degrading proteins. Consistent with the multiple tissues and diseases in which TGF-beta is

involved, the cellular targets are not restricted to any lineages or cell types. Any cell can express TGF-beta receptors and secrete TGF-beta ligand[53].

In humans there are three isoforms known, TGF-beta1, 2, and 3. TGF-beta binds to at least three membrane proteins, referred to as receptor type I, II, and III, that exist on virtually all cells. Type I and II are transmembrane serine-threonine kinases that interact with one another and facilitate each other's signalling. The type III receptor, also called betaglycan, is a membrane anchored proteoglycan that has no signalling structure but acts to present TGF-beta to other receptors[54]. The effects of TGF-beta on the synthesis and deposition of extracellular matrix are mediated by the type I receptor. The effects on cell growth and proliferation are mediated by the type II receptor[55].

TGF-beta production, secretion and storage are complex. After secretion, TGF-beta is associated with a "latency associated peptide" (LAP) forming the *small latent complex*. This association prevents binding of secreted TGF-beta to ubiquitously expressed receptors and assures an extracellular reservoir of TGF-beta that can be activated on demand. In most cells LAP is covalently linked to an additional protein, LTBP (latent TGF-beta binding protein, existing in four isoforms), forming the *large latent complex* [56]. LTBPs enhance the secretion of TGF-beta. LTBPs play a role in the targeting of the latent TGFbeta complex to the extracellular matrix. They are known to exist both as soluble molecules and in association with the ECM. LTBPs are associated into the matrix rapidly after secretion. TGF- beta can be released from the large latent complex by several activators, including integrins (integrin αvβ6), proteases and thrombospondin. TGF-beta acts both locally, by binding of the TGF-beta large latent complex to the extracellular matrix through the latent TGF-beta-binding protein (LTBP), and distally, through proteolytic release of latent complex from the ECM[57]. LTBPs are structural ECM proteins for targeting TGF-beta action.

5.1 TGF-beta signaling pathway

TGF-beta is a 25kDa protein that exerts signaling through two receptors: a serine-threonine kinase type I and type II cell surface receptor. The TGF beta ligand binds to a type II receptor dimer, which recruits a type I receptor dimer forming a hetero-tetrameric complex with the ligand. TGF-beta receptor III has a high affinity for TGF-beta receptor I and II. TGF-beta

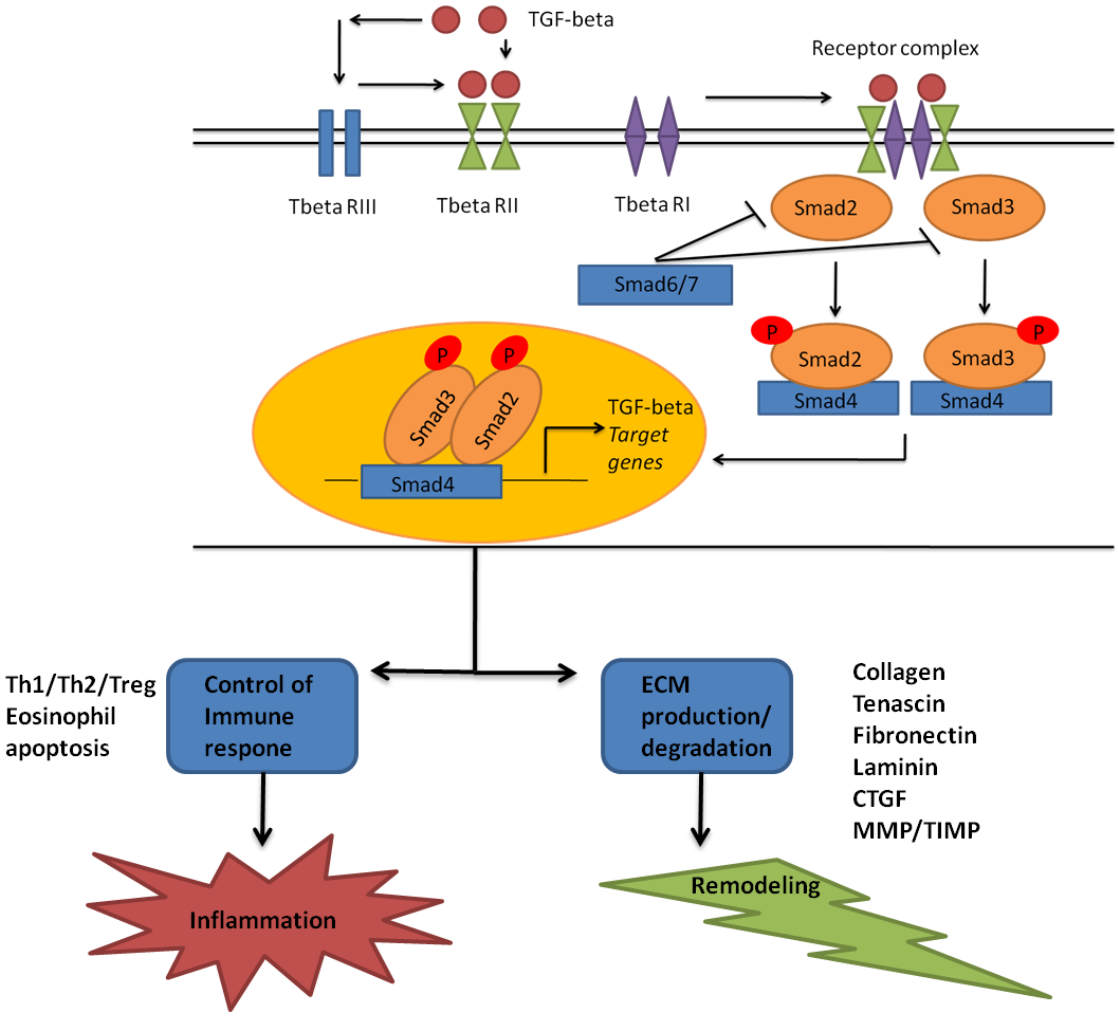
receptor III has a non-signaling role and functions as a co-receptor, but is able to enhance the binding of TGF-beta to the receptor II.

Upon binding of TGF-beta to the TGF-beta receptor II, TGF-beta receptor II recruits and transphosporylates TGF-beta receptor I. TGF-beta receptor I activates the ligand specific SMAD proteins Smad2 and Smad3.

SMADs are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream TGF-beta gene transcription[58].

The SMAD proteins are homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA. The name is a combination of the two.

Upon activation of Smad2 and Smad3, they form a trimeric complex with Smad4 and are translocated to the nucleus where they activate TGF-beta target genes. There exist inhibitory Smad proteins Smad6 and Smad7 that prevent either the dissociation of the Smad2/Smad3 from the TGF-beta receptor complex, and/or inhibit the binding of Smad2/Smad3 to Smad4[59].



TGF-beta signaling pathway and function

TGF-beta binds to Tbeta RII. This binding might be enhanced by the presence of Tbeta RIII. After binding to TGF-beta, Tbeta RII recruits and transphosphorylates Tbeta RI. The consequently activated type I receptors activate Smad 2 and Smad 3 by phosphorylation (P). This process is inhibited by Smad 6 and Smad 7. Activated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus. This results in the activation of target genes, influencing inflammation and extrecellular matrix remodeling.

5.2 Cellular Sources of TGF-beta

TGF-beta can be generated by many cells such as macrophages, epithelial cells, fibroblasts and eosinophils[60]. TGF-beta is usually secreted in its latent form. The highest amounts of TGF-beta are found in human platelets and mammalian bone[61].

TGF-beta and eosinophilic inflammation

The eosinophil, which plays a pivotal role in the pathogenesis of asthma, has also an important role in the inflammatory process of sinus disease. Eosinophils are a rich source of TGF-beta 1. Other cell types involved in inflammation are also potential sources of this fibrogenic factor. These include macrophages, T cells, mast cells, neutrophils, endothelial and epithelial cells, as well as smooth muscle cells and fibroblasts themselves[57,62].

5.3 TGF-beta in chronic rhinosinusitis: dual role

Among the growth factors that are possibly involved in chronic inflammatory diseases of the airways and therefore in chronic rhinosinusitis, TGF-beta could play a key role.

TGF-beta mediates a broad spectrum of biological activities, particularly airway remodeling in lower airways[62-64].

TGF-beta is a fibrogenic growth factor which stimulates extracellular matrix formation and chemotaxis of fibroblasts, but inhibits eosinophil survival and induces eosinophil apoptosis[65]. In particularly, it stimulates the production of extracellular matrix components such as Tenascin – C, collagen, fibronectin and laminin. Transforming growth factor beta (TGF-beta) is found in low levels in tissue homogenates from CRSwNP[66]. A possible mechanism of pseudocyst formation in CRSwNP could be the lack of TGF-beta and the overexpression of metalloproteinase 9 and metalloproteinase 7 without the upregulation of the tissue-inhibitor of matrix-metalloproteinase 1, which may account for the tissue destruction[67].

Remodeling in chronic rhinosinusitis will be discussed in **chapter 2.**

TGF-beta and immuneregulation

Many chronic diseases profit from the immunosuppressive effect of TGF-beta, however, this molecule has also been implicated in fibrosis formation and is suspected to play a major role in airway remodeling. In general, the function of TGF-beta could be understood as a counter regulatory cytokine to resolve inflammation and to initiate the repair process.

Besides the effect of TGF-beta on already differentiated T cells, effect during the development has also been noticed. Indirect immunomodulatory effects of TGF-beta occur through T regulatory cells. TGF-beta1 induces FOXP3 expression in CD25(-) naïve T cells to

enforce transition to T regulatory cells and is a critical factor in the development of peripheral T regulatory cells[68,69].

Moreover, T regulatory cells are able to suppress both Th1 and Th2 responses by producing suppressive cytokines such as IL-10 and TGF-beta.

Although TGF-beta can affect many cell types, however CD4+ T cells are of special interest since anti CD4 antibodies are protective in TGF-beta 1 knockout mice[70]. The effect of TGF-beta on CD4+ cells in a mouse model was of particular interest: a stimulating effect was found on Th 1 cells whereas inhibitory effects were observed on Th 2 cells[71].

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Chapter II

Tissue remodeling in chronic rhinosinusitis

Tissue remodeling in chronic rhinosinusitis

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Review in Curr Opin Allergy Clin Immunol. 2011 Feb;11(1):8-11

Purpose of review

The purpose of review is to summarize the current knowledge on remodeling in chronic sinus disease.

Recent findings

Chronic sinus disease is characterized by persistent inflammation of the nasal and paranasal mucosa and is currently classified into two major subgroups on the basis of the absence (CRSsNP) or presence (CRSwNP) of nasal polyps. TGF-beta and Matrix metalloproteinases are critical factors involved in the remodeling process.

Summary

Remodeling is clearly present in chronic sinus disease. TGF-beta has been implicated as an important factor in remodeling processes involved in chronic sinus disease, and serves as a main switch for different remodeling patterns in chronic sinus disease.

Keywords

Collagen

MMP

TGF-beta

1.Introduction

Remodeling is a critical aspect of wound repair in all organs, being defined as “modeling again”, or “modeling differently”. It is a dynamic process resulting in both extracellular matrix production and degradation. This may lead to a normal reconstruction processes with production of normal tissue, or may result in pathological reconstruction with formation of pathological tissue[1].

In lower airway disease remodeling has been extensively studied and reviewed[1-3]. It includes changes in airway epithelium, lamina propria and submucosa, resulting in airway wall thickening. The main histological features of remodeling are: macrophage and lymphocyte infiltration, fibroblast proliferation, angiogenesis, increased connective tissue formation (fibrosis) and tissue destruction. There is clear evidence that remodeling is also present in chronic sinus disease, and distinct remodeling features differentiate different subgroups of chronic rhinosinusitis.

Chronic rhinosinusitis is clinically a heterogeneous group of chronic inflammatory sinus diseases affecting up to 15% of the global population, with important socio-economical impact[4,5]. However, based on differential inflammatory and remodeling patterns, chronic rhinosinusitis can be divided in two major subgroups i.e. chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with polyp formation (CRSwNP)[6-8]. In Caucasians, CRSwNP is characterized by a predominant Th2 typed eosinophilic inflammation with high levels of IL-5, ECP and eotaxin, and high levels of local IgE. However, in Asian CRSwNP, a Th1/Th17 polarization was observed, and samples from Asian polyps demonstrated a more neutrophilic inflammation. Typical remodeling features in nasal polyps from both ethnic groups are albumin accumulation and oedema (pseudocyst) formation within the extracellular matrix. One striking feature is the relative lack of the transforming growth factor beta (TGF-beta 1) signaling in CRSwNP and lack of collagen production within the extracellular matrix. In contrast, CRSsNP is characterized by a mainly Th1 driven inflammation with high levels of IFN-gamma and active TGF-beta 1 signaling with subsequent excessive collagen deposition and fibrosis formation.

Other specific subgroups include nasal polyposis in patients with associated cystic fibrosis and allergic fungal sinusitis; these will not be addressed here[8].

The purpose of this review was to generally summarize the current knowledge on remodeling in chronic rhinosinusitis, using studies in human disease.

2. Review

2.1. Histomorphological features of remodeling in chronic sinus disease

The histology of chronic rhinosinusitis with polyp formation (CRSwNP) is typically characterized by the presence of pseudocyst formations consisting of albumin accumulation and oedema formation[9], the lack of collagen within the extracellular matrix[10], and the excessive infiltration of inflammatory cells mainly consisting of eosinophils in about 80% of the Caucasian polyps[11,9]. Other inflammatory cell types are lymphocytes and mast cells. No nervous structures can be found within nasal polyps[12,13]. “Early stage” polyps can be distinguished from “mature” polyps. The typical characteristic of an early polyp is the presence a pseudocyst in the core of the polyp, loose connective tissue with few inflammatory cells and an accumulation of inflammatory cells at the top of the early stage polyp. In contrast a mature polyp typically consists of a large amount of pseudocysts, with a less expressed cellular component[9]. Nasal polyps show a lack of vascular structures, and epithelial damage is often present.

In contrast, chronic rhinosinusitis without polyp formation is typically characterized by a more neutrophilic inflammation[14], together with fibrosis formation within the extracellular consisting of excessive collagen deposition and thickening of the collagen fibers, and the absence of pseudocysts[10].

These typical features of CRSsNP and CRSwNP have been confirmed in Asian patients[15], although the inflammatory patterns are different[16].

2.2. Factors influencing remodeling

Several factors have been implicated in remodeling, we here review the best studied factors involved.

2.2.1 TGF-beta

Transforming growth factor (TGF-) beta is a pleiotropic and multifunctional growth factor, with important immunomodulatory and fibrogenic characteristics. Many chronic diseases profit from the immunosuppressive effect of TGF-beta, however, this molecule has also been implicated in fibrosis formation and is suspected to play a major role in airway remodeling. In general, the function of TGF-beta could be understood as a counter regulatory cytokine to resolve inflammation and to initiate the repair process.

TGF-beta is considered as a master switch in the induction of the profibrotic program, and acts as chemoattractant and proliferation factor for fibroblasts[6]. It induces fibroblasts to synthesize ECM proteins and contract extracellular matrix. Three different isoforms (TGF beta 1, 2 and 3) have been described, which can bind to three membrane proteins, referred to as receptor type I, II, and III.

Further, TGF-beta regulates the function of immune cells; it is a strong suppressor of T cell activation and of antibody secretion by B cells. Recently, a deficit in FOXP3 expression (a specific transcription factor critical in Tregulatory cell differentiation and function) was demonstrated in CRSwNP, coinciding with low TGF-beta 1 protein levels. As TGF-beta acts both as an effector and an inductor of Treg function, the decreased expression of FOXP3 and TGF-beta1 protein, together with the upregulation of both Th1 (T-bet) and Th2 (GATA-3) transcription signals suggests defective T regulatory function in CRSwNP[6].

In a recent study, TGF-beta 1 protein expression was found increased together with TGF-beta RI expression and a high number of phospho-smad 2 positive cells, indicating an enhanced TGF-beta signaling in CRSsNP. In strong contrast, in CRSwNP a low TGF-beta 1 protein concentration, a decreased expression of TGF-beta RII and a low number of phospho-smad 2 positive cells indicate a low level of TGF-beta signaling in CRSwNP. These findings were reflected by the remodeling patterns observed, characterized by a lack of collagen in CRSwNP, and excessive collagen production with thickening of the collagen fibers in the extracellular matrix in CRSsNP[10].

Due to the regulatory function of TGF-beta in both inflammation and remodeling processes, TGF-beta could be an interesting therapeutic target for chronic rhinosinusitis treatment.

Long-term, low-dose macrolide therapy is currently suggested as treatment. It is believed that macrolides have an anti-inflammatory effect. Clarithromycin therapy has been shown to reduce cellular expression of TGF-beta 1 in *in vitro* biopsies (nasal mucosal cultures in the presence of clarithromycin or control) from CRS patients. *In vivo* however, nasal biopsies taken before and after clarithromycin treatment for three months showed no differences in cellular expression of TGF-beta[17].

2.2.2 Matrix metalloproteinases

The role of matrix metalloproteinases (MMPs) in the pathogenesis of lower airway diseases has been extensively studied[18-21]. In view of the united airway concept, MMPs have also been focus of research in upper airway disease. Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent endopeptidases that are known to be important to remodel the extracellular matrix.

One of the mechanisms proposed possibly leading to pathologic tissue remodeling in CRS, is the imbalance between MMPs and the tissue inhibitor of metalloproteinases (TIMPs).In CRSsNP, elevated levels of MMP-9 and TIMP-1 together with high levels of TGF-beta 1 are found. TGF-beta 1 induces the release of TIMP-1, inhibiting the proteolytic activity of MMP-9[22]. In contrast in CRSwNP, only MMP-9, but not TIMP-1, is up-regulated[23-26], due to the relative lack of TGF-beta 1. Within the pseudocysts present in CRSwNP, the inflammatory cells showed positive staining for MMP-9, suggesting a direct degradative function[27].

The lack of inhibition of MMPs by TIMP-1 in nasal polyp tissue can cause tissue destruction leading to pseudocyst formation. In contrast, up-regulation of TGF-beta 1 and TIMP-1 over MMP-9 could explain the prominent fibrosis found in CRSsNP[27]. Again, these findings were similar when comparing Caucasian and Asian disease[28]. In a recent case control study of Wang et al., where 203 cases of chronic rhinosinusitis with nasal polyposis and 730 controls were enrolled, evidence has been provided that MMP-9 gene polymorphisms may influence susceptibility to the development of chronic rhinosinusitis with nasal polyposis in Chinese population[29]. In contrast, the MMP2 gene does not play a crucial role in conferring risk for nasal polyps in a Taiwanese population[30].

MMPs could also be an interesting therapeutical target in chronic rhinosinusitis. Tetracycline-derivatives such as doxycycline are MMP inhibitors, which at regular or sub-antimicrobial dose exert systemic anti-inflammatory effects. In a double blind randomized placebo controlled trial, doxycycline has been shown to significantly reduce the levels of MMP-9 in nasal secretions, reducing the damage to nasal polyp tissue and eventually polyp size[31]. Methylprednisolone treatment did not change MMP-9 levels in nasal secretions. It was found that the effect of doxycycline on reduction of nasal polyp size was longer lasting (12 weeks) when compared to methylprednisolone (8 weeks)[31].

Of note, in asthma macrolide antibiotics have also shown an inhibitory function on MMPs[32].

MMP-9 expression in the extracellular matrix is increased during wound healing after sinus surgery. As inflammatory cells are the major source of MMP-9 expression, high secretion levels of MMP-9 after sinus surgery are linked to poor healing quality [33]. The frontal recess is especially vulnerable to restenosis, and frontal sinus stents have been used to overcome this problem. The use of doxycycline releasing stents have been studied in post-operative wound healing after sinus surgery. Doxycycline releasing stents significantly lowered MMP-9 concentrations and bacterial colonization locally, and improved postoperative healing quality after functional endoscopic sinus surgery[34].

2.2.3 Other factors involved in remodeling of chronic rhinosinusitis

Erbek et al. studied the expression of a disintegrin and metalloproteinase 33 (ADAM-33) protein in CRSwNP by immunehistochemistry. ADAM-33 is a member of the matrix metalloproteinases, and has a role in the angiogenesis and airway remodeling in asthma. In CRSwNP, it was found that the number of ADAM-33 positive cells was significantly higher in epithelial cells and in the mesenchymal cells of the vessels[35], pointing towards a similar role for ADAM-33 in upper airway remodeling.

In patients with CRS with asthma and CRS without asthma the role of platelet-derived growth factor (PDGF) was studied[36]. The study indicates that PDGF is produced by macrophages, eosinophils and epithelial cells in rhinosinusitis and that it acts on receptors in epithelial cells and fibroblasts. In the pathogenesis of rhinosinusitis PDGF may play a role in promoting tissue fibrosis and formation of nasal polyps. The role of the complement system

in CRSwNP has been studied, demonstrating significantly higher concentrations of the complement factors C3a desArg and C5a desArg in nasal secretions from CRSwNP patients when compared to controls. C3a and C5a cause an increased vascular permeability leading to plasma exudation and albumin accumulation as a consequence[37].

Very recent work by Sejima et al.[38] points towards the role of fibrinolytic components in tissue remodeling in chronic rhinosinusitis. Fibrinolytic components induce extracellular matrix (ECM) degradation and break-down. Plasmin degrades fibrin and converts inactive pro-matrix metalloproteinases into active MMPs. These activities are counteracted by plasminogen activator inhibitor-1 (PAI-1). TGF-beta1 is known to activate PAI-1. Plasminogen activators play an important role in the fibrinolytic system, as these proteins convert the proenzyme plasminogen into the active enzyme plasmin. Especially, urokinase plasminogen activator (uPA) binds to a specific uPA receptor (uPAR) and possesses proteolytic activity including tissue remodeling. The PAI-1/uPAR ratio of CRSwNP was significantly lower when compared to CRSsNP or controls, suggesting that the activity of uPA may be dominant in CRSwNP compared with the other groups. uPA convert proMMPs to active MMPs via plasmin, and uPA itself also activates MMPs. In CRSsNP, TIMP-1 is upregulated together with MMP-9, and high level of TGF-beta1 and low activity of uPA were observed, so that fibrosis is considered to proceed in the extracellular matrix. In contrast, in CRSwNP, TIMP-1 is not up-regulated, and high level of MMP-7/-9, high activity of u-PA, and low level of TGF-beta1 are observed, so that fibrinolysis is considered to proceed in the extracellular matrix.

3. Conclusion

Remodeling is a key feature of chronic rhinosinusitis, and distinct remodeling features clearly differentiate subgroups of chronic rhinosinusitis.

Of interest, when comparing Caucasian and Asian polyps, remodeling patterns are more consistent than the inflammatory pattern. One of the critical factors involved in remodeling of upper airway disease is TGF-beta, acting as a master switch for the development of either chronic rhinosinusitis with or without polyp formation. TGF-beta impacts fibrosis formation

through collagen production and the influence on the balance between MMPs and TIMP. Additionally, TGF-beta impacts the differentiation of T cells towards Tregs, allowing the different inflammatory patterns to establish.

