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Inflammation and remodelling patterns in early stage chronic rhinosinusitis

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Summary

Background A distinct set of inflammatory and remodelling factors have been found elevated in chronic rhinosinusitis.

Objective The investigation of their expression in early stage disease may reveal early events in this common disease.

Methods Sinonasal mucosal samples from nine 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 signalling collagen deposition was analysed. Inferior turbinates from patients undergoing (rhino-) septoplasty were collected as controls.

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. TGF-beta 1 mRNA expression in inferior turbinates of CRSsNP was significantly higher when compared to inferior turbinates of controls ($P = 0.017$). The pro-inflammatory cytokines and Th1-related cytokines did not show an upregulation in inferior turbinates of CRSsNP when compared to controls.

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 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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Keywords chronic rhinosinusitis, inflammation, remodelling, TGF-beta

Abbreviations CRSsNP, chronic rhinosinusitis without nasal polyps; IFN-γ, interferon gamma; MPO, myeloperoxidase; T-bet, T-box transcription factor; TGF-beta 1, transforming growth factor beta 1; Th, T helper (cell); PCR, polymerase chain reaction.

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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 [2], estimated to affect up to 14% of the global population in the United States. Its aetiology is probably multifactorial, including anatomical factors, allergic inflammation, immune deficiency, microbial factors, and immune-microbial interactions [3].

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 remodelling patterns, chronic rhinosinusitis with polyp formation (CRSwNP) can be distinguished from chronic rhinosinusitis without polyp formation (CRSsNP) [4]. 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 upregulation 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 and localize early events in the development of CRSsNP, using inferior turbinate mucosa as comparator [4–6].

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

Material and methods

Patients

Nasal tissue was obtained from nine 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 (n = 10, median age 27 years, range 18–45, 4F/6M). None of the control patients had a history of allergic rhinitis and/or asthma and all controls were skin prick test negative.

The diagnosis of chronic rhinosinusitis without polyps (CRSsNP) was based on history, clinical examination, and nasal endoscopy and computed tomography according to the current EP^{3}OS guidelines [3]. Sinus CT scans were scored according to the Lund-Mackay system [1]. Herein we included only patients with early stage bilateral disease, with a Lund-Mackay–Score of not higher than 12/24 after adequate treatment following the EPOS guidelines (Fig. 3). Solitary unilateral maxillary or sphenoidal sinus disease was excluded from this study, as these manifestations may not necessarily lead to bilateral CRSsNP.

The ostiomeatal complex and the anterior ethmoid were the most frequent sinuses demonstrating mucosal thickening. All patients had 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 washout 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 to 3 (no symptoms, mild, moderate, severe).

General exclusion criteria were based on the EP^{3}OS 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 and control subject before collecting samples.

PCR

Gene expression analysis by means of quantitative real-time PCR

The cDNA was synthesized from 2 μg of RNA with the iScript cDNA synthesis kit (Bio-Rad 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) 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) 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 min followed by 40 cycles at 95°C for 30 s and at 60°C for 1 min and for reactions using TaqMan probes of 1.5 min at 95°C followed by 50 cycles: 15 s at 95°C and 1 min 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

Table 1. Patient characteristics and symptom scores

N	9
Gender: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.

geNorm software [7, 8]. 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/30 ng 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 min. 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, MN, 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 [9]. Tissue was fixed in formalin (Fluka, Belgium) and embedded in paraffin. Paraffin sections were prepared (thickness 4–5 μm) and air dried for 24 h at 37°C. Sections were deparaffinized, hydrated and stained with picrosirius red (direct red 80; Sigma–Aldrich, St. Louis, MO, USA) for 60 min. 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, Elkhart, IN, USA). The sections were analysed 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 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.

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

The 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.