The importance of studying the mediators and cytokines lies in choosing the best therapeutic target.

Doxycyclin has been shown to significantly reduce the levels of MMP-9 in nasal secretions, and to provide a more sustained effect on reduction of nasal polyp volume when compared to methylprednisolone. In contrast, remodeling appears to be corticosteroid resistant.

Clarithromycin is capable of inhibiting pro-inflammatory cytokines in vitro, and reductions of TGF-beta and MMP-9 concentrations may represent additional mechanisms by which macrolides reduce inflammation in chronic airway disease, but failed to show effects in humans.

Further studies are needed to unravel the complicated pathway of tissue remodeling in chronic rhinosinusitis.

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Chapter III

Aims of the studies

Aims of the study

The aim of this thesis was to analyze the role of TGF-beta in inflammation and the relation with remodeling processes in chronic rhinosinusitis with or without nasal polyps, and the potential of TGF-beta as a new target for treatment.

Specific aims of the thesis were:

Chapter 4: T-cell regulation in chronic paranasal sinus disease

To analyze the role of TGF-beta in the T-cell mediated immune response, more specifically the direct tissue expression of transcription factors for T-cell subpopulations (including Th1, Th2, Th 17 and T regulatory cells), in relation to the cytokine expression patterns in the different disease subgroups.

Chapter 5: TGF-beta signaling and collagen deposition in chronic rhinosinusitis

The objective was to analyze the presence of TGF-beta isoforms, receptors and intracellular SMAD signaling, in relation to tissue remodeling in chronic rhinosinusitis.

Chapter 6: Inflammation and remodeling patterns in early-stage chronic rhinosinusitis

To analyze pro-inflammatory cytokines and remodeling factors in early-stage chronic rhinosinusitis at different anatomical locations within the nose and sinuses.

Chapter 7: Mepolizumab, a humanised anti-IL-5 monoclonal antibody, as treatment option for severe nasal polyposis.

To investigate the therapeutic potential of inhibiting IL-5 using a humanized monoclonal antibody as treatment of severe nasal polyposis.

Chapter IV

T cell regulation in chronic sinus disease

T cell regulation in chronic paranasal sinus disease

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ABSTRACT

Background

Chronic rhinosinusitis is an inflammatory disease with distinct cytokine and remodeling patterns. Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a Th2 skewed eosinophilic inflammation, whereas chronic rhinosinusitis without nasal polyps (CRSsNP) represents a predominant Th1 milieu.

Objective

We aimed to study the direct tissue expression of transcription factors for T-cell subpopulations, including T regulatory cells, in relation to the cytokine expression patterns in the different disease subgroups.

Methods

The expression of FOXP3, T-bet, GATA-3, RORc, the suppressive cytokines TGF-β1, IL-10 and Th1/ Th2/ Th17 cytokines (IFN-γ , IL-4, IL-5, IL-13, IL17) were analyzed by means of RT-PCR in 13 CRSsNP, 16 CRSwNP and 10 control samples. Additional protein measurements were performed for TGF-β1 and IFN-γ.

Results

In CRSwNP, we observed a significantly lower FOXP3 mRNA and TGF-β1 protein expression, but a significantly higher T-bet, GATA-3, IL-5 and IL-13 mRNA expression compared to controls, whereas RORc was not significantly different compared to controls. In CRSsNP, FOXP3, T-bet, GATA-3 and RORc expression was not significantly different from controls,

whereas TGF-β1 mRNA, IFN-gamma mRNA and protein were significantly higher in CRSsNP compared to controls. For Il-17, no significant differences were noted between all groups.

Conclusion

We demonstrate for the first time a decreased FOXP3 expression, accompanied by an up-regulation of T-bet and GATA-3 and a down-regulation of TGF-β1 in CRSwNP versus control and CRSsNP.

Clinical implications

This study reveals a new understanding in chronic sinus disease, pointing towards a deficient T regulatory cell function in CRSwNP, but not in CRSsNP.

Capsule summary

Chronic rhinosinusitis with nasal polyps is a difficult to treat and often relapsing disease. Here we report an impaired FOXP3 expression as a likely mechanism in the pathogenesis of nasal polyp disease.

INTRODUCTION

Chronic rhinosinusitis without nasal polyps (CRSsNP) and with nasal polyps (CRSwNP) are chronic sinus diseases, both characterized by persistent inflammation of the nasal and paranasal mucosa. Recent research has demonstrated that these pathologies can be differentiated into distinct subgroups, based on the expression of inflammatory and remodeling mediators [1,2]. CRSsNP is characterized by a predominant Th1 milieu with high IFN-γ and TGF-β1 concentrations [1], whereas CRSwNP typically show a Th2 skewed eosinophilic inflammation with high levels of IL-5, IgE and low TGF-β1 [3,4]. However, little is known regarding the intracellular mechanisms behind this initial T cell polarization. Naive T-cells differentiate towards different T cell subtypes based on the expression of certain transcription factors. T-bet (T-box transcription factor) involves commitment towards Th1 cells [5]; GATA-3 (gata binding protein 3) is critical for commitment towards Th2 cells, and controls the expression of interleukin IL-4 and IL-5 [6,7]. Moreover, the balance between Th1 and Th2 is controlled by an intriguing subset of T cells, called T regulatory cells (T reg cells) [8]. A number of recent studies indicate that T reg cells play an important role in diseases characterized by Th2 biased immune responses such as asthma and atopic dermatitis [9-11]. Up to now, no data are available regarding expression and regulation in CRSsNP, in particular CRSwNP. It was tempting to speculate that in CRSwNP, characterized by a massive Th2 driven eosinophilic inflammation, a de-regulated T regulatory function might be involved. Knowledge on T reg cells in human disease is scarce so far, and studies mostly are based on *in vitro* experiments using peripheral blood derived T reg cells.

In this study we analyzed T cell transcription factors and downstream events at the level of the sinonasal mucosa in controls and chronic sinus disease.

Two main populations of T regulatory cells have been defined. One comprises the naturally occurring T reg cells (nT reg), characterized by the CD4(+)CD25(+)Foxp3(+) phenotype. They functionally mature in the thymus, and their development is controlled by the transcription factor FOXP3. It is hypothesized that nT reg cells can migrate to sites of inflammation at mucosal surfaces and inhibit Th2 and Th1 cells via cell-cell contact [11]. Another group of T reg cells comprises the “induced” T reg cells (i T reg), generated from naïve T-cells in the periphery. The most important subsets of iT reg cells are Tr1 and Th3 cells. They suppress immune function by secretion of predominantly IL-10 and TGF-β1, respectively [12]. It is probable that both natural and inducible populations have complementary and overlapping

functions. Although FOXP3 was initially thought to be a specific marker for nT reg cells and could not be activated in peripheral T-cells [13-15], recent studies demonstrated the induction of FOXP3 in induced T reg cells [16-18], positioning this transcription factor as a marker for both, nT reg cells and iT reg cells. As indirect markers for Tr1 and Th3 activity, we measured IL-10 and TGF-β1 mRNA expression.

In this study we aimed to investigate the expression of key transcription factors for T regulatory and Th1/ Th2/ Th17 cells, in relation to the mRNA and protein expression of representative cytokines, in a Th2- and Th1-biased sinus disease.

MATERIAL AND METHODS

Patients

Sinonasal mucosa from 13 patients suffering from CRSsNP, 16 patients suffering from CRSwNP, and 10 control patients was obtained at the department of Otorhinolaryngology of the Ghent University Hospital, Belgium. Inferior turbinates from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls. For CRSsNP, tissue samples originated from ethmoidal mucosa. For CRSwNP samples of ethmoidal polyp tissue were used.

None of the control and CRSsNP patients had a history of asthma or a positive skin prick test to common inhalant allergens. In the CRSwNP group, five of the sixteen patients had asthma in history, two of these patients were skin prick test positive, with one patient reporting aspirin intolerance. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy and computed tomography (CT) of the paranasal cavities according to the current European EP³OS [19] and American [20] guidelines. General exclusion criteria were based on the EP³OS definition for research (cystic fibrosis, gross immunodeficiency, congenital mucociliary problems, non-invasive fungal balls and invasive fungal disease, systemic vasculitis and granulomatous diseases). Patients with non-allergic rhinitis with and without eosinophilia and vasomotor rhinitis were also excluded. All patients stopped oral and topical application of corticosteroids for at least one month before surgery. Patients did not take any other relevant medication. Patients who underwent prior sinus surgery were excluded. The study was approved by the local Ethical committee of the University Hospital Ghent, Belgium. An informed consent was obtained from each patient and control subject before collecting material.

Gene expression analysis - Quantitative real time PCR

cDNA was synthesized from 2 µg of RNA with the iScript cDNA synthesis kit (BioRad Laboratories, CA, USA) following the manufacturer's instructions. Levels of the transcription factors FOXP3, GATA-3, T-bet, RORc and cytokines IL-4, IL-5, IL-10, IL-13, IL-17, TGF-β1 and IFN-γ were determined by real time PCR. Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad laboratories, CA, USA) using specific primer sequences (see online repository, Table 1). PCR reactions contained 30 ng cDNA (total RNA equivalent), 250 nM of primer pairs , 1X SYBR Green I Master mix (Bio-Rad laboratories, CA, USA) or 1X TaqMan mix with 100 nM of the TaqMan probe in a final volume of 20 µl. PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute and for reactions using TaqMan probes of 1,5 minutes at 95 °C followed by 50 cycles: 15 seconds at 95 °C and 1 minute at 60 °C.

The expression of three housekeeping genes Beta actin (ACTB), Hydroxymethyl-bilane synthase (HMBS) and EF1 was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software [21,22]. The relative expression units of each gene per 30 ng of cDNA sample, was determined by using the qBase program (version 1.3.5, UGent, Belgium) and results are expressed as the logarithm of normalized relative expression units / 30ng cDNA.

Table 1.

*Primer sequences used for real-time PCR amplifications. * Sequences were obtained from the Real-Time PCR primer and probe database (http://medgen.ugent.be/rtprimerdb/)*

*** Sequences were provided by the Swiss Institute of Allergy and Asthma Research (SIAF)*

	Forward (5'→ 3')	Reverse (5'→ 3')	Amplicom size (bp)	Accession number
TGF-beta1	CAGCAACAATTC-CTGGCGATA	AAGGCGAAAGCCCTCAATTT	135	NM_000660.3
FOXP3**	GAAACAGCACATTCCCAGAGTTC	ATGGCCCAGCGGATGAG	100	NM_014009
T-bet**	GATGCGCCAGGAAGTTTCAT	GCACAATCATCTGGGTCACATT	83	NM_013351
GATA-3**	GCGGGCTCTATCACAAAATGA	GCTCTCCTGGCTGCAGACAGC	79	NM_002051
EF-1	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT	59	NM_001402
IFN-γ	ACTGACTTGAATGTCCAACGCA	ATCTGACTCCTTTTTCGCTTCC	101	NM_000619
ACTB *	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	139	NM_001101
HMBS *	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	154	NM_00319

TGF-β 1/ IFN-γ Elisa

Tissue homogenates were assayed for total TGF-β1 and IFN-γ using commercially available ELISA kits from R&D Systems (Minneapolis, USA). All data were expressed as ng/ml. For TGF-β, acid was added during ELISA procedure, resulting in physicochemical activation of latent TGF-β. Total TGF-β concentrations are reported including both active and latent forms.

Immunohistochemistry

CD3 staining

Sections were immunohistochemically stained with the mouse monoclonal antibody CD3 (clone UCHT1, Dako, Glostrup; Denmark). For immunohistochemical stainings specimens were fixed in acetone and incubated with primary antibody or isotype control for 1 hour and detected using the LSAB+ kit (Dako).

The number of positive cells was analyzed using a magnification of 400x and scored by two independent observers who did not know the diagnosis and clinical data. A grading scale from 0 to 3 was applied, ranging from absent to numerous stained cells. Score 0 represents no positive cells, score 1 <10 positive cells/field, score 2: 10-100 positive cells/field and score

3: >100 positive cells/field. All areas of the section were analyzed and for each sample 10 high power fields were scored.

FOXP3 staining

Tissue frozen sections were permeabilized with FOXP3 Fix/Perm solution (320501, BioLegend) and blocked with 10% normal goat serum (X0907, Dako Cytomation, Glostrup, DK) and incubated with primary polyclonal rabbit anti-human FOXP3 Ab (ab10563, Abcam, Cambridge, UK) overnigth at 4^{o}C. Slides were incubated with peroxidase labelled polymer followed by 3-amino-9-ethyl carbazole (DAB). Sections were counterstained with hematoxylin (Sigma, St. Louis, MO) and permanently mounted with Ultramount (S1964, Dako Cytomation, Glostrup, DK). FOXP3 blocking peptide (ab14151, Abcam, Cambridge, UK) was used as a control to block anti-FOXP3 binding. Human tonsil sections were used as a positive control on each staining run. Counting of 10 random high power fields was performed by two independent observers. For more information on material and methods, please visit the on-line repository.

Statistical analysis

Statistical analysis was performed with MEDCALC software v 9.2.0.1 (F. Schoonjans, Belgium). Data are expressed in Box-and-Whisker plots. When comparisons were made between groups, the Kruskall-Wallis test was used to assess significant inter-group variability. The Mann-Whitney *U* two tailed test was used for between-group comparison. The significance level was set at $\alpha = 0.05$.

RESULTS

Immunohistochemistry for FOXP3 and CD3

Immunohistochemical staining was used to determine the presence of FOXP3 expressing cells in healthy and diseased sinonasal mucosal tissue. Representative sections of control, CRSsNP and CRSwNP samples stained for FOXP3 are shown in figure 1 (n=6 per group). Tonsil sections were used as positive control. FOXP3 expressing cells were detectable in both healthy nasal mucosa and CRSsNP, but not in CRSwNP tissue (Figure 1). The median (IQR) counts for FOXP3 positive cells were significantly lower in CRSwNP (0; 0-4) compared to controls (39; 21-41; P<0.0001) and CRSsNP (52; 37-85; P<0.0001). Additionally, in order to estimate the total number of T cells present in tissues, we quantified the number of CD3 positive cells present in the tissues. The median (IQR) of CD 3 positive cell number (sum of ten high power fields) was similar in CRSsNP (22; 20-26) and CRSwNP (23; 19-30), but significantly higher compared to controls (20; 18-21; P=0.01 and 0.001, respectively).

Tissue expression of T reg and Th1/ Th2/ Th17 transcription factors

The expression levels of transcription factors for T reg (FOXP3) and Th1 (T-bet)/ Th2 (GATA-3)/ Th17 (RORc) populations were directly analyzed on tissue biopsies by means of quantitative real time-PCR. This revealed a significantly lower FOXP3 mRNA expression level in nasal polyps (CRSwNP) compared to controls (P=0.026), whereas the expression of T-bet (P=0.005) and GATA-3 (P=0.035) was significantly higher in CRSwNP compared to controls(Figure 2). RORc was not significantly different between the latter two groups, but was significantly lower in CRSwNP compared to CRSsNP (P < 0.001). In contrast, in chronic rhinosinusitis without polyps (CRSsNP), FOXP3 mRNA expression was higher compared to controls, and significantly higher compared to CRSwNP (P=0.005). No significant differences were noted for T-bet, GATA-3 and RORc compared to controls (median values and interquartile ranges for all transcription factors are specified in the online repository, Table 2).

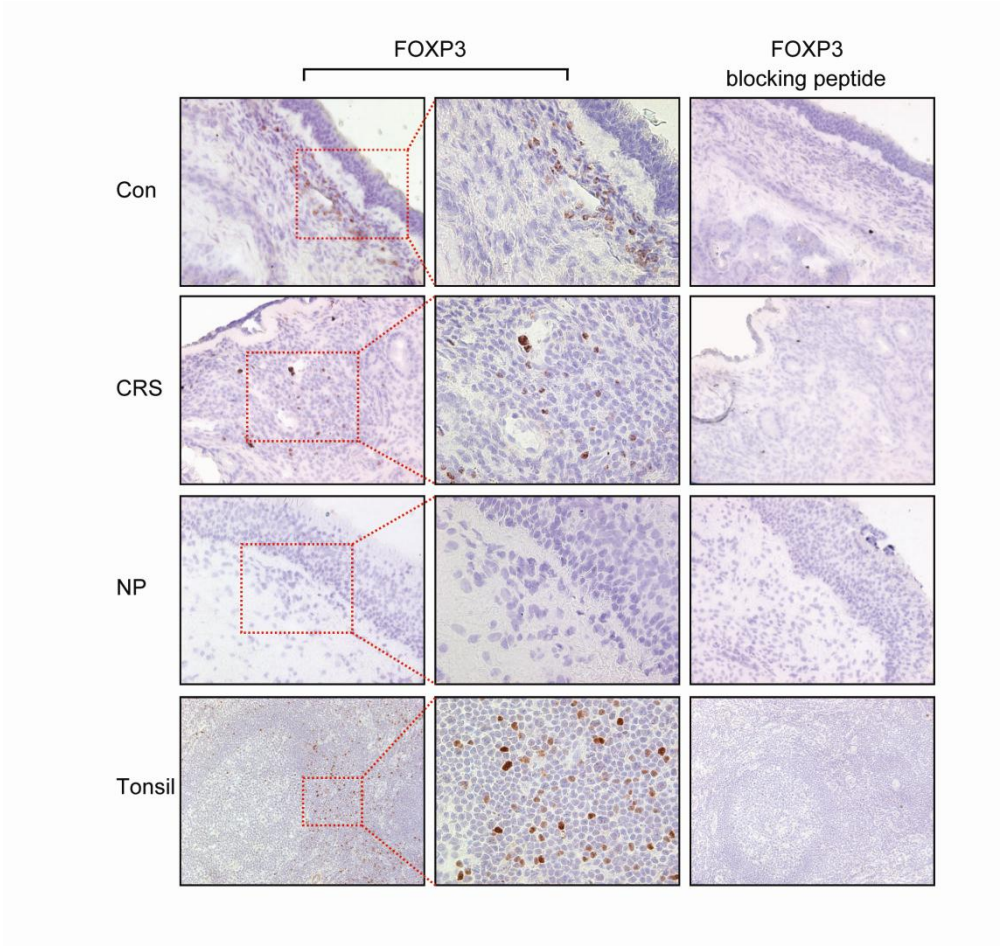

Figure 1

Expression of FOXP3+ cells in nasal mucosa from healthy individuals (controls, CON) and subjects with chronic rhinosinusitis without nasal polyps (CRSsNP) or with nasal polyps (CRSwNP). Lack of staining is observed by blocking with FOXP3 immunizing peptide. Human tonsil sections served as a positive control with FOXP3+ cells located in the parafollicular cortex (T cell zone). Original magnification was x200 or x400. For human tonsil magnification was x100 or x400. Representative slides are shown (n=6 per group).

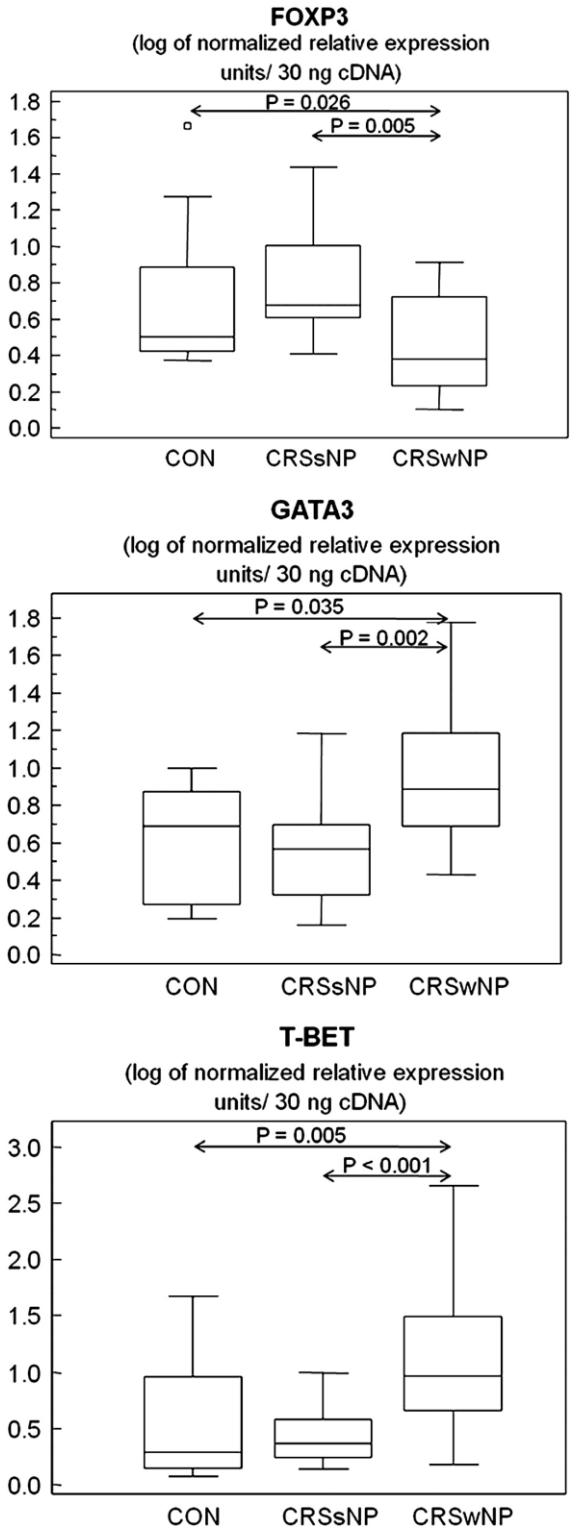

Figure 2

mRNA expression of transcription factors (FOXP3, T-BET, GATA3) involved in naïve T-cell differentiation in controls (CON), nasal polyps (CRSwNP) and chronic rhinosinusitis (CRSsNP) expressed as logarithm of normalized relative expression units/ 30ng cDNA.

Th1/ Th2/ Th17 and iT reg related cytokine expression

Differences in Th1/ Th2/ Th17 cytokine pattern, and iT reg related TGF-β1/ IL-10 were assessed at the mRNA level between CRSsNP, CRSwNP and controls (figure 3 A and B). Because of the relevance of post-translational regulation mechanisms, additional protein measurements were performed for TGF-β1 and IFN-γ (figure 3 B).

In CRSwNP, we found a significantly higher mRNA expression of the Th2 cytokine IL-5 compared to controls (P<0.001) and to CRSsNP (P<0.001), and a significantly higher mRNA expression of IL-13 in CRSwNP compared to controls (P=0.029). For IL-4, no significant differences were noted between controls, CRSsNP and CRSwNP (Figure 3A). In contrast, both mRNA and protein levels of the Th1 cytokine IFN-γ were significantly higher in CRSsNP compared to controls (P=0.009 for mRNA, P=0.001 for protein) and to CRSwNP (P=0.001 for mRNA, P=0.01 for protein). mRNA expression of TGF-β1 was significantly higher in CRSsNP compared to controls (P=0.033). At protein level, TGF-β1 showed higher concentrations in CRSsNP compared to controls and to CRSwNP, reaching statistical significance for the latter (P<0.001). Furthermore, TGF-β1 protein levels were significantly lower in CRSwNP compared to controls (P=0.015), although this difference was not observed at mRNA level (Figure 3B). For IL-10 mRNA expression, no significant differences were noted between groups (Figure 3A).

The mRNA expression of IL-17 was not significantly different between all groups (see table 2 in the online repository).

Within the CRSwNP group, patients with and without comorbid asthma were compared for the expression of FOXP3, T-bet, GATA3, RORc and IL-4, IL-5, IL-13, IL-10, IL-17, IFN-gamma and TGF-β1. This revealed no significant differences between both nasal polyp subgroups.

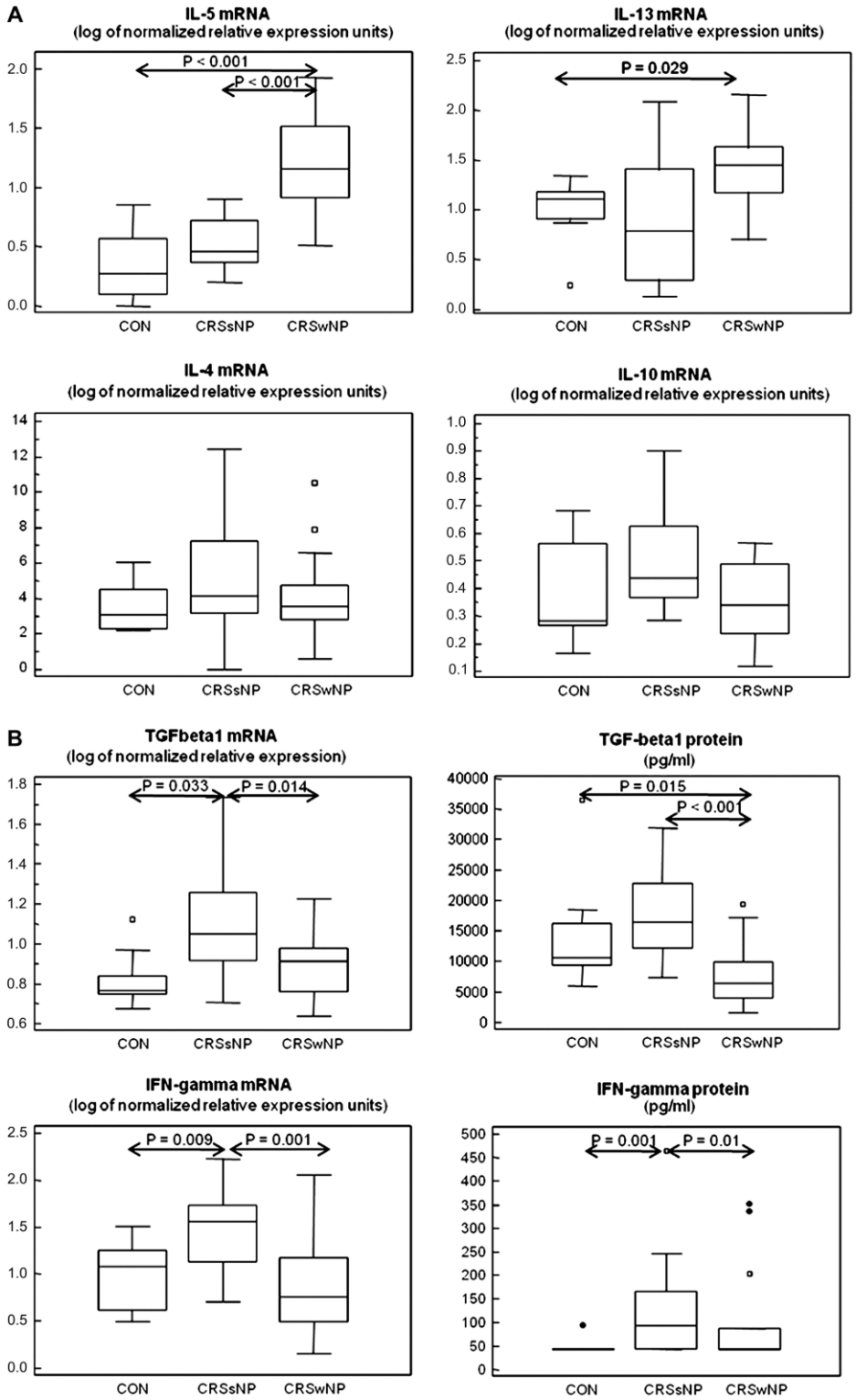

Figure 3 A and B

mRNA expression of key cytokines IFN-γ (Th1) and IL-4, IL-5, IL-13 (Th2), and iT reg related TGF-β1 and IL-10 in controls (CON), chronic rhinosinusitis (CRSsNP), and nasal polyps (CRSwNP), expressed as logarithm of normalized relative expression units/ 30 ng cDNA. For TGF-β1 and IFN-γ, additional protein measurements were performed, expressed as pg/ml.

DISCUSSION

In this study we analyzed at sinonasal mucosal tissue level, the expression of the transcription factors FOXP3, T-bet, GATA-3 and RORc in relation to Th1/ Th2/ Th17 cytokines and suppressive iT reg related cytokines.

We here demonstrate for the first time a significantly lower expression of FOXP3 in CRSwNP compared to controls and to CRSsNP. The reduced expression of FOXP3 in CRSwNP at mRNA level was consistent with the immunohistochemical findings. FOXP3 positive cells were almost undetectable in CRSwNP tissue sections, and appear to be focally present.

We used FOXP3 as the most widely accepted marker for T regulatory cells which, although still controversial, also has been associated with suppressive function. It was demonstrated in a mouse model of colitis that retroviral transfer of FOXP3 converts naïve T-cells into functional CD25(+) Treg cells [23]. Importantly, mutations in FOXP3 result in the absence or dysfunction of T reg cells and lead to the human IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and its murine homolog, the scurfy mouse [24,25]. Moreover, the role of T regulatory cells in controlling allergic diseases became apparent. In allergic rhinitis a reduced number of FOXP3 positive cells was reported compared to controls [26]. T regulatory cells play an important role in controlling Th2 immune responses, and an impaired expansion of natural and/ or inducible T reg cells has been suspected to result in the development of allergy and asthma [11]. In similarity with asthma, CRSwNP are typically associated with a Th2 driven eosinophilic inflammation, characterized by high concentrations of ECP, eotaxin and IL-5, but not correlated to atopy [4]. We suggest that a defective suppressive function of T regulatory cells in CRSwNP, indicated here by the low FOXP3 expression, might account for the often severe persistent eosinophilic inflammation. As T regulatory cells are assumed to control the pathogenic Th1 and Th2 cells, the reduced expression of FOXP3 in CRSwNP together with the up-regulation of T-bet and GATA-3 suggests either a deficiency or a dysfunction of T regulation in nasal polyp disease. However, this statement can only be of general nature at this moment. The exact functional implications of these findings need further investigation.

In contrast, in CRSsNP, adequate expression of FOXP3 and unchanged expression of the transcription factors for Th1 and Th2 lymphocyte populations compared to control mucosa suggests active T regulatory function. Since corticosteroids are known to up-regulate FOXP3

and T regulatory cells in asthma, only samples of patients who ceased oral and topical steroids for at least one month prior to surgery were selected.

GATA-3 is both necessary and sufficient for commitment towards Th2 cells and controls the expression of IL-5[6,7]. Moreover, GATA-3 also has the capacity to directly inhibit IFN-γ promoter activity, resulting in repression of Th1 and development of a Th2 phenotype [27-29]. The up-regulation of GATA-3 in CRSwNP was reflected by the subsequent increase of the IL-5 mRNA signal. These findings at mRNA level confirm previous protein data [30]. In CRSsNP, IL-5 mRNA was not up-regulated compared to controls, in line with the unchanged expression of GATA-3. Additionally, Th2 cytokines IL-4 and IL-13 were analyzed. IL-4 mRNA was not found significantly different between CRSsNP, CRSwNP and controls, confirming previous data [31]. However, IL-13 was significantly increased in CRSwNP compared to controls, which under certain circumstances has been described to replace IL-4 [32]. It should be noted that other cell types such as eosinophils and mast cells may contribute to GATA-3 expression [33]. IL-4, IL-5 and IL-13 levels can also variably be produced by eosinophils and mast cells. Although the coincidence of low FOXP3 levels and the upregulation of the GATA-3, together with the IL-5 and IL-13 signal suggest a functional relation, the contribution of individual cell types to GATA-3 and cytokine expression could not be quantified at whole tissue level.

T-bet is a Th1 specific T box transcription factor that controls the expression of the hallmark Th1-cytokine IFN-γ, and T-bet expression correlates with IFN-γ expression in Th1 cells [5]. The up-regulation of T-bet in CRSwNP was not reflected by a significant up-regulation of IFN-γ at mRNA expression and protein level in CRSwNP, confirming previous protein data[1]. Differently to T-bet which expression is mainly restricted to Th1 cell type, IFN-γ can be produced by several cell types including CD4+-Th1 cells, CD8, NK cells but also B-cells. Additionally, IFN- γ expression can also be influenced by exogenous and endogenous factors (IL-12/IL-12R and IL-18) that can act together or independently of the T-bet signaling pathway. Accordingly, the expression of T-bet was not expected to directly correlate with IFN- γ expression.

As a general remark we wish to mention that inferior turbinates from healthy persons were used as control tissue, since it is unethical to resect ethmoidal tissue from healthy persons. It should however be noted that the differences observed between controls and CRS with or without NP might be influenced by comparing these different tissue localizations. However, clear differences are observed between ethmoidal tissue from CRSsNP and CRSwNP.

As marker for Th17 cells, we analyzed RORc, a transcription factor that controls the differentiation towards pro-inflammatory Th17 cells and regulates IL-17 production [34,35]. The expression of RORc was not significantly different in diseased tissue compared to controls, although there was a significantly lower expression of RORc in CRSwNP compared to CRSsNP. For IL-17 no significant differences were found at mRNA level between CRSwNP, CRSsNP and controls.

In CRSsNP, IFN-γ mRNA expression was significantly up-regulated compared to controls. At protein level, this was also true when compared to CRSwNP, confirming the previously described Th1 polarized inflammation. However, the transcription signal of T-bet, critical for commitment towards the Th1 phenotype was not found up-regulated in CRSsNP.

As markers for induced T regulatory activity of Th3 cells, we analyzed the expression of the suppressive cytokine TGF-β1. Th3 cells are known to exert their immune-suppressive effect via the production of this growth factor. However, numerous other cell types can express TGF-β, such as macrophages, mast cells, neutrophils, eosinophils, endothelial and epithelial cells and fibroblasts. Furthermore, TGF-β1 induces FOXP3 expression in CD25(-) naïve T cells to enforce transition to T regulatory cells and is a critical factor in the development of peripheral T regulatory cells[36]. There was no significant difference in TGF-β1 mRNA expression in CRSwNP tissue vs. controls. However, regulation of TGF-β1 mainly occurs at the post-transcriptional level. TGF-β1 is secreted from cells as small latent complexes, preventing binding of TGF-β1 to ubiquitously expressed receptors, assuring an extracellular reservoir of TGF-β that can be activated on demand[37]. Therefore we performed additional protein quantification by means of ELISA, measuring both active and latent forms of TGF-β1. Latent forms were released from extracellular matrix by adding acid. This confirmed previously published findings of our group [4] and others [38,39] of a down-regulation of TGF-β1 protein in polyp tissue. Although the coincidence of low levels of TGF-β1 protein and the down-regulation of FOXP3 may imply a functional link, the impact of local TGF-β1 on T regulatory function has to be confirmed in CRSwNP.

In contrast, we found a significant up-regulation of TGF-β1 at mRNA level in CRSsNP, just not reaching significance at protein level, compared to controls. Furthermore, normal TGF- β1 protein levels coincided with adequate FOXP3 expression in CRSsNP tissue.

IL-10, a cytokine that is deeply involved in the regulation of inflammatory and immune responses, was measured at mRNA level. The induction of peripheral regulatory T cells by IL-

10 points towards a crucial role in the establishment of peripheral tolerance[40]. Specifically, IL-10 is instrumental in Tr1 mediated suppression of proliferation and cytokine production of naïve CD4(+)CD25(−) T, Th1 and Th2 cells. Activation of T cells in the presence of IL-10 induces a long lasting state of non responsiveness or anergy[41]. Here, no significant differences in IL-10 mRNA expression were found between controls, CRSsNP and CRSwNP.

TGF-β and IL-10 are both suppressor cytokines that frequently occur together at sites of inflammation, and both cytokines cooperate in the resolution of inflammation. TGF-β can induce IL-10, and IL-10 facilitates TGF-β regulatory activity[42]. The striking coincidence of low TGF-β1 protein levels, together with basal levels of IL10 mRNA (not higher compared to controls) in CRSwNP, points to a lack of regulatory effect in the resolution of inflammation, hence contributing to the chronicity of this disease.

CONCLUSION

In this study, we demonstrate a decreased expression of FOXP3 in nasal polyp tissue, reflecting a deficiency or a dysfunction of T regulatory cells in an often persistent, severely inflamed sinus disease, CRSwNP. In line with the low FOXP3 expression in CRSwNP, we describe low levels of TGF-β1 protein expression and an up-regulation of the transcription signals for Th1 (Tbet) and Th2 (GATA-3) subpopulations, pointing towards a defective suppression of their up-regulation by T reg cells. We suggest that this lack in T regulatory cell function may contribute to the severe persistent Th2-skewed airway inflammation often observed in CRSwNP patients. In contrast, up-regulated TGF-β1 protein levels compared to CRSwNP coincide with an adequate expression of FOXP3 and maintained control over T-bet and GATA-3 expression in CRSsNP, suggesting adequate T regulatory cell function in this sinus disease subgroup.

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Chapter V

TGF-beta in chronic sinus disease

TGF-beta signaling and collagen deposition in chronic rhinosinusitis

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ABSTRACT

Background: Chronic rhinosinusitis is an inflammatory disease with distinct cytokine and remodeling patterns.

Objective: The objective was to analyze the presence of TGF-beta isoforms, receptors, intracellular signaling and collagen deposition in chronic rhinosinusitis.

Methods: Sinonasal mucosal samples obtained from CRSwNP (n=13), CRSsNP (n=13) and controls (n=10) were analyzed for TGF-beta isoforms 1 and 2 by means of ELISA and IHC, and for TGF-beta receptor 1, 2 and 3 by RT-PCR and IHC. As downstream proteins phospho-Smad 2 (pSmad 2) and collagen were analyzed by performing immunostaining and picrosirius red staining, respectively.

Results: TGF-beta 1 and 2 protein concentrations, TGF-beta RI and TGF-beta RIII mRNA expression, the number of pSmad 2 positive cells and total collagen amount were significantly higher in CRSsNP versus controls. In CRSwNP, TGF-beta 1 protein concentration, TGF-beta RII and TGF-beta RIII mRNA expression, the number of pSmad 2 positive cells and total collagen amount were significantly lower versus controls. Only TGF-beta 2 protein was found higher in CRSwNP versus controls.

Conclusions: A high TGF-beta 1 protein expression, increased TGF-beta RI expression, and a high number of phospho-smad 2 positive cells all indicate an enhanced TGF-beta signaling in CRSsNP, whereas a low TGF-beta 1 protein concentration, a decreased expression of TGF-beta RII and a low number of phospho-smad 2 positive cells in CRSwNP indicate a low level

of TGF-beta signaling in CRSwNP. These findings are compatible with the remodeling patterns observed, reflected by a lack of collagen in CRSwNP, and excessive collagen production with thickening of the collagen fibres in the extracellular matrix in CRSsNP.

Clinical implications

A better understanding of the roles of TGF-beta isoforms and receptors in health and disease can provide more specific targets for therapeutic intervention.

Capsule summary

Chronic rhinosinusitis without nasal polyps and chronic rhinosinusitis with nasal polyps represent distinct diseases with clear differences in TGF-beta signalling pathway. These differences are compatible with the remodeling patterns observed in these disease subgroups.

Key words

Chronic rhinosinusitis, collagen, inflammation, nasal polyposis, phospho-Smad 2, remodeling, TGF-beta

INTRODUCTION

Transforming growth factor (TGF-) beta is a pleiotropic and multifunctional growth factor, with important immunomodulatory and fibrogenic characteristics. Many chronic diseases profit from the immunosuppressive effect of TGF-beta, however, this molecule has also been implicated in fibrosis formation and is suspected to play a major role in airway remodeling. In general, the function of TGF-beta could be understood as a counter regulatory cytokine to resolve inflammation and to initiate the repair process.

Chronic sinus disease is characterized by chronic inflammation of the nasal and paranasal mucosa, accompanied by tissue remodeling that includes changes in the ECM protein deposition and tissue structure[1]. Distinct disease entities can be distinguished within the group of chronic sinus diseases, based on different inflammation and remodeling patterns. Chronic rhinosinusitis without nasal polyps (CRSsNP) is characterized by high levels of IFN-gamma and TGF-beta 1[1,2]. In contrast, chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a predominant Th2 biased eosinophilic inflammation with high levels of IL-5, ECP and eotaxin, high levels of local IgE, but low levels of TGF-beta[1-3]. Nasal polyps typically consist of albumin accumulation and oedema formation within the extracellular matrix (pseudocyst formation)[4].

TGF-beta could play a crucial role in both suppression of airway inflammation and remodeling. TGF-beta either acts through direct suppression of the activation of T-cells and antibody secretion of B cells, or indirect through the induction of T-regulatory cell types [5]. Moreover, TGF-beta is considered as a master switch in the induction of the profibrotic program, and acts as chemoattractant and proliferation factor for fibroblasts[6]. It induces fibroblasts to synthesize ECM proteins and contract extracellular matrix. Three different isoforms (TGF beta 1, 2 and 3) have been described, which can bind to three membrane proteins, referred to as receptor type I, II, and III.

Until now the majority of investigations in upper airway disease have focused on the TGF-beta 1 isoform, although recent studies in lower airway disease suggest a distinctive role for TGF-beta 2, predominantly expressed in severe asthma and related to eosinophils[7]. Additionally, only few data are available on TGF-beta receptor expression and intracellular signaling in chronic sinus disease. TGF-beta receptor signaling is a very complex mechanism and is mediated by several signaling steps involving dimerization and posphorylation of receptor and intermediate molecules. One of the most important proteins that modulate

TGF-beta ligand activity are the Smad proteins. After activation of TGF-beta receptor I, phosphorylated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus. This process is inhibited by Smad 7. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates target gene expression[8].

The aim of this work was to study the expression of TGF-beta isoforms 1 and 2 and receptor expression in CRS with or without polyp formation, and to link this to the number of phosphorylated Smad 2 (pSmad 2) positive cells as downstream marker for active TGF-beta signaling. As outcome parameter for remodeling, picrosirius red stainings were performed to quantify collagen content, and viewed with polarized light to asses fiber thickness[9].

METHODS

Patients

Patients were selected at the department of Otorhinolaryngology of the Ghent University Hospital, Belgium. Inferior turbinate samples from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls (controls n=10, median age 27, range 18-45, 4F/6M). Samples from patients suffering from chronic rhinosinusitis (CRSsNP n=13, median age 42, range 34-78, 6F/7M) and nasal polyposis (CRSwNP n=13, median age 46, range 34-78, 5F/8M) were obtained during functional endoscopic sinus surgery (FESS) procedures. For CRSsNP and CRSwNP, tissue samples originated from the ethmoidal sinuses. All patients underwent a skin prick test to common inhalant allergens. None of the control and CRSsNP patients had a history of asthma or a positive skin prick test. In the CRSwNP group, four of the thirteen patients had a history of asthma, two of these patients were skin prick test positive, one patient had aspirin exacerbated respiratory disease.The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy and computed tomography (CT) of the paranasal cavities according to the current European EP³OS[10] and American[11] guidelines. General exclusion criteria were based on the EP³OS definition for research. Patients with non-allergic rhinitis with and without eosinophilia and vasomotor rhinitis were also excluded. All patients stopped oral corticosteroids for at least one month and topical application for at least two weeks before surgery. Patients did not take any other relevant medication. Subjects with concurrent asthma were maintained on no more than 1000 mcg/day beclomethason diproprionate or the equivalent. Patients who underwent prior sinus surgery were excluded. The study was approved by the local Ethical committee of the University Hospital Ghent, Belgium. An informed consent was obtained from each patient and control subject before collecting material.

ELISA for TGF-beta 1 and 2

Tissue homogenates were assayed for total TGF-beta 1 and 2 using commercially available ELISA kits from R&D Systems (Minneapolis, USA). Acid was added during ELISA procedure, resulting in physicochemical activation of latent TGF-β. Total TGF-β concentrations are reported including both active and latent forms. All data were expressed as ng/ml.

mRNA Gene expression analysis

Quantitative real time PCR

cDNA was synthesized from 2 µg of RNA with the iScript cDNA synthesis kit (BioRad Laboratories, CA, USA) following the manufacturer's instructions. mRNA levels of TGF-beta receptors I, II and III were determined by real time PCR. Amplification reactions were performed on an iQ5 Real-Time PCR Detection System (Bio-Rad laboratories, CA, USA) using specific primer sequences (see online repository, Table 1). PCR reactions contained 30 ng of cDNA (total RNA equivalent) of unknown samples, 1X SYBR Green I Master mix (Bio-Rad laboratories, CA, USA) and 250 nM of primer pairs in a final volume of 20 µl. PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute. The expression of three housekeeping genes Beta actin (ACTB), Hydroxymethyl-bilane synthase (HMBS) and Elongation Factor 1 (EF-1) was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software[12,13]. The relative expression units of each gene per 30 ng of cDNA sample, was determined by using the qBase program (version 1.3.5, Ghent University, Belgium).

Immunohistochemistry for TGF-beta 1, 2 and TGF-beta R I and II

Tissue was fixated in formalin (Fluka, Belgium) and embedded in paraffin. Paraffin sections were prepared (thickness 4-5 µm) and air dried for 24 hours at 37°C. After deparaffinization in parasolve and antigen retrieval by heating in citrate buffer (pH=6), endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxidase (VWR International, Belgium) in TBS (pH 7.8) containing 0.001 % NaN_3 (VWR International, Belgium) for 20 minutes at room temperature. The sections were then washed with TBS for 10 minutes, polyclonal anti-human antibodies TGF-beta 1 and 2 were added and incubated overnight at 4 °C. Polyclonal anti-human TGF-beta receptor 1 and 2 antibodies were incubated for 60 minutes at room temperature. All polyclonal anti-human TGF-beta antibodies (1, 2) and polyclonal anti-human TGF-beta receptor (1, 2) were purchased from R&D Systems (Minneapolis, USA) and diluted to 2 µg/l in TBS/0.5 % BSA. Negative controls consisted of an antibody of the similar isotype. Following the overnight incubation for TGF-beta 1 and 2, and the 60 minutes incubation for TGF-beta receptor 1 and 2, the slides were washed for 10 minutes in TBS.