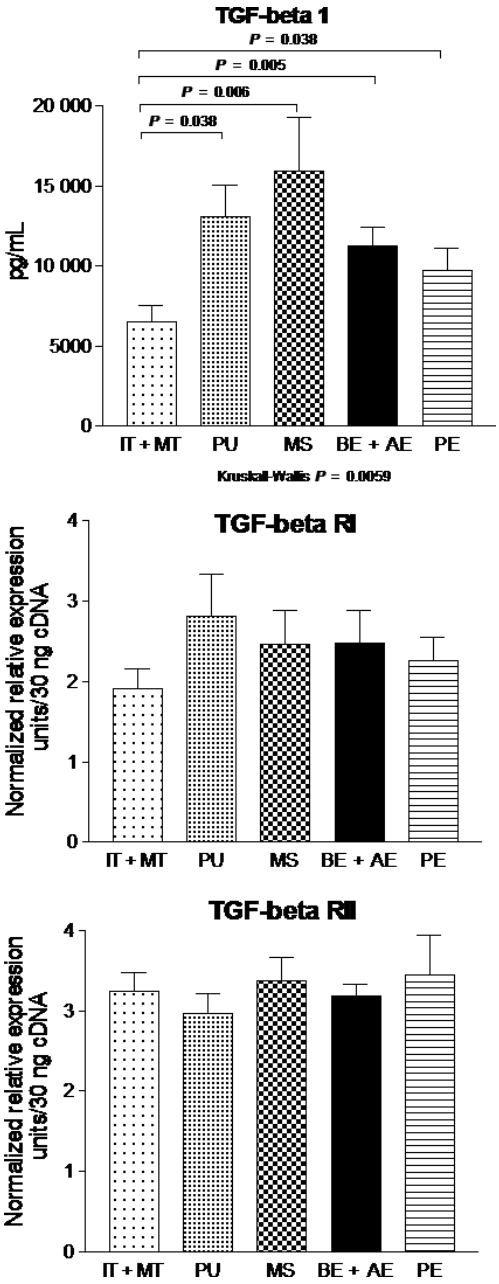

Fig. 1. Expression of TGF-beta 1 protein, and TGF-beta receptors I and II mRNA in sinunasal mucosal tissue. Inferior and middle turbinates served as control. IT, inferior turbinate; MT, middle turbinate; PU, processus uncinatus; MS, maxillary sinus; BE, bulla ethmoidalis; AE, anterior ethmoid; PE, posterior ethmoid.

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

Transcription factors T-bet and IFN-gamma, markers of a Th1driven 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 protein concentrations in inferior turbinates of CRSsNP vs. control patients showed no significant difference. The pro-inflammatory cytokines IL-1 and TNF-alpha and Th1-related cytokines did neither show an upregulation in inferior turbinates of CRSsNP when compared to control patients.

Picrosirius red staining for collagen (Fig. 4)

Picrosirius red staining was performed to assess collagen content in the extracellular matrix. Sections were examined through crossed polars (see Fig. 4a). Larger collagen fibres light up in bright orange and thinner fibres show green. This birefringence, also called double refraction is highly specific for collagen. Orange collagen fibres were present in significantly higher amount present in the maxillary sinuses (median percentage of area 41.17%) when compared to inferior turbinates (33.49%, $P = 0.008$), as presented in Fig. 4b.

Discussion

Inflammatory mucosal disease in the sinuses shows specific remodelling 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 [4, 6]. Moreover, it was recently shown that TGF-beta 1 and its receptors TGF-beta RI and RIII are strongly upregulated in CRSsNP, resulting in a high number of phospho-Smad 2-positive cells that indicate pro-fibrotic signalling [4–6]. This is reflected by a typical remodelling process characterized by a higher collagen deposition in CRSsNP together with the presence of thick collagen fibres when compared to healthy controls [5].