A labelled polymer HRP (Dako EnvisionTM + System, Peroxidase (AEC) kit, Dako Denmark) was applied and incubated for 30 minutes at room temperature. After washing in TBS for 10 minutes staining was completed by a 10 minute incubation with AEC (amino-ethylcarbazole) substrate-chromogen which results in a red-colored precipitate. Finally, sections were counterstained with Heamatoxyline (VWR International, Belgium) for 2 minutes, washed extensively in running tapwater and mounted in Aquatex (VWR International, Belgium).

Immunohistochemistry for phospho-Smad 2

Paraffin sections were prepared as described before. Aspecific binding was blocked for 30 minutes with 2% of BSA/TBS. Slides were then incubated with polyclonal Phospho-Smad 2 (Ser 465/467) antibody (Cell Signaling Technology, Beverly, MA, USA) or control serum (rabbit serum (DAKO, Belgium), 1/100 in 2% BSA/TBS) for one hour. After washing in TBS for 10 minutes, a labelled polymer HRP (Dako Envision^{TM} + System, Peroxidase (AEC) kit, Dako Belgium) was applied and incubated for 30 minutes at room temperature. After washing in TBS for 10 minutes, the staining was completed by a 10 minute incubation with amino-ethylcarbazole (AEC) substrate-chromogen which results in a red-colored precipitate. Finally, sections were extensively washed in running tap water and mounted in Aquatex (VWR International, Belgium).

Image analysis

Positive intranuclear pSmad 2 staining was analyzed in 6 samples per group. Images from the entire tissue section were obtained with a 40X objective lens (final magnification 400X) and recorded on a digital camera (Olympus C-5050) with no overlapping zones. Positive intranuclear cell staining was quantified using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). Original images were converted to 8-bit grayscale. Using the threshold function, pSmad 2 positive areas were converted to saturated black areas and all other areas where then rendered white to result in a binary image. The threshold setting was manually validated comparing the binary image to the original image. Positive nuclei were scaled by the function “analyze particles” (with a minimum size of 50 to a maximum of 500 pixels), and a report was generated for each image presenting the count of pSmad 2 positive cells.

Picrosirius red stainings

Collagen was measured by means of picrosirius red staining, a technique to identify collagen superior to trichrome masson stainings[9]. Paraffin sections were prepared as described

before. Sections were deparaffinized, hydrated, and stained with picrosirius red (direct red 80, Sigma-Aldrich, St. Louis, USA) for 60 minutes. The sections were then washed in two changes of acidified water, dehydrated in three changes of 100% ethanol, and mounted in Tissue-Tek (Miles Inc, USA). The sections were analyzed using an Olympus microscope (CX-40) equipped with filters to provide circularly polarized illumination. The lower filter was placed above the microscope's field iris diaphragm ring, while the upper filter was placed below the linear polarizer aligned such that its transmission axis was at 45°. Tissue images viewed under bright-field and polarized light were obtained with a 40X objective lens (final magnification 400X) and recorded on a digital camera (Olympus C-5050).

Image analysis

Total collagen was quantified under bright-field microscopy. Image analysis was carried out with Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). Briefly, the entire section of a slide was captured by consecutive fields under bright-field at a final magnification of 400X, with no overlapping zones. The total collagen amount was calculated for each image after subtraction of background and conversion to 8-bit images. The total collagen content was calculated for each section expressed as percentage of the total area.

Statistical analysis

Statistical analysis was performed with MEDCALC software version 9.4.2.0 (F. Schoonjans, Belgium). Data are expressed as median and interquartile ranges. When comparisons were made between groups, significant between-group variability was first assessed using Kruskall-Wallis test. The Mann Whitney U-test two tailed was then used for between-group comparison. Exact P-values are reported. The significance level was set at $\alpha = 0.05$.

RESULTS

Elisa for TGF-beta 1 and 2

TGF-beta 1 protein concentration was significantly lower in CRSwNP (30767 pg/ml; IQR 18669-35096) when compared to controls (39814 pg/ml; IQR 36131-45750), but significantly higher in CRSsNP (50135 pg/ml; IQR 45397-69554) compared to controls and CRSwNP. TGF-beta 2 protein was significantly higher in both CRSwNP (3091pg/ml; IQR 2662-3845) and CRSsNP (3068 pg/ml; IQR 2825-4395) compared to controls (1852 pg/ml; IQR 1298-2663) (see Figure 1).

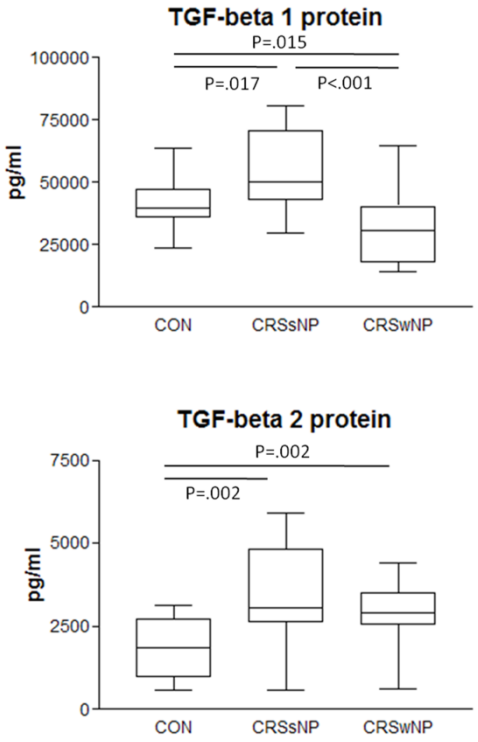

Figure 1. *ELISA measurements for TGF-beta 1 and 2, expressed as pg/ml. Tissue homogenates were prepared from control tissue (inferior turbinate), CRSsNP and CRSwNP.*

RT-PCR data for TGF-beta RI, II and III

In CRSsNP, transcript levels expressed as normalized relative expression units (NREU) of TGF-beta RI (1.52 NREU; IQR 1.08-1.91) and TGF-beta RIII (1.31 NREU; IQR 0.97-2.39) mRNA were significantly higher when compared to controls (0.84 NREU; IQR 0.76-1.11 and 0.955 NREU; IQR 0.55-1.11). In CRSwNP, TGF-beta RII (0.43 NREU; IQR 0.248-0.86) and TGF-beta RIII

(0.495 NREU; IQR 0.27-0.79) was significantly lower when compared to controls (1.02 NREU; IQR 0.86-1.31and 0.955 NREU; 0.55-1.11) (see Figure 2).

Within the CRSwNP group, patients with and without comorbid asthma were compared for TGF-beta 1 and TGF-beta 2 protein and mRNA expression of TGF-beta RI, II and III. This revealed no significant differences between both nasal polyp subgroups.

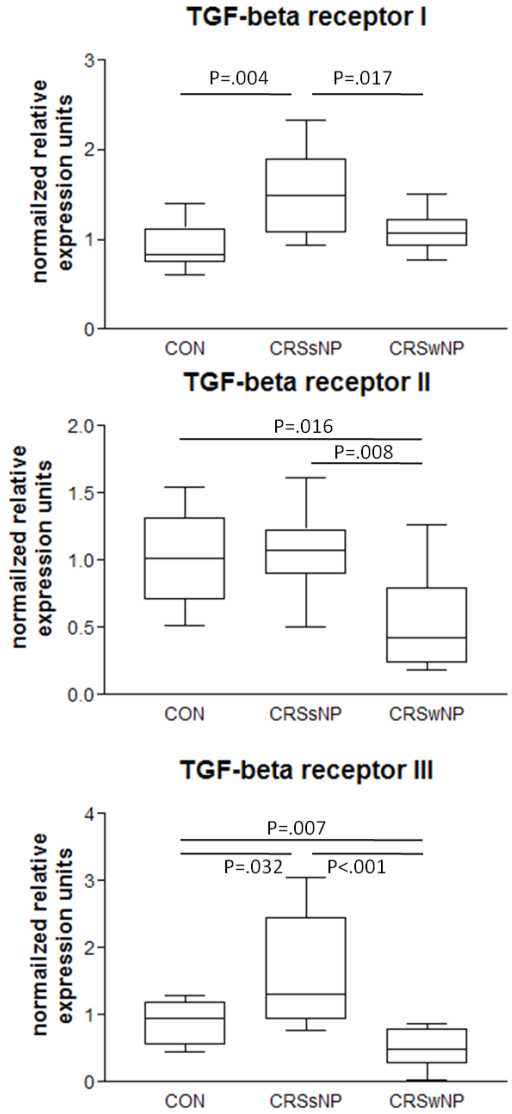

Figure 2. *TGF-beta receptor I, II and III mRNA expression measured by means of RT-PCR in controls, CRSsNP and CRSwNP. Data are expressed normalized relative expression units/ 30ng cDNA.*

Immunohistochemistry for TGF-beta 1 and 2, and for TGF-beta receptor I and II

Immunohistochemical staining was used to determine the presence of TGF-beta 1 and 2 isoforms in healthy and diseased sinonasal mucosal tissue. Representative sections of control, CRSsNP and CRSwNP are shown in figure 3.

In control tissue, staining for TGF-beta 1 and 2 was detected in basal epithelial cells, the basal membrane was negative. Few TGF-beta 1 and TGF-beta 2 positive cells were detected subepithelially. Endothelial cells of blood vessels were found positive for TGF-beta 1 and 2, and TGF-beta 1 deposition could be observed within the lumen of the vessel representing accumulation of platelets and blood cells. TGF-beta RI and RII positive cells were detected in the ciliary and basal cells of the epithelium, and in inflammatory cells.

In CRSsNP, the extracellular matrix stained more intensely for TGF-beta 1 when compared to normal mucosa. Some TGF-beta 1 positive cells were also detected within the epithelium. For TGF-beta 2, many positive cells were detected in ciliary and basal cells of the epithelium. TGF-beta RI and RII positive cells were more abundantly present in the epithelium of CRSsNP when compared to normal mucosa. Positive inflammatory cells in the subepithelial region were also detected.

In CRSwNP, TGF-beta 1 staining was not detected in the epithelium. Few TGF-beta 1 positive inflammatory cells were detected subepithelially. Connective tissue surrounding pseudocyst zones was found positive. In contrast, TGF-beta 2 was detected in the epithelial cells of CRSwNP, and in inflammatory cells, some of which were eosinophils. The epithelium of CRSwNP showed less TGF-beta RI and RII positive cells when compared to controls and CRSsNP.

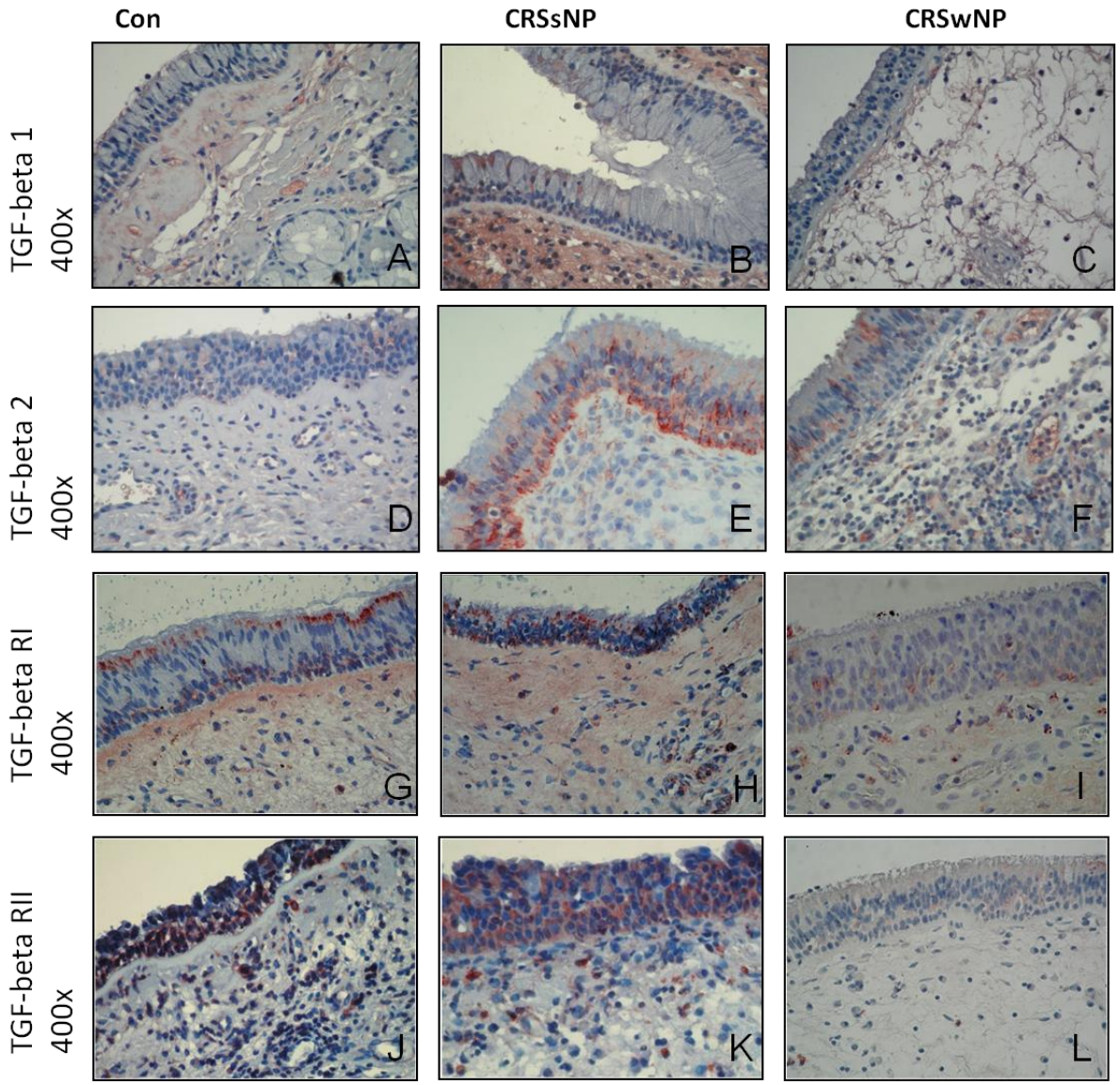

Figure 3. *Immunostaining for TGF-beta 1, 2 and TGF-beta RI and II in controls, CRSsNP and CRSwNP. Epithelium, basal membrane and subepithelial region are shown at a final magnification of 400X.*

Immunohistochemistry for pSmad 2

Representative sections of immunohistochemical stainings for pSmad 2 performed in controls, CRSsNP and CRSwNP are shown in figures 4 A-F. The number of pSmad 2 intranuclear positive cells was significantly higher in CRSsNP (108.5; IQR: 53.5-164) versus controls (68.5; IQR: 42-110), and significantly lower in CRSwNP (32.5; IQR: 17-59.5) compared to controls (68.5; IQR: 42-110), as presented in figure 5A.

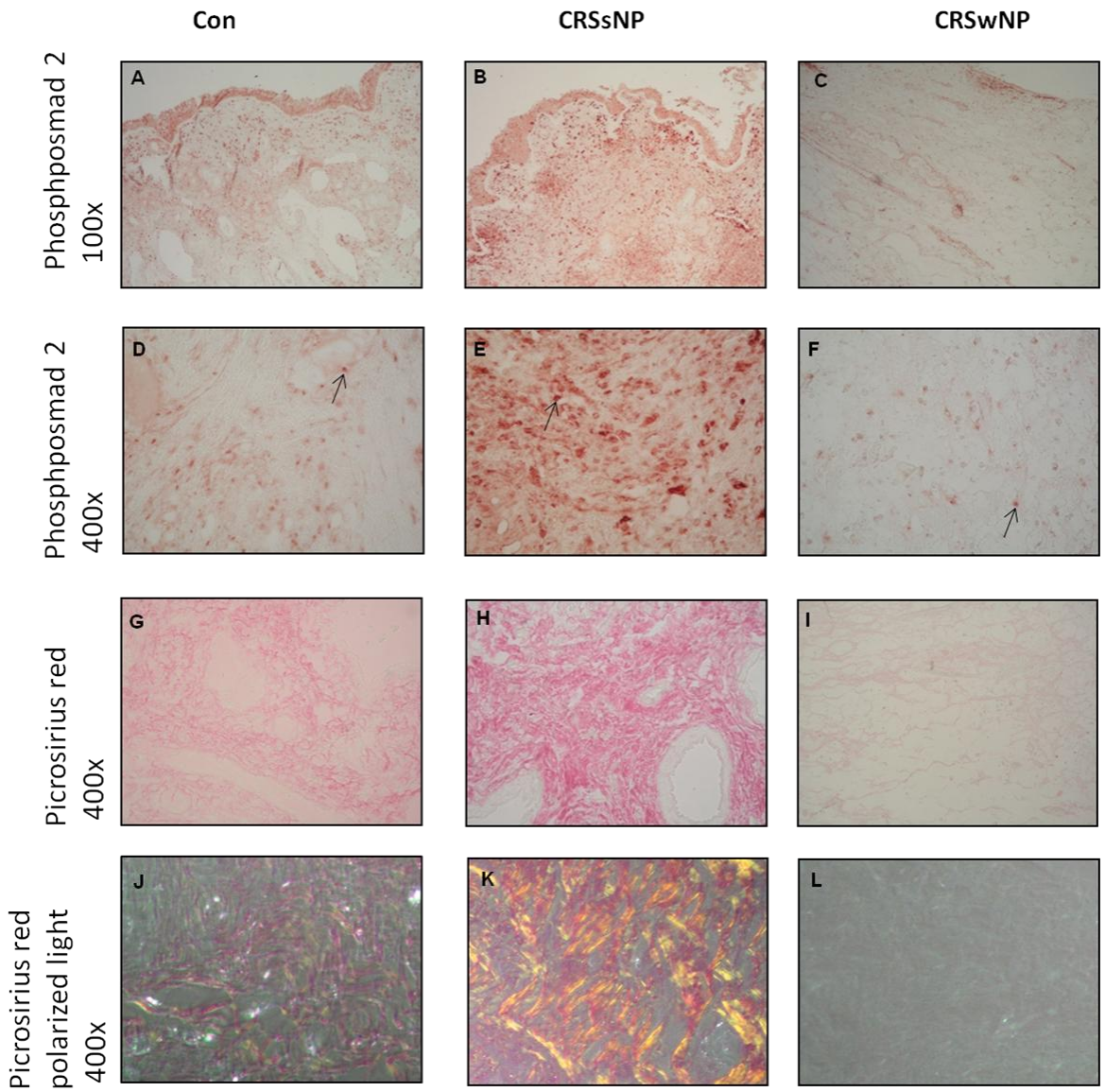

Figure 4. *Immunostaining for pSmad 2 in controls (A and D), CRSsNP (B and E) and CRSwNP (C and F). Postive intranuclear staining is indicated by an arrow.*

Picrosirius red stainings for collagen in controls, CRSsNP and CRSwNP, first viewed in bright-field microscopy (G, H, I), and viewed under polarized light (J, K, L) to assess fiber thickness.

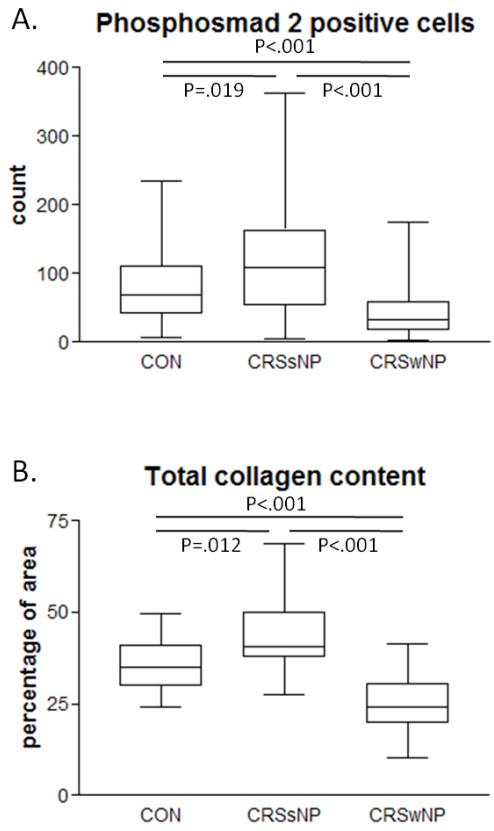

Figure 5. A. *Quantification by means of image analysis of the number of pSmad 2 positive cells in controls, CRSsNP and CRSwNP.* **B.** *Quantification by means of image analysis of total collagen content in controls, CRSsNP and CRSwNP.*

Picrosirius red stainings for collagen

Picrosirius red stainings were performed to assess collagen content in the extracellular matrix. Sections were first viewed in bright-field microscopy, shown in Figure 4 G,H and I. Collagen stains red on a pale yellow background. The total collagen amount in the extracellular matrix was found significantly higher in CRSsNP (median percentage of area 40.8) and significantly lower in CRSwNP (median percentage of area 24) when compared to controls (median percentage of area 35.1), as presented in Figure 5B.

Sections were additionally examined through crossed polars (see Figure 4 J, K and L). Larger collagen fibers light up in bright orange, and thinner fibers show green. This birefringence is highly specific for collagen[14]. Orange collagen fibers were present in CRSsNP, which is characteristic for thick collagen fibers. In contrast, almost no thick orange fibers could be detected in CRSwNP (Figure 4 J, K and L).

DISCUSSSION

TGF-beta is a multifunctional and ubiquitously expressed growth factor, of major interest in airway disease. TGF-beta has important anti-inflammatory effects, but it also acts as a master switch in the induction of fibrosis. We here demonstrate clear differences in the local tissue concentration of TGF-beta 1 between CRSsNP and CRSwNP, confirming previous studies [1,4,15]. Low TGF-beta 1 protein levels appear to be a constant finding in nasal polyp disease (CRSwNP), whereas TGF-beta 1 up-regulation is characteristic for CRSsNP. However, post-translational modifications might complicate the interpretation of TGF-beta measurements at tissue level. The regulation of TGF-beta is a complex mechanism. TGF-beta is secreted in an inactive form to prevent binding to ubiquitously expressed receptors. Moreover, TGF-beta can be associated to extracellular matrix proteins to assure an extracellular reservoir of TGF-beta, which can be activated on demand. This association to the matrix was observed on immunohistochemical stainings in CRSsNP, demonstrating intense extracellular matrix staining for TGF-beta 1. Additionally the TGF-beta protein findings were linked to receptor expression and intracellular signaling.

TGF-beta 1 first binds to TGF-beta RII, this complex then recruits TGF-beta RI. TGF-beta RIII acts as a facilitator to the binding of TGF-beta RI and TGF-beta RII, and thus has no direct signaling role[16]. After binding to TGF-beta, TGF-beta RII recruits and phosphorylates TGF-beta RI, leading to phopshorylation of Smad 2 and Smad 3. Phosphorylated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus. This process is inhibited by Smad 7. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression of TGF-beta target genes[8]. As TGF-beta RI and RII are both necessary for TGF-beta signaling, a decreased expression of TGF-beta RII observed in CRSwNP together with low TGF-beta 1 protein concentration suggests suppressed TGF-beta signaling. In strong contrast, in CRSsNP, the increased TGF-beta RI expression was observed together with high TGF-beta 1 protein expression. TGF-beta RIII, which act as an enhancer for TGF-beta RI and RII binding, was found decreased in CRSwNP and higher in CRSsNP compared to controls, further supporting the decreased TGF-beta receptiveness in CRSwNP, and increased TGF-beta susceptibility in CRSsNP.

As a downstream signal of active TGF-beta, the expression of pSmad 2 was analyzed. Smad 2 proteins are a family of transcription factors and are the only TGF-beta receptor substrates with a demonstrated ability to propagate signals[8,17,18]. Clear differences were observed in

the number of pSmad 2 positive cells. The number of positive cells was higher in CRSsNP and lower in CRSwNP compared to controls, pointing towards increased active TGF-beta signaling in CRSsNP, and suppressed signaling in CRSwNP.

Remarkably, little is known about the presence of TGF-beta 2 in sinonasal tissue. In contrast to previous findings at mRNA level[19], we here show a differential expression of TGF-beta1 and 2 at protein level in CRSwNP. Recent studies in lower airway disease suggest a distinctive role for TGF-beta 2, predominantly expressed in severe asthma and mainly related to eosinophils[7]. Similarly CRSwNP, a disease characterized by a severe eosinophilic inflammation revealed a higher expression of TGF-beta 2 when compared to controls. However, no correlation could be found in biopsies of CRSwNP between ECP, an important end product of eosinophils, and TGF-beta 2 (data not shown). The lack of correlation could be explained by the fact that other cell types present in CRSwNP might also contribute to total TGF-beta 2 levels. Based on our immunohistochemical findings, we could detect TGF-beta 2 positive eosinophils, however epithelial cells and other inflammatory cell types also stained positive for TGF-beta 2. Moreover, total TGF-beta 2 protein levels were also increased in CRSsNP, a disease lacking tissue eosinophilia.

It was previously hypothesized that decreased TGF-beta expression in CRSwNP could be interpreted as a decreased T regulatory cell function, which has recently been confirmed[2]. On the other hand, TGF-beta plays a crucial role in the extracellular matrix metabolism, It stimulates the production of TIMP-1, a tissue inhibitor of metalloproteinases (TIMP) that prevents enzymatic breakdown of the ECM[20]. In CRSsNP, matrix metalloproteinase 9 (MMP-9) and TIMP-1 are found upregulated, whereas in CRSwNP, MMP-9, but not TIMP-1, is up-regulated. The lack of the upregulation of TIMP-1 can be related to the low TGF-beta 1 levels in CRSwNP[20-23].

We here show a low amount of collagen present in the extracellular matrix of CRSwNP when compared to controls, and an absence of thick collagen fibers when viewed under polarized light. The lack of TGF-beta 1 in CRSwNP can be interpreted as a lack of tissue repair, reflected by loose connective tissue and oedema formation in a severely inflamed tissue. In contrast, TGF-beta 1 levels are higher in CRSsNP when compared to controls, together with TGF-beta R I and R III, and with a higher number of pSmad 2 positive cells. A higher collagen

content was present in CRSsNP compared to controls. This is indicative for excessive tissue repair and fibrosis formation in CRSsNP.

We wish to mention that corticosteroids are currently the recommended treatment for both CRS with and without NP. In this study, almost all patients were treated with topical corticosteroids for three months. Treatment failure of corticosteroids indicated the surgery. A wash out period of 4 weeks was maintained prior to the actual surgery. To our knowledge, there are no data available on the effect of the prior use of topical steroids on TGF-beta and receptor expression in upper airways. Although the current use of steroids is clearly linked to changes in the cytokine and mediator profile[24], corticosteroids seem to be unable to modulate TGF-beta expression, as observed earlier in severe lower airway disease[25,26].

We also wish to stress that we used inferior turbinates from patients with turbinate hypertrophy, but no chronic rhinosinusitis, as control tissues, and compared those to ethmoidal tissue from CRSsNP and CRSwNP patients. Since it is considered unethical to perform sinus surgery in order to resect ethmoidal tissue from undiseased sinuses, we used inferior turbinates as control. However, clear differences were also observed between ethmoidal tissues from CRSsNP and CRSwNP patients, supporting our observations.

CONCLUSION

Clear differences in the TGF-beta signaling cascade are observed between CRSsNP and CRSwNP. This supports the hypothesis that CRSsNP and CRSwNP are two distinct disease entities. A low TGF-beta 1 protein concentration, a decreased expression of TGF-beta RII and a low number of phospho-smad 2 positive cells in CRSwNP all indicate a low level of TGF-beta signaling in nasal polyp disease. In strong contrast, in CRSsNP, high TGF-beta 1 protein expression, increased TGF-beta RI expression, and a high number of phospho-smad 2 positive cells all indicate an enhanced TGF-beta signaling in CRSsNP. This is reflected by the typical extracellular matrix remodeling patterns observed. CRSwNP is characterized by a lack of collagen and tissue repair, whereas CRSsNP demonstrated fibrosis with excessive collagen production and thickening of the collagen fibers.

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Chapter VI

Inflammation and remodeling patterns in early-stage chronic rhinosinusitis

Inflammation and remodeling patterns in early-stage chronic rhinosinusitis

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ABSTRACT

Background: A distinct set of inflammatory and remodeling factors have been found elevated in chronic rhinosinusitis; the investigation of their expression in early-stage disease may reveal early events in this common disease.

Methods: Sinonasal mucosal samples from 9 patients with early-stage CRSsNP were taken from the inferior and middle turbinates, the uncinate process, maxillary sinus, anterior ethmoid, bulla ethmoidalis and the posterior ethmoid and measured for TGF-beta 1 and it´s receptors, MPO protein as well as pro-inflammatory cytokines (TNF-alpha and IL-1beta) and the Th1 cell signature (IFN-gamma and T-bet). As outcome parameter for TGF-beta signaling collagen deposition was analyzed.

Results: TGF-beta 1 protein concentrations were significantly increased in the maxillary sinuses (P = 0.006), the uncinate process (P = 0.01), the anterior ethmoid including the bulla ethmoidalis (P = 0.005) and the posterior ethmoid (P = 0.037) when compared to the inferior and middle turbinates. Collagen deposition was significantly increased in the maxillary sinus when compared to the inferior turbinates (P = 0.008). In contrast, mRNA for TGF-beta receptors, Th1 related markers (IFN-gamma and T-bet), pro-inflammatory cytokines (IL-1 beta and TNF-alpha) and MPO protein as neutrophil marker were expressed at all locations but showed no significant differences between the various locations.

Conclusions: In early-stage chronic sinus disease, TGF-beta protein is expressed in significantly higher concentrations within the paranasal sinuses when compared to

turbinates, whereas pro-inflammatory, neutrophilic and Th1 markers did not show any difference. These findings suggest that remodeling might exist before and independent from chronic inflammation.

Key words

Chronic rhinosinusitis, inflammation, remodeling, TGF-beta

INTRODUCTION

Chronic rhinosinusitis represents a common and often debilitating form of sinusitis with important impact on the quality of life of the patients. The prevalence is high and still increasing[1], estimated to affect up to 14% of the global population in the United States. Its etiology is probably multifactorial, including anatomical factors, allergic inflammation, immune deficiency, microbial factors and immune-microbial interactions[2].

Chronic rhinosinusitis clinically represents a spectrum of disorders that share chronic inflammation of the nose and paranasal sinuses; however it is today considered a heterogenous group of diseases. Based on the differential expression of inflammatory cytokines and remodeling patterns, chronic rhinosinusitis with polyp formation (CRSwNP) can be distinguished from chronic rhinosinusitis without polyp formation (CRSsNP)[3]. Clinically late-stage CRSwNP in Caucasians is characterized by a reduced expression of members of the TGF-beta family and its receptors, a preferentially Th2 driven eosinophilic inflammation and a deficit in T regulatory cells, whereas CRSsNP shows an increased expression of Th1 cytokines with a consequently neutrophilic inflammation, and an up-regulation of TGF-beta and its receptors vs. inferior turbinate mucosa. The focus of this study was restricted to early stage CRSsNP disease and aimed to define early events in the development of CRSsNP, using inferior turbinate mucosa as comparator[3-5].

The aim of the present study was to analyze the inflammation and remodeling parameters in the different paranasal sinuses in early stage CRSsNP patients, who were selected on the basis of their history and CT scan, to define the mediators and location of early changes in this frequent disease.

MATERIAL AND METHODS

Patients

Nasal tissue was obtained from 9 patients with chronic rhinosinusitis without polyp formation (CRSsNP) during routine endoscopic sinus surgery at the department of Otorhinolaryngology at the Ghent University Hospital, Belgium. Biopsies of the mucosa were taken at the following anatomical locations: inferior turbinate, middle turbinate, uncinate

process, maxillary sinus, anterior ethmoid including bulla ethmoidalis, and posterior ethmoid. Inferior turbinate samples from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls.

The diagnosis of chronic rhinosinusitis without polyps (CRSsNP) was based on history, clinical examination, nasal endoscopy and computed tomography according to the current EP³OS guidelines[2]. Sinus CT scans were scored according to the Lund-MacKay system. The Lund-MacKay staging system scores each sinus (anterior ethmoid, posterior ethmoid, maxillary, frontal, and sphenoid sinuses) according to the following scale: 0, no opacification; 1, partial opacification; 2, complete opacification. The ostiomeatal complex was scored as 0 (not occluded) or 2 (occluded). The left and right sides were staged separately. The scores were summed so that the total Lund score may range from 0 to 24 for each patient. We only included patients with early stage bilateral disease, lasting shorter than 4 years according to their clinical history. For the CT scan, the involvement of not more than 3 sinuses was allowed which persisted after adequate treatment following the EPOS guidelines.

The ostiomeatal complex and the anterior ethmoid were the most frequent sinuses demonstrating mucosal thickening. All patients have been treated with a combination of topical corticosteroids and clarithromycine 250 mg per day for at least 2 months, but still suffered from or again developed symptoms justifying functional endoscopic sinus surgery. A wash out period of 4 weeks before surgery was maintained for oral and topical corticosteroids and antibiotics. Patients underwent a skin prick test for common inhalant allergens, and were asked about asthma symptoms and smoking habits. Rhinosinusitis symptoms were pre-operatively scored by a physician on a scale from 0-3 (no symptoms, mild, moderate, severe).

General exclusion criteria were based on the EP³OS definition for research (cystic fibrosis, gross immunodeficiency, congenital mucociliary problems, non-invasive fungal balls and invasive fungal disease, systemic vasculitis and granulomatous diseases). Patients who underwent prior nasal or sinus surgery were excluded. The study was approved by the local Ethical committee of the University Hospital Ghent, Belgium. An informed consent was obtained from each patient before collecting samples.

PCR

Gene expression analysis by means of quantitative real time PCR

cDNA was synthesized from 2 µg of RNA with the iScript cDNA synthesis kit (BioRad Laboratories, CA, USA) following the manufacturer's instructions. Levels of the transcription factor T-bet, the cytokines IFN-gamma, TNF-alpha, IL1beta, and TGF-beta receptor 1 and 2 were determined by real time PCR. Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad laboratories, CA, USA) using specific primer sequences (see online repository, Table 1). PCR reactions contained 30 ng cDNA (total RNA equivalent), 250 nM of primer pairs , 1X SYBR Green I Master mix (Bio-Rad laboratories, CA, USA) or 1X TaqMan mix with 100 nM of the TaqMan probe in a final volume of 20 µl. PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute and for reactions using TaqMan probes of 1.5 minutes at 95 °C followed by 50 cycles: 15 seconds at 95 °C and 1 minute at 60 °C.

The expression of the housekeeping genes Beta actin (ACTB) and Hydroxymethyl-bilane synthase (HMBS) was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software[6,7]. The relative expression units of each gene per 30 ng of cDNA sample, was determined by using the qBase program (version 1.3.5, UGent, Belgium) and results are expressed as the logarithm of normalized relative expression units / 30ng cDNA.

Protein concentrations of TGF-beta 1 and MPO

Surgical samples were snap frozen in liquid nitrogen and stored at – 80 °C until homogenization. The tissue was thawed, weighed and 1 ml of 0.9 % NaCl with protease inhibitor Complete (Roche, Mannheim, Germany) was added per every 0.1 g of tissue. The tissue was then homogenized using a B. Braun homogenizer for 5 minutes. The homogenates were centrifuged at 3000 g, 4 °C for 10 min. After centrifugation 250 µl aliquots were made and stored at –80 °C until needed for ELISA. To release latent TGF-beta from the extracellular matrix, samples were treated with acid prior to the ELISA. TGF-beta 1 and MPO levels were determined using commercially available ELISA kits from R&D Systems (Minneapolis, USA). All data were expressed as ng/ml.

Collagen deposition by means of picrosirius red stainings

Collagen was measured by means of picrosirius red staining[8]. Tissue was fixed in formalin (Fluka, Belgium) and embedded in paraffin. Paraffin sections were prepared (thickness 4-5 µm) and air dried for 24 hours at 37°C. Sections were deparaffinized, hydrated, and stained with picrosirius red (direct red 80, Sigma-Aldrich, St. Louis, USA) for 60 minutes. The sections were then washed in two changes of acidified water, dehydrated in three changes of 100% ethanol, and mounted in Tissue-Tek (Miles Inc, USA). The sections were analyzed using an Olympus microscope (CX-40) equipped with filters to provide circularly polarized illumination. The lower filter was placed above the microscope's field iris diaphragm ring, while the upper filter was placed below the linear polarizer aligned such that its transmission axis was at 45°. Tissue images viewed under bright-field and polarized light were obtained with a 40X objective lens (final magnification 400X) and recorded on a digital camera (Olympus C-5050).

Image analysis

Collagen content was quantified under polarized light microscopy. Image analysis was carried out with Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). Briefly, the entire section of a slide was captured by consecutive fields under bright-field at a final magnification of 400X, with no overlapping zones. The total collagen amount was calculated for each image after subtraction of background and conversion to 8-bit images. The total collagen content was calculated for each section expressed as percentage of the total area.

Statistical analysis

Statistical analysis was performed with MEDCALC software version 9.4.2.0 (F. Schoonjans, Belgium). Data are expressed as median and interquartile ranges. When comparisons were made between groups, significant between-group variability was first assessed using Kruskall-Wallis test. The Mann Whitney U-test two tailed was then used for between-group comparison. Exact P-values are reported. The significance level was set at $\alpha = 0.05$.

RESULTS

Patient characteristics (table 1)

Nine patients with early stage CRSsNP were included, with a median disease duration of 24 months. Symptom scores showed that nasal obstruction and post-nasal drip were predominant. The median age of our study group was 46 years. Three of the nine patients had a positive skin prick test to common aeroallergens, one patient was asthmatic, and one patient was a smoker. None of the patients had previous sinus surgery. The median Lund and Mackay CT score was 6/24.

Table 1: patient characteristics and symptom scores

N	*9*
Female/male	5/4
Median age	46 (39,5-56)
Duration of the disease (months)	24 (18,5-42)
SPT positive	3/9
Asthma in history	1/9
Aspirin hypersensitivity in history	0/9
Smoking	1/9
COPD	0/9
Previous FESS	0/9
Median CT score (Lund/Mackay)	6/24
Nasal obstruction	2 (1,75-3)
Rhinorrhea	0 (0-2)
Sneezing	0(0-0,25)
Anosmia	1 (0,75-2,25)
Post nasal drip	2 (1-2,25)
Headache	1 (0-2,25)
Dyspnoea	0 (0-1)
Cough	1 (0-1,5)

N, number of included patients

Data are reported as median and interquartile ranges.

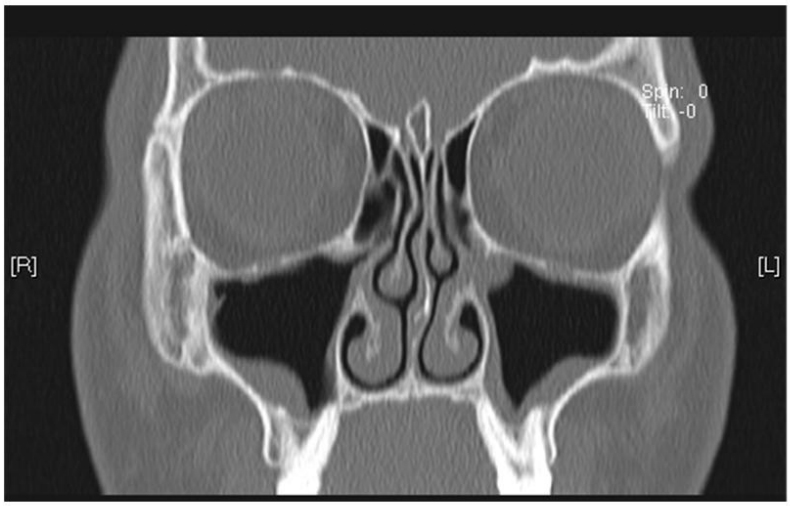


Figure : *A typical CT scan of a patient with early-stage CRSsNP, showing some opacification of the OMC-area and the maxillary sinuses.*

TGF-beta 1 protein expression, mRNA expression of TGF-beta receptors I and II (Figure 1)

TGF-beta 1 protein concentrations were significantly higher – in this order - in the maxillary sinuses (14281 pg/ml; IQR 7766-23349 and P = 0.006) , the uncinate process (14048 pg/ml; IQR 8690-16236 and P = 0.01), the anterior ethmoid including the bulla ethmoidalis (10645 pg/ml; IQR 9515-14415 and P = 0.005) and the posterior ethmoid (10130 pg/ml; IQR 5780-12988 and P = 0.038) when compared to the inferior and middle turbinates (5027 pg/ml; IQR 3852-8880). No significant differences were noted in TGF-beta1, RI and II mRNA expression between the different locations.

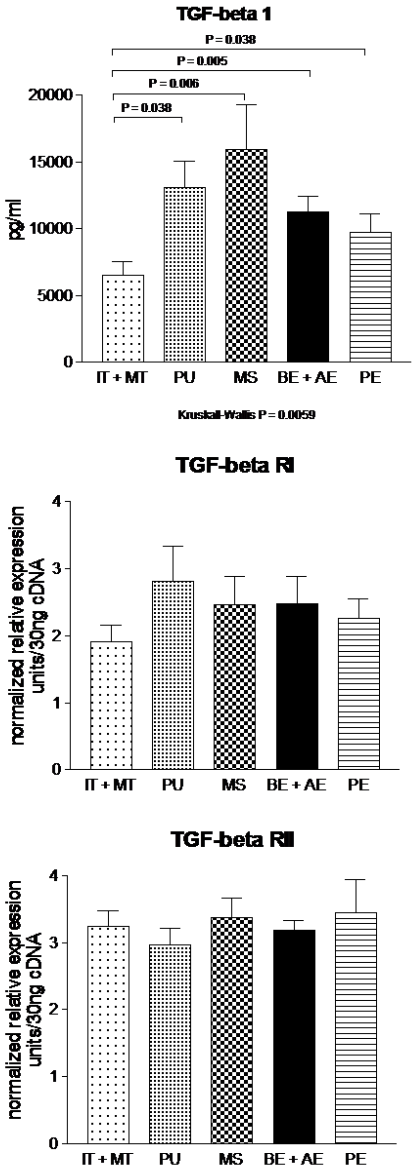


Figure 1: *Expression of TGF-beta 1 protein, and TGF-beta receptors I and II mRNA in sinunasal mucosal tissue. Inferior and middle turbinates served as control.*

IT: inferior turbinate, MT: middle turbinate, PU: processus uncinatus, MS: maxillary sinus, BE: bulla ethmoidalis, AE: anterior ethmoid, PE: posterior ethmoid

Th1 and pro-inflammatory cytokines, MPO protein (Figure 2)

T-bet and IFN-gamma, markers of a Th1 driven inflammation, and TNF-alpha and IL-1 beta, representing pro-inflammatory cytokines, were expressed in all nasal and sinus locations, with no significant differences between the sites. The same was true for MPO protein, a marker of neutrophil inflammation, which could be detected in all anatomical locations without significant differences between the sites.

Comparison of TGF-beta 1 concentrations in inferior turbinates of CRSsNP versus control patients showed a significant increase of TGF-beta 1 ($p=0,017$ on mRNA data). The proinflammatory cytokines IL-1 beta and TNF-alpha and Th1 related cytokines did not show an upregulation in inferior turbinates of CRSsNP when compared to control patients.