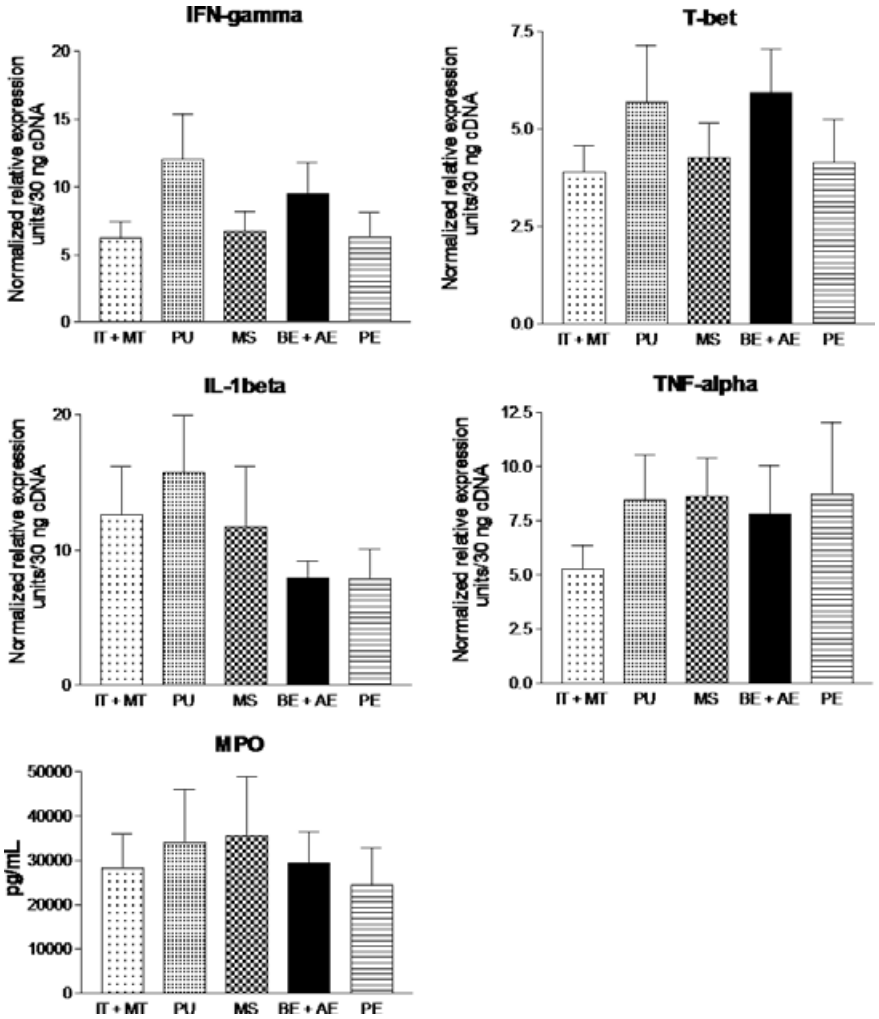

Fig. 2. Expression of mRNA for Th1 (IFN-gamma and T-bet) and pro-inflammatory (IL-1 beta and TNF-alpha) markers and protein of the neutrophil marker MPO in turbinates and sinus mucosa. No significant differences were noted between all groups. IT, inferior turbinate; MT, middle turbinate; PU, processus uncinatus; MS, maxillary sinus; BE, bulla ethmoidalis; AE, anterior ethmoid; PE, posterior ethmoid.

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

We could not find significant differences for TGF-ß at mRNA level between the various locations. However, it is well known that 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. 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 prior to the ELISA measurements. Subsequently, we could demonstrate that the upregulation 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 upregulated, we suggest that TGF-beta plays a crucial role in the regulation of remodelling 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 [4, 6]. As markers for a Th1 biased

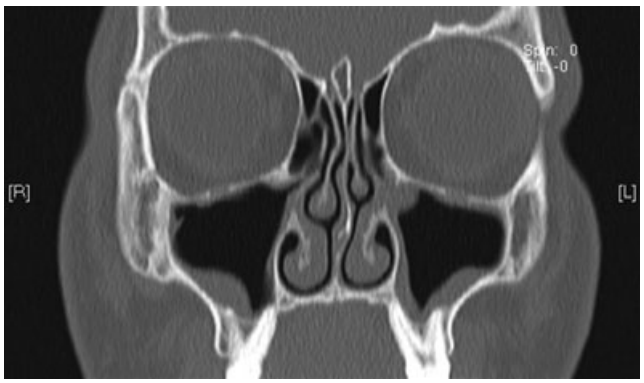

Fig. 3. CT scan: A typical CT scan of a patient with early stage CRSsNP, showing some opacification of the OMC-area and the maxillary sinuses.