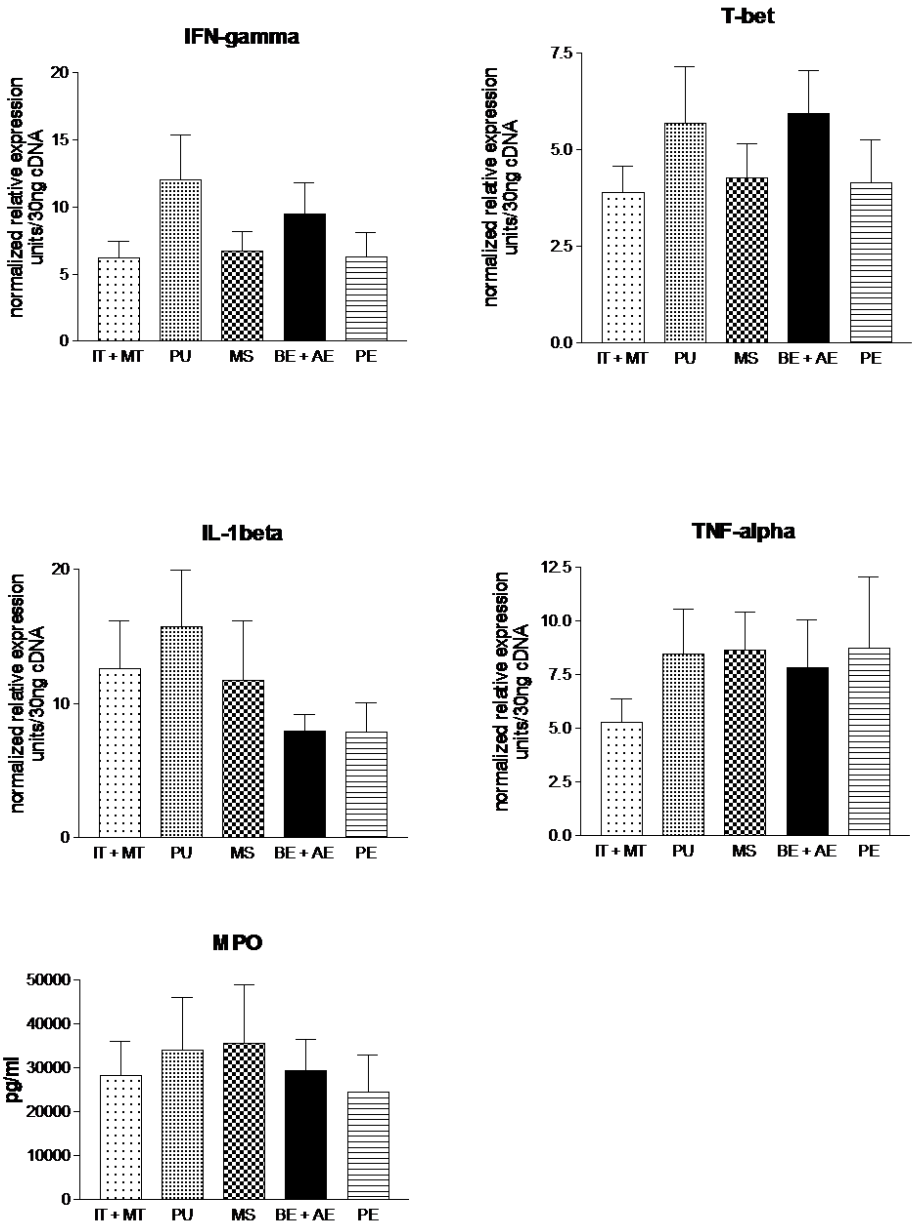
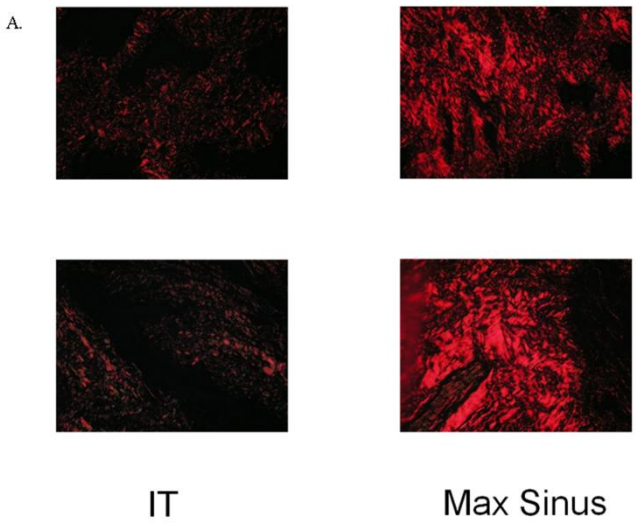


Figure 2: *Expression of mRNA for Th1 (IFN-gamma and T-bet) and pro-inflammatory (IL-1 beta and TNF-alpha) markers and protein of the neutrophil marker MPO in turbinates and sinus mucosa. No significant differences were noted between all groups.*

IT: inferior turbinate, MT: middle turbinate, PU: processus uncinatus, MS: maxillary sinus, BE: bulla ethmoidalis, AE: anterior ethmoid, PE: posterior ethmoid

Picrosirius red stainings for collagen (Figure 4)

Picrosirius red stainings were performed to assess collagen content in the extracellular matrix. Sections were examined through crossed polars (see Figure 4A). Larger collagen fibers light up in bright orange and thinner fibers show green. This birefringence is highly specific for collagen. Orange collagen fibers were present in significantly higher amount in the maxillary sinuses (median percentage of area 41.17%) when compared to inferior turbinates (33.49%, P = 0.008), as presented in Figure 4B.



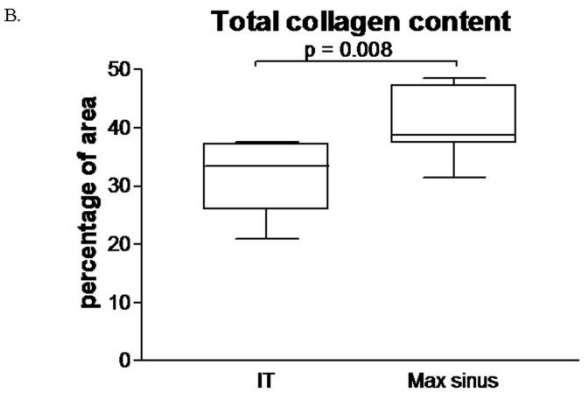


Figure 4: *A. Picrosirius red staining for collagen in inferior turbinates (IT) and maxillary sinus viewed under polarized light*

Figure 4: *B. Quantification by means of image analysis of total collagen content in inferior turbinates and maxillary sinuses demonstrating significantly higher collagen deposition in maxillary sinuses when compared to inferior turbinates*

Discussion

Inflammatory mucosal disease in the sinuses shows specific remodeling and inflammatory patterns. CRSsNP has previously been described as being a predominant Th1 mediated neutrophilic disease, characterized by increased levels of IFN-gamma and MPO[3,5]. Moreover, it was recently shown that TGF-beta 1 and it´s receptors TGF-beta RI and RIII are strongly up-regulated in CRSsNP, resulting in a high number of phospho-Smad 2-positive cells to indicate pro-fibrotic signalling[3-5]. This is reflected by a typical remodeling process characterized by a higher collagen deposition in CRSsNP together with the presence of thick collagen fibers when compared to healthy controls[4].

Here we show that in early stage CRSsNP disease, surprisingly little mucosal inflammation in the sinuses can be shown, whereas there already is a manifest up-regulation of TGF-beta protein expression. TGF-beta 1 was significantly over-expressed in the paranasal sinuses when compared to turbinates, with the highest expression in the maxillary sinuses; concentrations of TGF-beta 1 were three-fold higher compared to nasal turbinates. Although we were not able to demonstrate a significant up-regulation of the TGF-beta RI, we noted the presence and a marginal, but insignificant increase in the expression of this receptor.

Subsequently, we could demonstrate that the up-regulation of TGF-beta 1 in the presence of the receptor was accompanied by an increased deposition of collagen within the maxillary sinuses. As TGF-beta 1 protein shows a higher expression within the paranasal sinuses, whereas the inflammatory and Th1 cytokines appear not to be up-regulated, we suggest that chronic rhinosinusitis is a TGF-beta mediated disease with subsequent remodeling and fibrosis formation, which only secondarily may be associated with inflammation of the mucosa.

The selection of markers for inflammation was based on previous studies[3,5]. As markers for a Th1 biased inflammation, T-bet and IFN-gamma were analyzed. T-bet is a Th1 specific T box transcription factor that controls the expression of the hallmark Th1-cytokine IFN-γ[9]. T-bet and IFN-gamma were found to be up-regulated in CRSsNP in previous studies in patients who suffered from more severe disease with a median disease duration of 4.2 years [3-5]. Strikingly, in this study involving patients with a median duration of 24 months, these

markers are ubiquitously expressed in the turbinates and sinuses, but did not show any significant topological differences.

TNF-alpha and IL-1beta mRNA expression were measured as major pro-inflammatory cytokines[3], reflecting pro-inflammatory responses against e.g. bacterial infection. We here detect these cytokines in both paranasal sinuses and turbinates, but were unable to find up-regulation in the sinuses. Finally, MPO (myeloperoxidase) was used as a marker for neutrophilic granulocyte activation, which also demonstrated no difference between turbinates and sinuses. Thus, to our surprise, we could not find any sign of inflammation in early stage CRSsNP in the sinuses. Th1-related and pro-inflammatory cytokines did not show an up-regulation in inferior turbinates of chronic rhinosinusitis versus control patients.

As TGF-beta 1 protein showed a higher expression within the paranasal sinuses, whereas the inflammatory and Th1 cytokines appear not to be up-regulated, we suggest that chronic rhinosinusitis is a TGF-beta mediated disease with subsequent remodeling and fibrosis formation, which only secondarily may be associated with chronic inflammation of the mucosa. The increased expression of TGF-beta 1 is in line with previous findings where we detected an up-regulation of TGF-beta 1 in advanced CRSsNP when compared to CRSwNP[5], coinciding with adequate expression of the Tregulatory cell marker FOXP3[5]. We have already demonstrated that inflammation and remodeling may be separate processes in upper airway disease, specifically in nasal polyps, which are likely to develop independently from each other. Whereas remodeling patterns in Chinese and Caucasian CRSwNP disease appear similar[10], inflammatory patterns in those polyps are clearly different between the ethnic groups[11], showing a Th2- versus a Th17-biased inflammation. These observations underline the dissociation of inflammation and remodeling.

In comparison to lower airway disease, there was so far a clear lack of knowledge regarding the natural history of the upper airway inflammatory response. In early stage asthma mucosal inflammation seems consistently present, and remodeling may develop in parallel[12,13]. The central role of TGF-beta in airway fibrosis has been described extensively[12,14]. It is often assumed that there is a linear progression between an initiating stimulus leading to inflammation, which in turn leads to remodeling. However this paradigm has recently been challenged also in lower airway disease[15,16]. Based on studies on airway

biopsies in children, it has been suggested that remodeling may occur very early in asthma and may in some cases even precede clinical symptoms[17]. Similarly, we found an initial up-regulation of TGF-beta with subsequent collagen deposition in upper airway disease.
We wish to mention that this study is limited to cross sectional data on inflammation and remodeling patterns in a group of patients with limited chronic sinus disease existing for approximately 24 months. Frontal sinus tissue and sphenoidal tissue was not obtained on a regular basis in these patients, who had no involvement of those sinuses and thus no indication for surgery. We therefore limited the investigation to the mentioned locations. Furthermore, biopsies had to be limited in size, as the preservation of sinus mucosa showing no relevant alterations is mandatory in functional sinus surgery; this restricted the number of possible investigations.

Still, these findings provide a new view on the natural course of CRSsNP, and suggest further research on the regulation of TGF-beta in the initiation and maintenance of the disease. The role of inflammation in the persistence of disease and its role in tissue remodeling need to be investigated in depth. Furthermore, these findings underline the importance of TGF-beta as target for therapeutic intervention, may it be early or late stage disease.

Conclusion

In early chronic sinus disease, TGF-beta is up-regulated within the paranasal sinuses, initiating the production of collagen and initiating a remodeling processes, whereas signs of inflammation are still lacking. We suggest that TGF-beta plays a central role in the initiation of CRSsNP, and represents a major target for further research and future intervention.

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Chapter VII

ANTI IL-5 treatment

Mepolizumab, a humanised anti-IL-5 monoclonal antibody, as treatment option for severe nasal polyposis.

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ABSTRACT

BACKGROUND**:** Approximately 85% of Caucasian nasal polyps are characterized by prominent eosinophilia. IL-5 is the key driver of eosinophilic differentiation and survival. OBJECTIVE: To investigate the therapeutic potential of inhibiting IL-5 using a humanized monoclonal antibody as treatment of severe nasal polyposis. METHODS**:** 30 patients with severe nasal polyposis (grades 3-4 or post-surgery recurrent) refractory to corticosteroid therapy were randomized in a double blind fashion to receive either 2 single IV injections (28 days apart) of 750 mg mepolizumab (n=20) or placebo (n=10). Change from baseline in nasal polyp score was assessed monthly until 1 month post last dose (week 8). CT scans were also performed at week 8. RESULTS**:** 12/20 patients on mepolizumab showed a significantly improved nasal polyp score and CT-scan score compared to 1/10 on placebo at week 8 versus baseline. CONCLUSION**:** Mepolizumab achieved a statistically significant reduction of the nasal polyp size for at least 1 month post dosing in 12/20 patients. IL-5 inhibition is a potential novel therapeutic approach in patients with severe eosinophilic nasal polyposis.

(supported by GSK, EUDracCT No 2005-005113-11)

CLINICAL IMPLICATIONS

Two intravenous injections with mepolizumab (anti-IL-5) significantly reduce the size of chronic rhinosinusitis with nasal polyps based on endoscopic scoring and blinded CT scan assessment.

CAPSULE SUMMARY

Two injections of mepolizumab were well tolerated and significantly reduced the size of nasal polyps for at least 1 month post last dose.

KEY WORDS

Anti-IL-5, mepolizumab, eosinophils, Chronic rhinosinusitis, nasal polyposis

INTRODUCTION

Chronic sinus disease covers a multitude of different entities, such as chronic rhinosinusitis without nasal polyps (CRSsNP) and CRS with NP (CRSwNP). Although in the recent position paper for sinus disease of the EAACI, the difference between CRSsNP and CRSwNP is made by clinical investigation and endoscopy,[1] other studies have suggested that these two entities have distinct pathways of inflammation.[2,3] CRSwNP in Caucasian subjects is characterized by a TH2 eosinophilic inflammation with high levels of IL-5 and IgE,[4-6] whereas CRSsNP shows a TH1 milieu with high IFN-γ and TGF-β1 concentrations.[3]

In Caucasian patients, 80-90% of the nasal polyps (NP) are characterized by prominent eosinophilia.[1,7] It is assumed that through release of toxic products, eosinophils lead to tissue damage and growth of polyps.[8] The accumulation and activation of eosinophils is favoured by low concentrations of TGF-β1 and by overproduction of IL-5 and eotaxin in NP tissue.[3] High amounts of IL-5 were detected in patients with NP, both at mRNA and protein level.[9,10] This cytokine seems to play a key role in chemotaxis, activation and survival of eosinophils.[11,12] Treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 mAb results in eosinophil apoptosis and decreases tissue eosinophilia in vitro.[10] Concerning the raised IgE level, there is increasing evidence that Staphylococcus aureus derived enterotoxins stimulate the eosinophilic inflammation by production of TH2 cytokines and local IgE formation.[13]

Interestingly, NP of Chinese patients are clinically indistinguishable from polyps of their Caucasian counterparts, but they lack IL-5 and eotaxin expression in the tissue resulting in lower numbers of tissue eosinophils.[14,15] The direct comparison of Belgian and Chinese polyps shows that there is a shared but still to be clarified pathway of mucosal edema formation, T-effector cell activation and regulatory T-cell impairment.[16] Moreover, Caucasian patients showed comorbid asthma more frequently than Chinese patients.16 Inflammation in asthma shares many features with the eosinophilic inflammation in NP, such as an increased number of mucosal eosinophils, IgE formation and a TH2 profile with raised IL-5 and eotaxin.[17]

These findings suggest that different types of polyps may require different treatments, based on the respective pathophysiology. Tailored medication schemes based on phenotyping have to be developed. In Caucasian patients, IL-5 is a key driver of maintaining

polyps, namely: eosinophilic differentiation and survival. The objective of the current study was to investigate the therapeutic potential of inhibiting IL-5 using a humanized monoclonal antibody as treatment of severe nasal polyposis. Our group has been able to demonstrate shrinkage of NP in over half of the patients treated with a single intravenous injection of an anti-human IL-5 monoclonal antibody in the past.[18] Moreover, local IL-5 concentrations at baseline were significantly higher in responders, in contrast to non-responders. We suggested that nasal IL-5 levels could predict the response to anti-IL-5 treatment.18 However, the primary endpoint of this study was safety, and efficacy was only studied by nasal endoscopy. In the current study, we wanted to determine the efficacy of two injections of mepolizumab on nasal polyp volume in subjects with severe CRSwNP by nasal endoscopy and CT-scan imaging. In addition, markers of biological activity such as IL-5 and nasal eosinophilia were assessed over a period of eleven months post last dose.

METHODS

Patients

Thirty subjects suffering from chronic rhinosinusitis with primary (grade 3 or 4, see outcome measures) or post-surgery recurrent (grade 1 to 4) NP were included. The inclusion criteria specified that subjects must have failed standard care for CRSwNP and the diagnosis of this condition was based on the European position paper on rhinosinusitis and nasal polyps.[1] The use of systemic corticosteroids and surgical intervention were not allowed from one month prior to treatment until the end of the study, and subjects were not permitted to use nasal corticosteroids, nasal antihistamines, nasal atropine, nasal cromolyn, nasal saline and antibiotic treatment for two months after first dosing. The study was conducted at the Department of Otorhinolaryngology of the University Hospital in Ghent, Belgium. The local ethics committee approved the study and all volunteers gave a written informed consent before participation in the study.

Study Design

This is a randomized, double-blind, placebo controlled study of mepolizumab in patients suffering from CRSwNP. After signing the informed consent and a 4 to 12 week run-in period, subjects were randomized to receive 2 single IV injections (28 days apart) of 750 mg mepolizumab (20 subjects) or placebo (10 subjects). Follow up visits were scheduled 1, 4, 8, 12, 24, 36 and 48 weeks after first dosing. During the follow up visit after 4 weeks, the second injection of mepolizumab was administered. All randomized patients were included in the analysis. The study was double-blind up to 48 weeks.

Outcome measures

The primary endpoint of this study was the reduction in nasal polyp score[19,20] at 8 weeks after the first dosing (one month post second dose). This total polyp score (TPS) is the sum of the right and left nostril scores, evaluated by nasal endoscopy. CRSwNP was graded based on polyp size: 0 = no polyps; 1 = small polyps in the middle meatus not reaching below the inferior border of the middle concha; 2 = polyps reaching below the lower border of the middle turbinate; 3 = large polyps reaching the lower border of the inferior turbinate or

polyps medial to the middle concha; 4 = large polyps causing complete obstruction of the inferior meatus.

Secondary endpoints included changes in CT scans and assessments such as nasal peak inspiratory flow (nPIF), symptom score (sum of individual symptoms: anterior rhinorrhea, nasal obstruction, postnasal drip (PND) and loss of sense of smell; 0 = no symptoms, 1 = mild, 2 = moderate, 3 = severe). CT scans were assessed for improvement versus worsening or no change after 8 weeks with respect to baseline. This was done independently by three different observers. Biological activity was evaluated by peripheral blood eosinophil counts and measurement of cytokines and mediators in serum and nasal secretion. Blood eosinophils were counted automatically using a 2 ml heparinized blood sample. Nasal secretions were obtained by placing sinus packs (IVALON® 4000 plus) in both nasal cavities for exactly 5 minutes immediately processed as previously described.[12] Serum and nasal secretions were assayed by ELISA for IL-1ß, IL-5 (R&D Systems, Minneapolis, USA), MPO (BioCheck, Foster City, USA) and SOL-IL-5Rα (Innogenetics, Ghent, Belgium). ECP concentrations were obtained using the Uni-CAP system (Pharmacia & Upjohn, Upsala, Sweden), while IL-6 concentrations were measured with a Fluorokine MAP cytokine multiplex kit (R&D Systems, Minneapolis, USA) using the BioRad Bio-plex 200. The lower detection limits (LDLs) before diluting were 2 µg/l for nasal ECP, 3.9 pg/ml for nasal IL-5, 7.8 pg/ml for nasal IL-5Rα, 1.8 pg/ml for nasal IL-6, and 0.2 kU/l for nasal total IgE and 0.1 kU/l for serum total IgE.

Safety was assessed by adverse event reporting, vital signs, symptom check, physical examination and blood analysis.

Statistical analysis

The primary endpoint of this study is the change from baseline (CFB) in TPS at week 8. This was analysed using the exact Mann-Whitney U test. As a supporting analysis, improvement in TPS (defined as a negative CFB) was analysed using the Fisher Exact test. Because of the large number of dropouts, we did not interpret any observations after week 8.

Regarding the CT-scans, we checked inter-rater reliability using the Fleiss kappa coefficient. The Fisher Exact test of CT score improvement in the treated versus placebo groups was done for each rater. Symptom scores, blood eosinophils, serum ECP and serum IL-5Rα were

analysed using the exact Mann-Whitney U test, and nPIF via the AUC (area under curve). For the markers in nasal secretions, there were a lot of observations below LDL. Because of this, the Peto-Peto-Prentice test was used as it utilizes all data, acknowledging the unobserved values below LDL, without imputing an exact value for them.[21] For nasal MPO there was no LDL issue and we have tested its CFB using the exact Mann-Whitney U test.

Because the large number of dropout, time to withdrawal was compared using a Kaplan-Meier plot and log rank test. We also looked at the reasons for dropout and their implications in more detail. In order to deal with the missing data problem, we performed a last observation carried forward imputation (LOCF) and an available case analysis (AC). Concerns exist regarding whether it is appropriate to use LOCF or AC.[22] For brevity, throughout the manuscript only the LOCF results are stated, but the AC results are also calculated.

Within the treated group a distinction could be made between responders (people with an improved TPS of at least 1 unit at week 8 versus baseline) and non-responders. We investigated whether there were baseline differences between responders and non-responders, again using the exact Mann-Whitney U test and the Peto-Peto-Prentice test where appropriate.

We performed a post-hoc power calculation for the Mann-Whitney U test of the primary endpoint - TPS CFB at week 8 - based on the present study, using the O'Brien-Castelloe approximation. A post-hoc power of 68% was obtained by LOCF paradigm.

Data analysis was performed using SAS version 9.1 (http://www.sas.com/) and R version 2.11.1 (http://cran.r-project.org/). Error bars in the figures represent 95% confidence intervals of the mean based on normal approximation.

RESULTS

Patients

The baseline characteristics of the study patients are summarized in Table 1. The history and symptoms of the mepolizumab and placebo groups were compared. Age and gender were similar. Almost half of the patients was atopic (based on skin prick testing) and 43% had asthma. The number of patients that had sinus surgery in the past was high. At baseline, our patient population consisted of 3 people with grade 1, 6 with grade 2, 16 with grade 3, and 5 with grade 4 as maximal unilateral nasal polyp size, equally divided into the different groups. Consequently, the mean TPS in both groups was comparable.

Table 1: Baseline characteristics of the study patients, divided into the groups 'Mepolizumab treated' and 'Placebo'.

Baseline characteristics	Mepolizumab treated	Placebo
N	20	10
Age in years (range)	51 (33-66)	46 (27-59)
Female/male	5/15	2/8
Atopy (SPT positive)	10/20	4/10
Asthma in history	10/20	3/10
Aspirin intolerance	3/20	0/10
Sinus surgery in history	12/20	8/10
Duration of disease in years (range)	10 (6-13.5)	12.5 (9-16)
Tobacco use	5/20	1/10
Median total symptom score (IQR)	6 (6-9)	8.5 (7-9)
Loss of smell	3 (2-3)	3 (2-3)
Congestion	2 (1-3)	2.5 (2-3)
Anterior rhinnorrhea	2 (1-2)	1.5 (1-3)
Postnasal drip	1 (0-2)	2 (1-2)

Safety and adverse events

Sixteen of the 30 subjects (53%) reported at least 1 adverse event over 48 weeks of follow up. One serious adverse event and 23 adverse events occured. The serious adverse event was a diverticulitis, due to a pre-existing condition and not considered to be related to the study drug. Of the adverse events, common cold was the most frequent, reported by 6 persons (5 episodes in the mepolizumab-treated group and 1 in the placebo group). Comparing the mepolizumab-treated patients with the placebo group, none of the adverse events reached significance. We observed no meaningful changes in vital signs, physical examination and blood analysis.

Primary endpoint: total polyp score

The primary endpoint was the difference in TPS at week 8 (visit 5) versus baseline (visit 2). Using LOCF, the CFB on mepolizumab was -1.30 (SD 1.72) and on placebo was 0.00 (SD 0.94), resulting in a treatment difference of -1.30 (SD 1.51; p value p=0.028, Mann-Whitney U test). Figure 1 shows the CFB at different time points and the baseline and week 8 TPS for each subject.