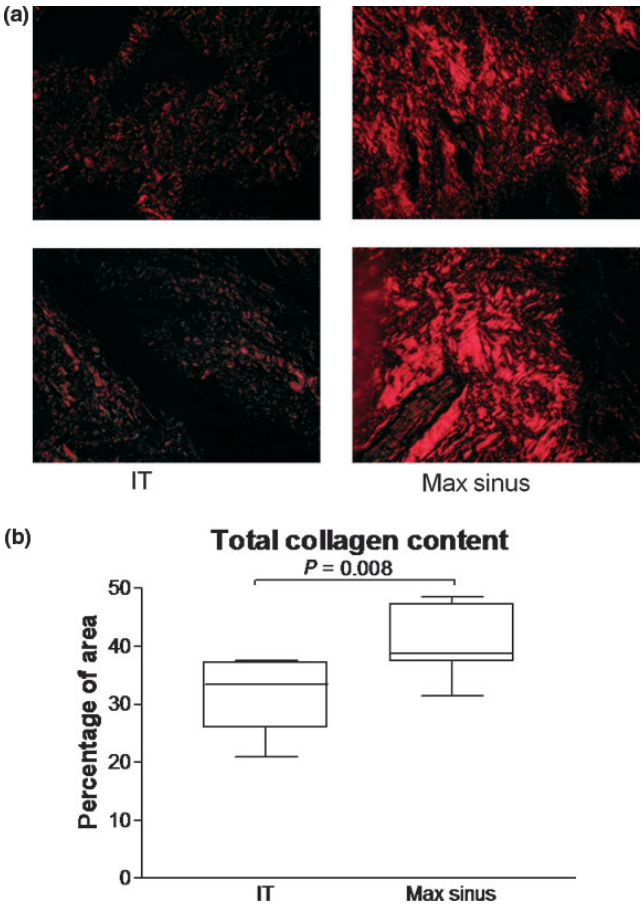

Fig. 4. (a) Picrosirius red staining for collagen in inferior turbinates (IT) and maxillary sinus (MS) viewed under polarized light. (b) Quantification by means of image analysis of total collagen content in inferior turbinates and maxillary sinuses demonstrating significantly higher collagen deposition in maxillary sinuses when compared to inferior turbinates

inflammation, T-bet and IFN-gamma were analysed. T-bet is a Th1 specific T box transcription factor that controls the expression of the hallmark Th1-cytokine IFN-γ [10]. T-bet and IFN-gamma were found to be upregulated in CRSsNP in previous studies in patients who suffered more severe disease with a median disease duration of 4.2 years [4–6]. 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.

The TNF-alpha and IL-1beta mRNA expression were measured as major pro-inflammatory cytokines [4], 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 upregulation 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 upregulation in inferior turbinates of chronic rhinosinusitis vs. 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 upregulated, we suggest that chronic rhinosinusitis is a TGF-beta plays a crucial role in remodelling 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 upregulation of TGF-beta 1 in advanced CRSsNP when compared to CRSwNP [6], coinciding with adequate expression of the T regulatory cell marker FOXP3 [6]. We have already demonstrated that inflammation and remodelling may be separate processes in upper airway disease, specifically in nasal polyps, which are likely to develop independently from each other. Whereas remodelling patterns in Chinese and Caucasian CRSwNP disease appear similar [11], inflammatory patterns in those polyps are clearly different between the ethnic groups [12], showing a Th2- vs. a Th17-biased inflammation. These observations suggest that different inflammatory patterns exist despite comparable remodelling patterns, questioning the association between inflammation and remodelling.

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 remodelling may develop in parallel [13, 14]. The central role of TGF-beta in airway fibrosis has been described extensively [13, 15]. It is often assumed that there is a linear progression between an initiating stimulus leading to inflammation, which in

turn leads to remodelling. However, this paradigm has recently been challenged also in lower airway disease [16, 17]. Based on studies on airway biopsies in children, it has been suggested that remodelling may occur very early in asthma and may in some cases even precede clinical symptoms [18]. Similarly, we found an initial upregulation of TGF-beta with subsequent collagen deposition in early stage chronic rhinosinusitis, however, further investigation is needed.

We wish to mention that this study is limited to cross-sectional data on inflammation and remodelling patterns in a small group of nine patients with limited chronic sinus disease existing for approximately 24 months. Indication for surgery in early stage chronic rhinosinusitis is an exception, and therefore it is difficult to obtain larger numbers of patients. However, despite small the number of cases, we could find significant differences in TGF-beta production and collagen deposition between different sites. 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.

The use of control tissue was restricted to inferior turbinates from patients undergoing septoplasty or rhinoseptoplasty with inferior turbinotomy, as it is considered unethical to surgically open healthy sinuses and resect sinusal mucosa in control subjects. In line with previous work of co-author Van Crombruggen et al. [19], no significant difference was noted at TGF-beta protein level between inferior turbinates of controls vs. chronic rhinosinusitis inferior turbinates. We wish to mention that the term 'early chronic rhinosinusitis' has been defined rather arbitrarily, as there is no current definition for early chronic rhinosinusitis. We here intended to include patients with bilateral, but limited disease showing persistent changes on CT scan after recommended medical therapy.

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 remodelling 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 upregulated within the paranasal sinuses, initiating the production of collagen and initiating a remodelling processes, whereas signs of inflammation are 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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Conflict of interest: The authors declare no conflict of interest.

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