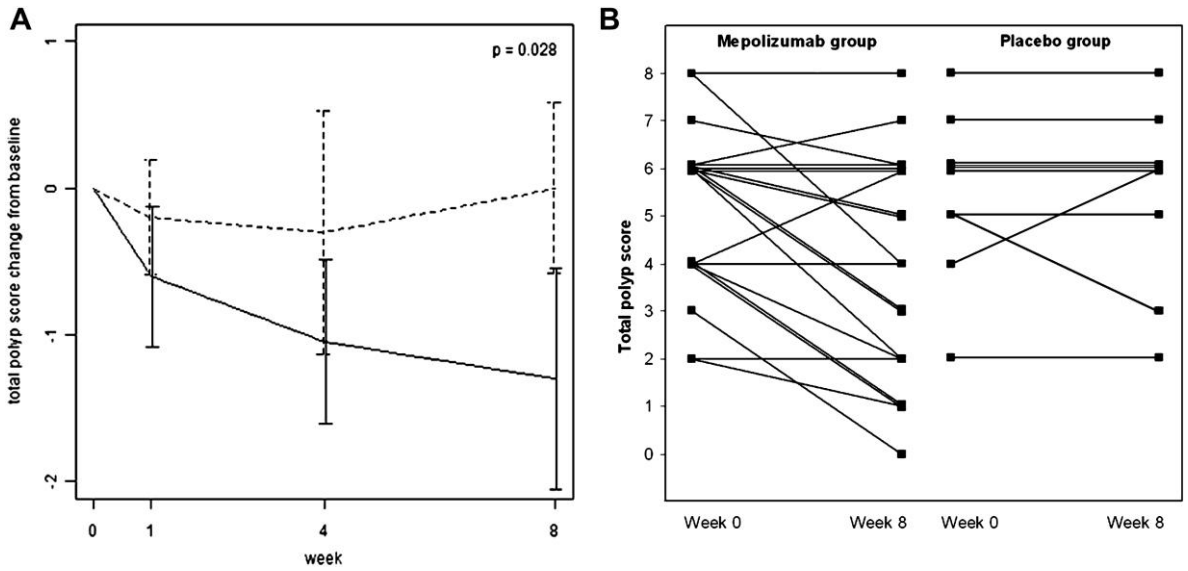

Figure 1: (A) Mean CFB in TPS based on LOCF for the treated (solid line) and placebo (dashed line) groups starting at the moment of first administration. Error bars indicate 95% CIs of the mean based on normal approximation. (B) Baseline and week 8 TPSs in absolute values

based on LOCF for each subject and divided into the mepolizumab-treated and placebo groups.

Again, using LOCF, the percentage improvement in TPS for mepolizumab was greater than placebo, with 60% versus 10% (odds ratio 13.5; p=0.018, Fisher Exact test).

CT score improvement

The Fleiss kappa coefficient of inter-rater reliability was 0.679 using LOCF, indicating good agreement between the three raters of the CT scans. Figure 2 shows the percentages improvement of CT scan. An improvement was seen in over half of the mepolizumab-treated patients compared to <20% of the placebo group, compared to the baseline scans (p=0.058, p=0.024 and p=0.049 for the different raters using LOCF, Fisher Exact test).

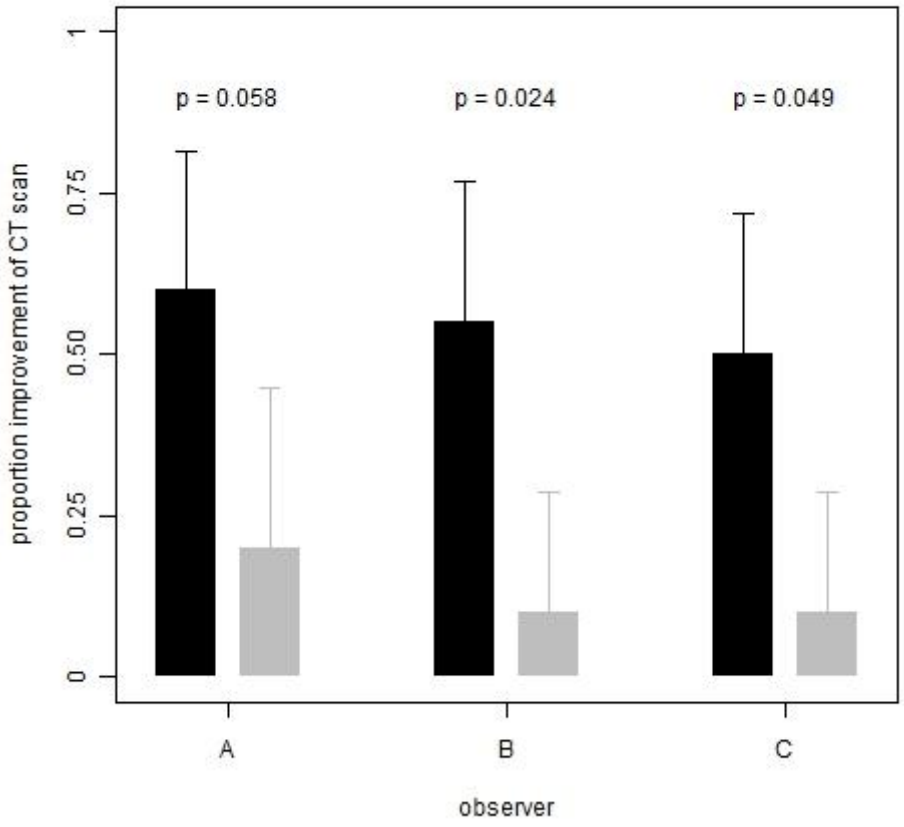

Figure 2: Proportional improvement in CT scan scores based on LOCF for the mepolizumab-treated (black columns) and placebo (gray columns) groups rated by 3 different observers (A, B, and C). Error bars indicate 95% CIs of the proportion based on normal approximation.

Symptom scores and nPIF

Reduction from baseline of loss of smell, postnasal drip and congestion at week 8 was greater in the treated than in the placebo group, but rhinorrhea stayed at the same level. Remarkable, the improvement of loss of smell stayed at the same level during the whole period of follow up (11 months post last dose), while the other symptoms normalized after a period of time. However, none of these differences was statistically significant.

Figure 3 shows the mean CFB in nPIF, resulting in a different AUC. This suggests better values of nPIF in the mepolizumab-treated group than in the placebo group. The nPIF AUC values were also formally compared, resulting in p=0.095 for LOCF.

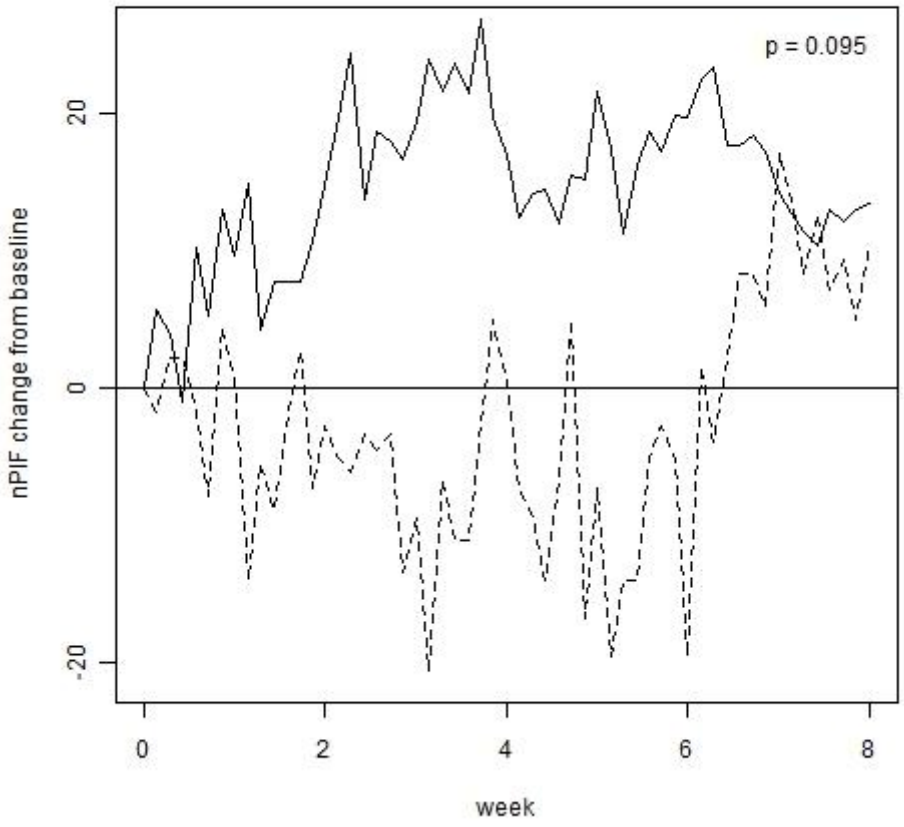

Figure 3: Mean CFB in nPIF based on LOCF for the treated (solid line) and placebo (dashed line) groups starting at the moment of first administration.

Blood and serum markers

CFB at week 8 of blood eosinophils ($p<0.001$ for LOCF), serum ECP ($p=0.022$ for LOCF) and serum IL-5Rα ($p<0.001$ for LOCF) showed a significant reduction in the verum versus the placebo group. Evolution of serum ECP, blood eosinophils and serum IL-5Rα is shown in Figure 4.

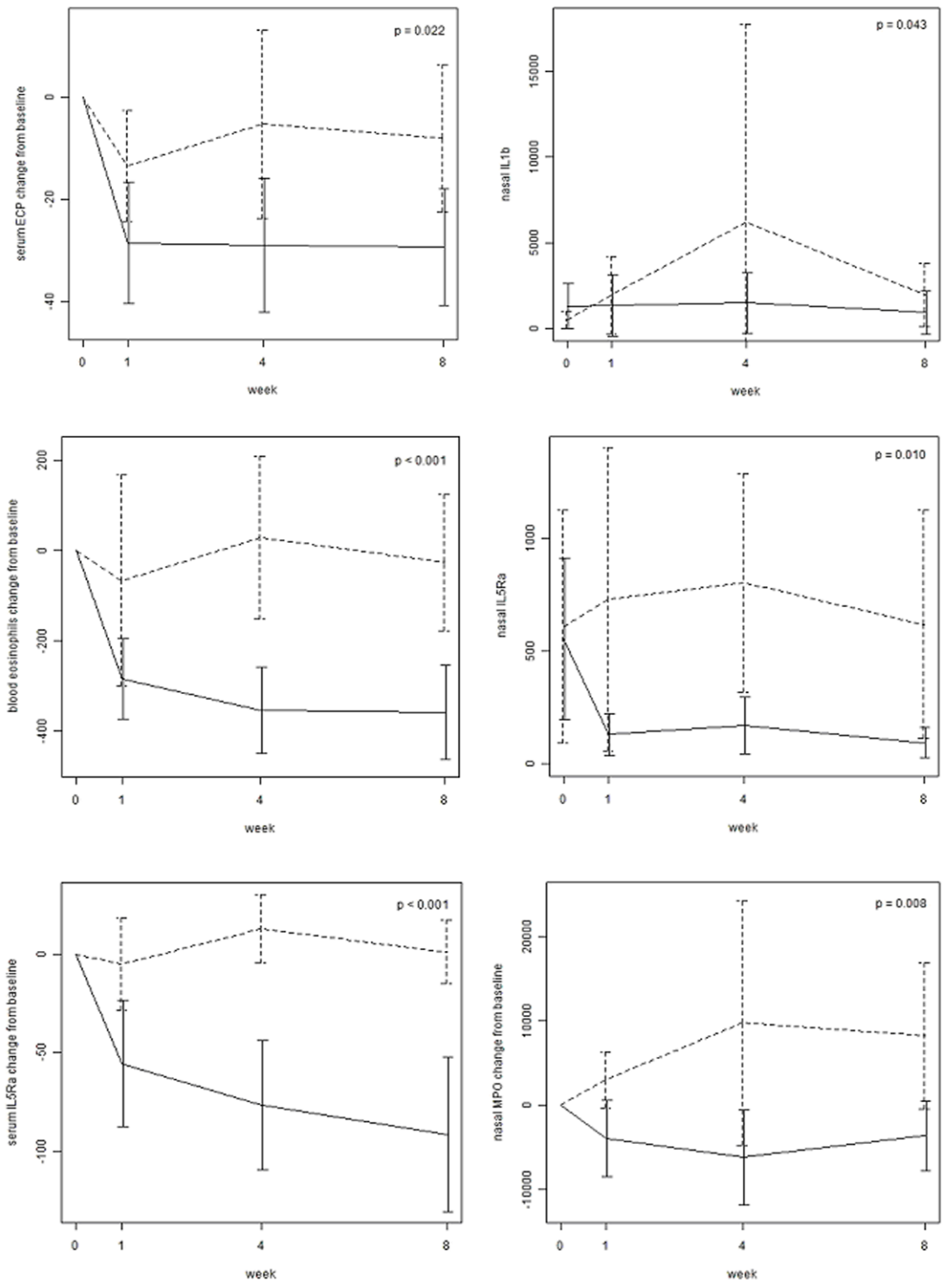

Figure 4: Fig 2. Mean CFB in serum ECP levels, blood eosinophil counts, serum IL-5Rα levels, and nasal MPO levels and mean nasal IL-1β and IL-5Rα levels (all in micrograms per liter), imputing 0 for observations of less than the LDL. These representations are based on LOCF and show the mepolizumab-treated (solid line) and placebo (dashed line) groups starting at the moment of first administration. Error bars indicate 95% CIs of the mean based on normal approximation.

Markers in nasal secretion

In contrast with nasal ECP (p=0.260 using LOCF), nasal IL-5 (p=0.094 using LOCF) and nasal total IgE (p=0.170 using LOCF) at week 8, which were not significantly different between groups, nasal IL-5Rα (p=0.010 for LOCF), nasal IL-6 (p=0.020 for LOCF) and nasal IL-1β (p=0.043 for LOCF) were significantly lower in the treated group. CFB at week 8 of nasal MPO (p=0.008 using LOCF) showed a significant reduction in the mepolizumab-treated group. Evolution of nasal IL-1β, IL-5Rα and MPO is also shown in Figure 4.

Dropouts

The proportions of treated and placebo patients still in the study at the different time points can be seen in Figure 5. There were 3 dropouts at the time of the primary endpoint (week 8), all of them in the placebo group. At the end of the study, there is a considerable dropout rate in both the mepolizumab and placebo arms. However, the time to drop out was significantly longer in the mepolizumab arm (p=0.005, log-rank test versus placebo). The reasons for dropout were comparable (Table 2). The most important were the need for rescue medication (5/20 in the mepolizumab-treated group and 3/10 in the placebo group) and nasal surgery with removal of NP (4/20 in the mepolizumab-treated group and 3/10 in the placebo group), which were said to be exclusion criteria.

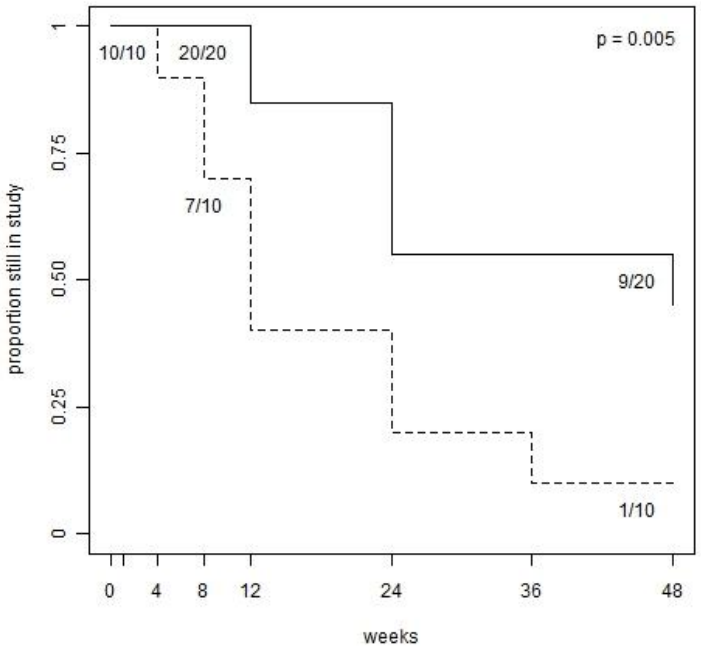

Figure 5: Proportion of patients still in the study in treated (solid line) and placebo (dashed line) groups.

Table 2: Overview of reasons for dropout in 'Mepolizumab treated' and 'Placebo' groups.

	Week 8 (primary time point)		Week 48 (end of study)	
	Mepolizumab treated	Placebo	Mepolizumab treated	Placebo
Still in study	20/20	7/10	9/20	1/10
Rescue operation	0/20	1/10	4/20	3/10
Rescue medication	0/20	1/10	5/20	3/10
Accidental medication	0/20	1/10	1/20	1/10
Did not show up	0/20	0/10	1/20	2/10

Responder analysis

The percentage of patients responding with an improvement in TPS at week 8 was 60% in the mepolizumab group. None of the baseline characteristics was significantly different between responders and non-responders. In particular for baseline TPS and local IL-5 levels we found no difference (p=0.97 and p=0.26).

DISCUSSION

In this double blind, randomized, placebo controlled study, we evaluated the effect of two injections of mepolizumab 750 mg IV in patients with severe CRSwNP. This treatment produced a significant reduction in TPS in 12/20 patients. These effects were confirmed by changes in CT scan evaluations. Together, the observations support a role for anti-IL-5 in a subgroup of patients with CRSwNP, and confirm previous results achieved with a single injection of a different anti-IL-5 antibody, reslizumab.[18] It is possible that additional doses of mepolizumab could lead to a larger impact on nasal polyposis or even resolution of the disease in a still to define subpopulation of polyp patients. Moreover, the rebound eosinophilia seen with reslizumab, was not observed using mepolizumab.

As previous studies showed, anti-IL-5 treatment is safe and well tolerated.[18,23,24] In our study, we did not observe significant differences of adverse events between the treatment and the placebo group.

Both groups had a mean TPS between 5 and 6 out of a potential maximum of 8 points at baseline, reflecting the severity of the disease as determined by the inclusion criteria. A higher proportion of patients in the treated group improved compared to placebo at week 4, and this number increased after the second dosing. A beneficial effect was seen in more than half of the treated patients one month post last dose. Because similar studies with anti-IL-5 treatment are lacking, we could only compare with our previous study.[18] This also showed a reduction in nasal polyp size in half of the patients. A meta-analysis testing the effect of intranasal steroids compared to placebo, found a decrease in nasal polyp assessment (score from 0 to 3, comparable to our TPS without grade 4) of 0.43-0.63,[25] while we observed a mean decrease of 1.30 (with 4 grades instead of 3), using mepolizumab.

Of importance, the changes in TPS were assessed objectively by repetitive CT scans, evaluated by 3 independent observers. CT scan imaging confirmed that more than half of the patients objectively profited from this potentially new therapeutic approach.

The typical symptoms that are so characteristic of CRSwNP, all showed trends towards improvement in the treated group, except rhinorrhea, but none of them reached statistical significance. Some of the effects were long lasting; the reduction of loss of smell in the treated group lasted for the whole period of follow up. Nasal congestion seemed to improve temporarily without reaching significance. Furthermore, nPIF changes compared to baseline

were superior in the mepolizumab-treated group, suggesting a decrease in nasal obstruction.

When analysing systemic and local markers of eosinophilic inflammation, we found a significant decrease in blood eosinophils in the treated group compared to the placebo group, also reflected by ECP in the serum. This is in line with the results of other studies in asthmatics and is considered the most important effect of the treatment in the hypereosinophilic syndrome.[26-28] The decrease in blood eosinophils was paralleled by a decrease in serum and nasal secretion IL-5Rα concentrations. Furthermore, nasal IL-6, MPO and IL-1b were significantly decreased, suggesting effects of treatment also on parameters of the neutrophilic inflammation present in CRSwNP.

In contrast to reslizumab, there was no reactive eosinophilia with mepolizumab; this counterregulation clearly was of concern in former studies.[18,23] However, increasing blood eosinophils with associated deterioration of the clinical condition is also reported with mepolizumab.[29] The rebound eosinophilia after anti-IL-5 treatment is a result of a serum factor that enhances eosinophil survival. Reversal of this effect by the addition of anti-IL-5 suggests that this factor may be IL-5 itself.[30] We suggest that rebound eosinophilia could be avoided by the administration of multiple doses of anti-IL-5 treatment. This effect was also seen before in studies with more than one injection.[23,28,29] Monthly administration of this treatment is supposed to be most appropriate, stabilizing the clinical course and preventing rebound eosinophilia.[29] However, one study found that improvement in symptoms and eosinophilia lessened with each subsequent dose.[23] It remains unclear whether prolonged treatment with anti-IL-5 could be used and what the effect would be.

As these patients suffer from severe and disabling disease, we observed clearly more dropouts in the course of the study in the placebo compared to the treatment group. Figure 5 shows that, at any point, the dropout was larger in the placebo group than in the mepolizumab-treated group. This difference was significant, indicating that dropout depends on treatment. In fact, the main reasons for exclusion were the need for systemic steroids and the need for surgery in the follow up period, both higher in the placebo group, although each individual reason for dropout was not statistically significant.

The comparison between responders and non-responders did not provide the expected proof of the relationship between response to treatment and concentrations of IL-5 in nasal

secretion at baseline, as seen in our previous study.[18] We also tested the effect of mepolizumab in the responder group. The decrease of TPS in responders was significantly maintained until 36 weeks after treatment, implying a long-term effect.

One of the major study limitations is the small sample size (n=30). This is probably the reason why we do not observe significant changes in symptom scores, although the nasal polyp score and CT scan significantly improved. Another study limitation is the long-term dropout rate, which makes interpretation of long-term follow up data difficult. Moreover, we only tested the administration of 2 injections of mepolizumab. More studies with a larger sample size and long-term treatment are required to determine the optimal treatment scheme for clinical use. Attention should be paid to parameters predicting treatment success, as this will be of clinical relevance. We believe anti-IL-5 treatment has a great potential, especially when we succeed in predicting the patients who would respond to the treatment.

In summary, two injections of 750 mg anti-IL-5 mAb (mepolizumab) showed a significant improvement over placebo of the endoscopic TPS. The TPS was decreased at week 8 in 12/20 patients with mepolizumab in contrast to 1/10 patients with placebo. In addition, 11/20 mepolizumab-treated patients showed an improvement in CT scan. Furthermore, the injection of two doses of mepolizumab was well tolerated, and no rebound eosinophilia was observed. IL-5 inhibition seems to be a promising novel therapeutic approach in patients with severe CRSwNP, but we require more long term studies to assess its full possibilities and indications. Better phenotyping could help to select the patients that would benefit from this treatment.

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Chapter VIII

Discussion and perspectives

TGF-beta in chronic sinus disease: dual role

Based on our findings, TGF-beta plays a key role in the pathophysiology of chronic rhinosinusitis, since its functions relate to both inflammation and remodeling processes.

A repeatable finding is the difference in TGF-beta protein concentrations in chronic rhinosinusitis with and without nasal polyps. Lower TGF-beta levels are a key feature of nasal polyps, whereas higher levels of TGF-beta are a persistent finding in chronic rhinosinusitis. Chronic rhinosinusitis without nasal polyps is characterized by a Th1 biased inflammation, with high levels of IFN-gamma, and fibrosis formation as a consequence of the up-regulation of TGF-ß. In contrast, Caucasian nasal polyps typically display a Th2 type inflammation, with high levels of IL5, total IgE and tissue eosinophilia, and edema formation as a consequence of the down-regulation of TGF-beta.

I. T-cell regulation in chronic paranasal sinus disease

In chapter 4, we have focused on the involvement of TGF-beta in T regulatory cell development and function. Chronic sinus diseases all show a T-cell mediated immune reponse[1], however divergence of T cell polarization is observed towards a Th1 cytokine profile in CRSsNP with high IFN-γ and TGF-beta1 concentrations, or a Th2 profile in CRSwNP characterized by eosinophilic inflammation with high levels of IL-5, IgE and low TGF-beta1. To date, there is no knowledge available on the intracellular mechanism behind this initial T cell polarization. Therefore, we analyzed the transcription factors that are critical in the development of the different T cell subtypes. T-bet skews differentiation towards Th1 cells, whereas GATA-3 skews towards Th2 cells, and regulates the expression of IL-4 and IL-5.

In recent years, evidence grew on the existence of a dedicated population of regulatory T cells (Tregs), a subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.

Tregs can inhibit Th1 and Th2 cells directly by cell-cell contact. These cells are called naturally occurring Tregs. Induced Tregs are generated in the periphery and have suppressive capacities via the production of TGF-beta and IL-10.

Shortly after the discovery of Treg cells, another subset of T cells, called Th17 cells filled the gaps in our understanding of T cell biology. Th17 cells have, via IL-17 production, proinflammatory properties through the induction of cytokines such as TNF-alpha, IL-1 beta

and IL-6. Experimental models suggest that TH17 cells may be important for neutrophilic inflammation in acute airway inflammation[2]. The transcription factor involved in Th17 differentiation is called RORγt, equivalent to RORc in humans[3].

We have analyzed the expression of TGF-beta in relation to T cell subsets including Th1, Th2, T regulatory and Th17 cells. To evaluate the role of Foxp3+ T regulatory cells (Tregs) in the pathogenesis and management of chronic rhinosinusitis, we investigated the location and expression of Foxp3 in CRSsNP and CRSwNP.

Knowledge on T reg cells in human disease is scarce so far, and studies mostly are based on *in vitro* experiments using peripheral blood derived T reg cells. We aimed to study the direct tissue expression of transcription factors that are responsible for differentiation towards the different T-cell subpopulations, in relation to the cytokine expression patterns in the different disease subgroups.

The expression of the transcription factors FOXP3, T-bet, GATA-3, RORc, the suppressive cytokines TGF-beta1, IL-10 and Th1/ Th2/ Th17 cytokines (IFN-γ , IL-4, IL-5, IL-13, IL17) were analyzed in CRSsNP, CRSwNP and controls. Additional protein measurements were performed for TGF-beta1 and IFN-γ.

We could show a deficit in FOXP3 expression in CRSwNP accompanied by an up-regulation of T-bet and GATA-3, coinciding with low TGF-beta 1 protein levels. In CRSsNP, FOXP3, T-bet, GATA-3 and RORc expression was not significantly different from controls, whereas TGF-beta1 mRNA, IFN-gamma mRNA and protein were significantly higher in CRSsNP compared to controls. For Il-17, no significant differences were noted between all groups.

As TGF-beta acts both as an effector and an inducer of Treg function, the decreased expression of FOXP3 and TGF-beta1 protein, together with the upregulation of both Th1 (T-bet) and Th2 (GATA-3) transcription signals suggests defective T regulatory function in CRSwNP.

Perspectives

These data improve the understanding of pathophysiology of CRSsNP and CRSwNP, opening new perspectives for therapeutic approaches, suggesting that Tregs might represent a specific therapeutic target.

Relevance to lower airway disease

Recent observations in asthma revealed comparable findings, demonstrating a decreased FOXP3 expression in CD4(+)CD25(high)T regulatory cells of peripheral blood mononuclear cells (PBMC) from asthmatics. There was also a tendency observed for increased FOXP3 expression in glucocorticosteroids treated asthmatics[4].

Hartl et al could demonstrate that T reg cells were decreased in broncho-alveolar lavage fluid of asthmatic children compared with values in children with cough or control subjects. In children with asthma, inhaled corticosteroid treatment was associated with increased percentages of CD4(+)CD25(hi) T cells in peripheral blood and broncho-alveolar lavage fluid[5].

Relevance in therapeutic monitoring and management

Li et al confirmed an increased number of FOXP3+ Tregs in nasal polyps (CRSwNP) after treatment with intranasal steroid (mometasone 50 micrograms/day for 4 weeks). They confirmed that FOXP3 and IL-10 were downregulated in CRSwNP compared to the control mucosa. FOXP3 and IL-10 expression were increased significantly after intranasal steroid treatment. These data confirm our results that FOXP3 is downregulated in CRSwNP and that intranasal steroids attenuate the chronic inflammatory response at least partially by enhancing the expression and function of Foxp3 in NP**[6].**

Relevance in phenotyping

Another study of our group extended this knowledge to a Chinese population. Th1/Th2 and Treg associated transcription factors were analyzed in Chinese NP and compared to western CRSwNP. Chinese, predominantly neutrophilic polyps share the downregulation of FOXP3 and TGF-beta1 with Caucasians; however, strikingly, Chinese nasal polyps have a mixed Th1 and Th17 pattern with significantly lower GATA3 expression and higher IL-17 concentrations compared to Caucasian polyps.

In western nasal polyps we repeatedly found low IFN-γ concentrations, whereas T-bet signaling was present. GATA-3 also has the capacity to directly inhibit IFN-γ promoter activity, resulting in repression of Th1 and development of a Th2 phenotype. The up-regulation of GATA-3 in CRSwNP was reflected by the subsequent increase of the IL-5 mRNA signal. Moreover, it is probable that posttranslational mechanisms suppress IFN-gamma expression.

II. TGF-beta signaling and collagen deposition in chronic rhinosinusitis

In chapter 5, we aimed to analyze the presence of TGF-beta isoforms and its receptors in chronic rhinosinusitis with or without polyp formation, and linked this to downstream intracellular signaling by measuring the number of phosphosmad 2 positive cells. TGF-beta 1 first binds to TGF-beta RII, this complex then recruits TGF-beta RI. After binding to TGF-beta, TGF-beta RII recruits and phosphorylates TGF-beta RI, leading to phopshorylation of Smad 2 and Smad 3. Phosphorylated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus. This process is inhibited by Smad 7. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression of TGF-beta target genes[7].

As outcome parameter for remodeling we analyzed collagen deposition within the extracellular matrix.

Inappropriate functioning of TGF-beta receptors has been implicated in several pathological conditions, such as carcinogenesis, rheumatoid arthritis, and fibrotic diseases. A better understanding of the relative roles of the three TGF-beta isoforms therefore seems crucial for a better understanding of the typical inflammatory and extracellular matrix changes observed in CRS.

Clear differences in the TGF-beta signalling cascade were observed between CRSsNP and CRSwNP, supporting the hypothesis that CRSsNP and CRSwNP are two distinct disease entities. Low levels of TGF-beta 1 protein concentration, a downregulation of its receptor TGF-beta RII and a low number of phosphosmad 2 positive cells in CRSwNP all indicate a low level of TGF-beta signalling in nasal polyp disease. In strong contrast, in CRSsNP, high TGF-beta 1 protein expression, increased TGF-beta RI expression, and a high number of phosphosmad 2 positive cells all point towards an enhanced TGF-beta signaling in CRSsNP. This is reflected by the typical extracellular matrix remodeling patterns observed. CRSwNP is characterized by a lack of collagen and tissue repair, whereas CRSsNP demonstrated fibrosis with excessive collagen production and thickening of the collagen fibres.

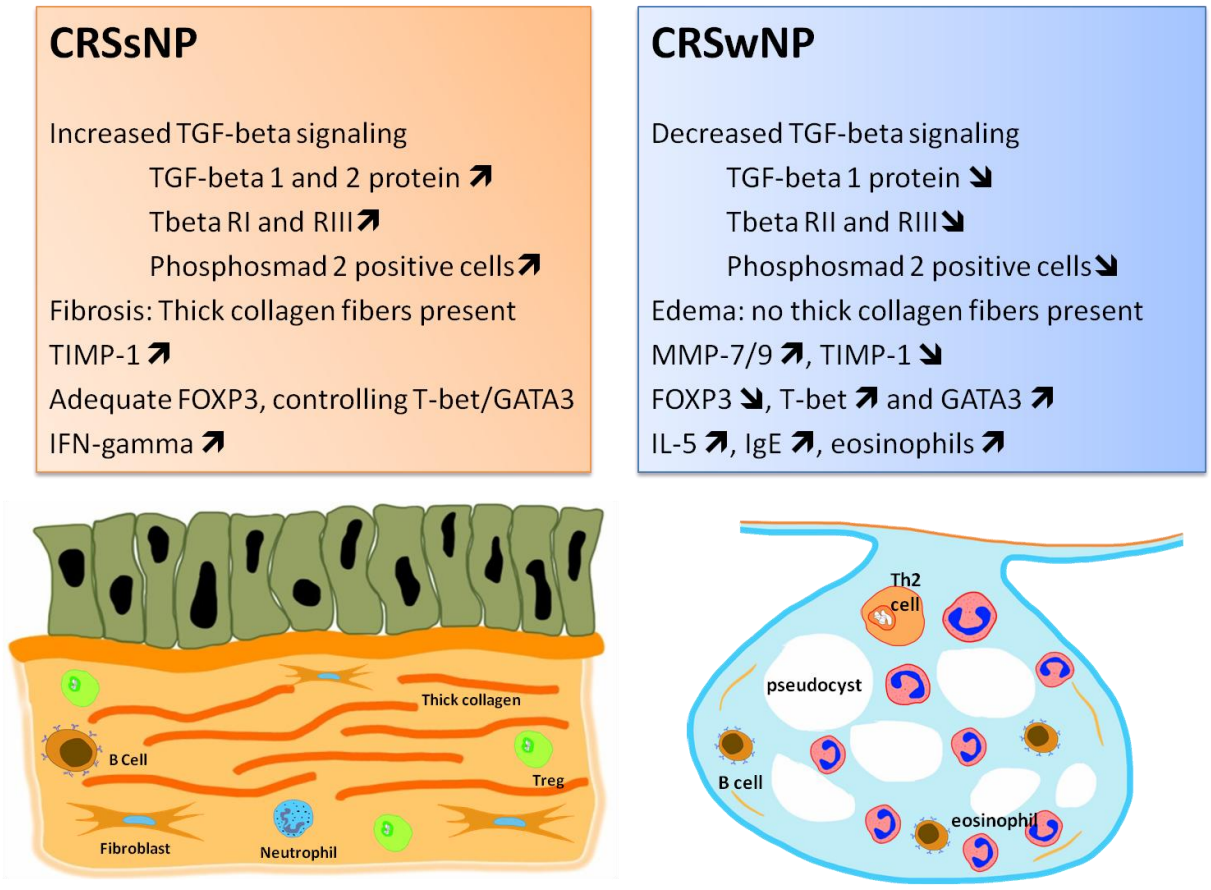

Figure: Differences in TGF-beta protein and receptor expression, downstream signaling (phosphosmad 2) and remodeling features.

Perspectives

Besides the impact of TGF-beta on T cell differentiation as described in chapter number 4, TGF-beta appears to be one of the critical factors involved in remodeling of upper airway disease.

TGF-beta acts as a master switch for the development of either chronic rhinosinusitis with or without polyp formation. TGF-beta impacts fibrosis formation through collagen production and the influence on the balance between MMPs and TIMP.

Even when looking at different ethnic populations, this finding seems to be consistent. Nasal polyps (CRSwNP) in the Caucasian population are commonly characterized by prominent tissue eosinophilia[8], (even more abundant present when comorbidities such as asthma and/or aspirin hypersensitivity are associated) and low levels of TGF-beta1 in former studies[1]. Recent studies in CRSwNP from South China, however, suggested that clinically equivalent NP disease also may exist with lack of tissue eosinophilia, and a lack of

IL-5 and eotaxin expression[12]. The remodeling patterns are more consistent than the inflammatory pattern, indicating that TGF-beta1 and its signalling may be a well-conserved key marker for CRS differentiation[9].

These data need to be confirmed on larger scale. Therefore, a large European multicenter study has been conducted with help of the European Commission (6th framework programme GA²LEN) in order to characterize patients with upper airway diseases on the basis of clinical parameters, infectious agents, inflammatory mechanisms and remodeling processes, and to differentiate the term chronic rhinosinusitis further to smaller disease entities based on clinical and biological parameters[10]. A specific module has been dedicated to TGF-beta and remodeling in chronic rhinosinusitis.

Relevance in therapeutic management

There are a number of possible approaches to decrease the action of TGF-beta in fibrotic disease. Experiments with anti-TGF-beta antibodies showed reduced synthesis of matrix proteins in nephritic rats[11]. Treatment of dermal wounds with anti-TGF-beta showed a reduction of the collagen deposition. Another possible therapeutic approach could be soluble TGF-beta receptors, which inhibit the binding of TGF-beta to its membrane receptor. Similarly, the latency associated peptide that is released during activation of TGF- beta could be used to inhibit the action of TGF-beta.

III. Inflammation and remodeling patterns in early stage chronic sinus disease

Chronic rhinosinusitis without polyp formation (CRSsNP) is typically characterized by an increased expression of Th1 cytokines with subsequent neutrophilic inflammation, high levels of TGF-beta and IFN-gamma, and expression of FOXP3, controlling GATA3 and T-bet expression, suggesting balanced T cell homeostasis in CRSsNP. Typical remodeling features include collagen deposition within extracellular matrix and thickening of collagen fibers.

However, little is known regarding the initial events that lead to the development of CRSsNP.

In Chapter 6, we aimed to study early events in the development of CRSsNP.

We have analyzed inflammation and remodeling parameters at different sinusal locations (the inferior and middle turbinates, the uncinate process, maxillary sinus, anterior ethmoid, bulla ethmoidalis and the posterior ethmoid) within a group of 9 patients with recently developed early chronic sinus disease without nasal polyps.

Local intersinusal differences in the expression of pro-inflammatory and remodeling cytokines were investigated. Only patients with early stage bilateral disease were included, with a Lund-Mackay–Score not higher than 12/24 after adequate treatment following the EP³OS guidelines.

In early-stage chronic sinus disease, TGF-beta protein is expressed in significantly higher concentrations within the paranasal sinuses when compared to control mucosa from inferior turbinates, concentrations where highest in the maxillary sinuses. Pro-inflammatory, neutrophilic and Th1 markers did not show any difference between the sinuses and turbinates.

These findings question the relation between inflammation and remodeling. Remodeling may be preceding inflammation, or may develop independent from inflammation; also the findings in nasal polyps point in this latter direction, as the remodeling pattern is largely similar in neutrophilic and eosinophilic polyps. Airway remodeling so far was assumed to be a consequence of chronic inflammation, but this relationship between the remodeling and inflammatory components is now questioned.

Relevance to lower airway disease

When comparing with lower airway disease in newly diagnosed asthma it is assumed that mucosal inflammation as well as remodeling are always present. However, based on studies on airway biopsies in children, it has recently been suggested that remodeling may occur

very early in asthma and may in some cases even precede clinical symptoms[12]. Our data in upper airway disease would support this possibility.

Several studies have attempted to correlate the degree of remodeling and the severity of asthma, but conflicting data have been obtained[13].

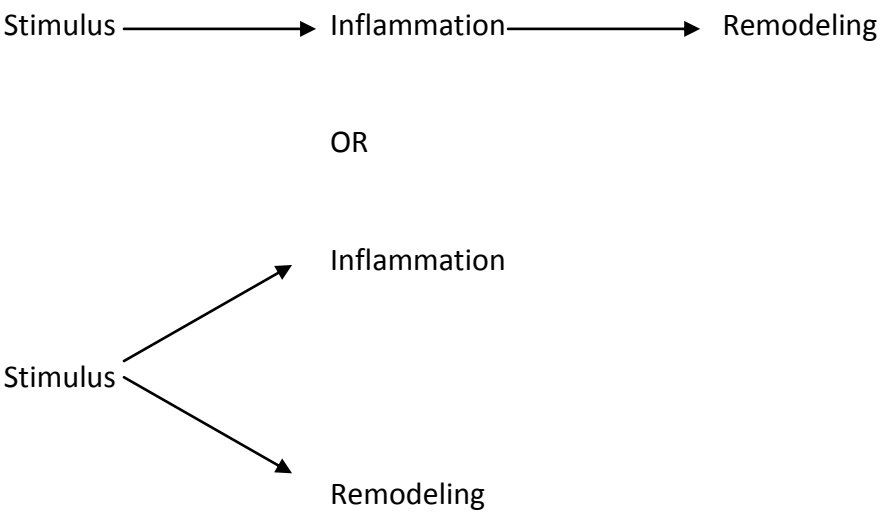

Figure 1. *Remodeling and inflammation*

It is often assumed that there is a linear progression between an initiating stimulus leading to inflammation, which in turn leads to remodeling. Alternatively, however, the same stimulus could independently lead both to inflammation and remodeling .

Perspectives

Despite the low number of patients in our study due to the fact that patients with early disease seldom would be indicated for surgery, it was striking to observe that no signs of inflammation were present. Although these findings need further investigation in larger numbers of patients, we can question the relation between inflammation and remodeling. In the case of a dissociation, both processes may need to be approached independently.

IV. Anti IL-5

Several therapies are utilized in the treatment of chronic rhinosinusitis, however, corticosteroids and antibiotics remain the basis of the current armentarium.

Research of the last years has led to better understanding of the pathogenesis and resulted in more tailored treatment options, in particular of nasal polyposis. The eosinophils have been suspected to play a key role in the pathogenesis of nasal polyposis. There is a clear relation between eosinophils and TGF-beta: TGF-beta is able to extinguish the prolonging effects of hematopoietins like IL-5, IL-3 and GM-CSF on eosinophil survival and to induce eosinophil apoptosis. The low TGF-beta levels observed in nasal polyposis may contribute to the massive eosinophilic inflammation observed in at least 80% of the Caucasian polyps[14].

In a first step, this has led to a safety and pharmacokinetic pilot study using humanized anti-IL-5 antibodies for the treatment of nasal polyps. In a double-blind, placebo-controlled, randomized, 2-center study, 24 subjects with bilateral nasal polyps were randomized to receive a single intravenous infusion of reslizumab, a humanized anti-human IL-5 mAb, or placebo. A single injection of reslizumab was safe and well tolerated; blood eosinophil numbers and concentrations of eosinophil cationic protein were reduced up to 8 weeks after treatment in serum and nasal secretions. However, individual nasal polyp scores improved only in half of the treated patients for 4 weeks. Responders had increased IL-5 concentrations in nasal secretions at baseline compared with non-responders, and logistic regression analysis revealed that increased nasal IL-5 levels predicted the response to anti-IL-5 treatment.

Therefore, a second randomized, double-blind, placebo controlled study with repeated injections of anti-IL-5 antibodies was needed. We aimed to determine the efficacy of two injections of mepolizumab on nasal polyp volume in subjects with severe nasal polyposis by using nasal endoscopy and CT-scan imaging as outcome parameters. In addition, markers of biological activity such as IL-5 and nasal eosinophilia were assessed over a period of eleven months post last dose.

Thirty subjects suffering from chronic rhinosinusitis with primary or post-surgery recurrent nasal polyps (grade 3 or 4, see outcome measures) were included.

Two injections of mepolizumab significantly reduced the size of nasal polyps for at least 2 months post dosing in 60% of the patients. These effects were confirmed by changes in the CT scan evaluations.

The comparison between responders and non-responders did not result in the expected relationship between response to treatment and nasal IL-5 at baseline, as seen in the first study. This discrepancy may be due to the fact that baseline IL-5 was identified as a predictor of response in a post-hoc analysis of the first study, which always should be considered with care. Furthermore, the number of patients in those studies does not allow firm conclusions.

We also wish to mention that the position of anti-IL5 treatment in comparison to the current armentarium needs further research, as in this current study we did not compare with conventional treatment.

However, these results underline the necessity of defining subgroups of patients for specific therapy based on clinical or biological parameters, even within a “clinical entity” such as nasal polyps.

Therefore, a large multicenter anti-IL5 study is currently conducted, in order to allow further differentiation into CRSwNP subgroups, based on inflammatory and remodeling parameters. This will allow a better fenotyping and probably predict response to treatment.

An issue that needs further attention is the long term safety. Mepolizumab has been utilized for the treatment of hypereosinophilic syndrome and asthma. The current clinical experience in the treatment of asthma patients has been recently reviewed by Busse et al. [15] These results point to no major safety concerns, in all cases mepolizumab was well tolerated and raised no major safety concerns.

General conclusions

Chronic rhinosinusitis represents an umbrella term for different disease phenotypes such as CRSwNP and CRSsNP, based on clearly distinguishable remodeling and inflammatory cell patterns. Remodeling and T regulatory cell activity are both subject to the regulation of TGF-beta, a major switch in sinus diseases, with a deficiency of T regulatory cells allowing for chronic inflammation. Both remodeling and inflammation are decisive for the expression of disease, and justify the introduction of specific phenotypes of CRS such as CRSsNP and CRSwNP; within CRSwNP, specific T cell cytokines such as IL-5 furthermore allow a further differentiation into endotypes responsive to novel treatment approaches by biologicals such as anti-IL5 humanized antibodies.

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Curriculum Vitae

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- "TGF-beta signaling in chronic sinus disease". XXIXth Congress of the European Academy for Allergy and Clinical Immunology, 7-11 juni 2008, Barcelona
- "Peak nasal inspiratory flow testing and sniff'n stick tests". GA²LEN survey meeting, Imperial college, 29 maart 2008, Londen
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- 9-16 juni 2007, XXVI Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Gothenburg, Zweden: "T-cell regulation in chronic sinus disease"
- 30 augustus 2007, First Scientific Interuniversitaire attractive polen (IAP) meeting, Gent: "T-cell regulation in chronic sinus paranasal sinus disease"
- 15-19 juni 208, 22nd ERS and the 27th ISIAN Congress, Crete, Griekenland: "TGF-beta signaling in chronic sinus disease"

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- Travel grant voor de abstract "TGF-beta signaling in chronic sinus disease". XXIXth Congress of the European Academy for Allergy and Clinical Immunology, 7-11 juni 2008, Barcelona
- Poster award voor "TGF-beta signaling and collagen deposition in chronic sinus disease". ERS and ISIAN 2008, 22nd congress of the European Rhinologic Society, 15-19 juni 2008, Kreta
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