

TGF- β signaling and collagen deposition in chronic rhinosinusitis

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

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

Methods: Sinonasal mucosal samples obtained from chronic rhinosinusitis with nasal polyps (CRSwNP; n = 13), chronic rhinosinusitis without nasal polyps (CRSsNP; n = 13), and controls (n = 10) were analyzed for TGF- β isoforms 1 and 2 by means of ELISA and IHC, and for TGF- β R1, 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- β 1 and 2 protein concentrations, TGF- β receptor (R) I and TGF- β RIII mRNA expression, the number of pSmad 2–positive cells, and total collagen amount were significantly higher in CRSsNP versus controls. In CRSwNP, TGF- β 1 protein concentration, TGF- β RII and TGF- β RIII mRNA expression, the number of pSmad 2–positive cells, and total collagen amount were significantly lower versus controls. Only TGF- β 2 protein was found higher in CRSwNP versus controls. **Conclusion:** A high TGF- β 1 protein expression, increased TGF- β RI expression, and a high number of pSmad 2–positive cells all indicate an enhanced TGF- β signaling in CRSsNP, whereas a low TGF- β 1 protein concentration, a decreased expression of TGF- β RII, and a low number of pSmad 2–positive cells in CRSwNP indicate a low level of TGF- β 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 fibers in the extracellular matrix in CRSsNP. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, collagen, inflammation, nasal polyposis, phospho-Smad 2, remodeling, TGF- β

TGF- β is a pleiotropic and multifunctional growth factor, with important immunomodulatory and fibrogenic characteristics. Many chronic diseases profit from the immunosuppressive effect of TGF- β ; 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- β could be understood as a counterregulatory 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 extracellular matrix (ECM) protein deposition and tissue structure.¹ Distinct disease entities can be distinguished within the group of chronic sinus diseases on the basis of different inflammation and remodeling patterns. Chronic rhinosinusitis without nasal polyps (CRSsNP) is characterized by high levels of IFN- γ and TGF- β 1.^{1,2} In contrast, chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a predominant T_H2-biased eosinophilic inflammation with high levels of IL-5, eosinophilic cationic protein, and eotaxin; high levels of local IgE; and low levels of TGF- β .¹⁻³ Nasal polyps typically consist of albumin accumulation and edema formation within the ECM (pseudocyst formation).⁴

TGF- β could play a crucial role in both suppression of airway inflammation and remodeling. TGF- β acts either directly through suppression of the activation of T cells and antibody secretion of B cells, or indirectly through the induction of regulatory T-cell types.⁵ Moreover, TGF- β is considered 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 ECM. Three different isoforms (TGF- β 1, 2, and 3) have been described that can bind to 3 membrane proteins, referred to as receptor (R) types I, II, and III.

Until now, the majority of investigations in upper airway disease have focused on the TGF- β 1 isoform, although recent studies in lower airway disease suggest a distinctive role for TGF- β 2, predominantly expressed in severe asthma and related to eosinophils.⁷ In addition, few data are available on TGF- β receptor expression and intracellular signaling in chronic sinus disease. TGF- β receptor signaling is a very complex mechanism and is mediated by several signaling steps involving dimerization and phosphorylation of receptor and intermediate molecules. Some of the most important proteins that modulate TGF- β ligand activity are the Smad proteins. After activation of TGF- β RI, phosphorylated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus. This process is inhibited by

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Abbreviations used

AEC:	Amino-ethylcarbazole
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
ECM:	Extracellular matrix
IQR:	Interquartile range
NREUm:	Normalized relative expression units
pSmad 2:	Phosphorylated Smad 2
TBS:	TRIS-buffered saline
TGF- β R:	TGF- β receptor
TIMP:	Tissue inhibitor of matrix metalloproteinase 1

Smad 7. Together with coactivators, corepressors, and other transcription factors, the Smad complex regulates target gene expression.⁸

The aim of this work was to study the expression of TGF- β isoforms 1 and 2 and receptor expression in chronic rhinosinusitis 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- β signaling. As outcome parameter for remodeling, picrosirius red stainings were performed to quantify collagen content and viewed with polarized light to assess 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 years; range, 18-45 years, 4 women, 6 men). Samples from patients with chronic rhinosinusitis (CRSsNP, $n = 13$, median age, 42 years; range, 34-78 years; 6 women, 7 men) and nasal polyposis (CRSwNP, $n = 13$; median age, 46 years; range, 34-78 years; 5 women, 8 men) were obtained during functional endoscopic sinus surgery 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 subjects or patients with CRSsNP had a history of asthma or a positive skin prick test. In the CRSwNP group, 4 of the 13 patients had a history of asthma, 2 of these patients had a positive skin prick test, and 1 patient had aspirin-exacerbated respiratory disease. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal cavities according to the current European EP³OS¹⁰ and American¹¹ guidelines. General exclusion criteria were based on the EP³OS definition for research. Patients with nonallergic rhinitis with and without eosinophilia and vasomotor rhinitis were also excluded. All patients stopped oral corticosteroids for at least 1 month and topical application for at least 2 weeks before surgery. Patients did not take any other relevant medication. Subjects with concurrent asthma were maintained on no more than 1000 $\mu\text{g/d}$ beclomethasone dipropionate or the equivalent. Patients who underwent previous 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- β 1 and 2

Tissue homogenates were assayed for total TGF- β 1 and 2 by using commercially available ELISA kits from R&D Systems (Minneapolis, Minn). Acid was added during the 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 nanograms per milliliter.

mRNA gene expression analysis

Quantitative real-time PCR. cDNA was synthesized from 2 μg RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif) following the manufacturer's instructions. mRNA levels of TGF- β RI, II, and III were determined by real-time PCR. Amplification reactions were performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using specific primer sequences (see this article's Table E1 in the Online Repository at www.jacionline.org). PCR reactions contained 30 ng cDNA (total RNA equivalent) of unknown samples, 1X SYBR Green I Master mix (Bio-Rad Laboratories) and 250 nmol/L 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 3 housekeeping genes, β -actin, hydroxymethyl-bilane synthase, and elongation factor 1, was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software (Ghent University Hospital, Center For Medical Genetics, Ghent, Belgium).^{12,13} The relative expression units of each gene per 30 ng cDNA sample was determined by using the qBase program (version 1.3.5; Ghent University, Belgium).

Immunohistochemistry for TGF- β 1 and 2 and TGF- β RI and II

Tissue was fixed in formalin (Fluka, Sigma-Aldrich, Bornem, 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, Haasrode, Belgium) in TRIS-buffered saline (TBS; pH 7.8) containing 0.001% NaN_3 (VWR International) for 20 minutes at room temperature. The sections were then washed with TBS for 10 minutes. Polyclonal antihuman antibodies TGF- β 1 and 2 were added and incubated overnight at 4°C. Polyclonal antihuman TGF- β RI and II antibodies were incubated for 60 minutes at room temperature. All polyclonal antihuman TGF- β antibodies (1, 2) and polyclonal antihuman TGF- β receptors (1, 2) were purchased from R&D Systems and diluted to 2 $\mu\text{g/L}$ in TBS/0.5 % BSA. Negative controls consisted of an antibody of the similar isotype. After the overnight incubation for TGF- β 1 and 2 and the 60-minute incubation for TGF- β RI and II, the slides were washed for 10 minutes in TBS.

A labeled polymer horseradish peroxidase (Dako Envision + System, Peroxidase (amino-ethylcarbazole; AEC) kit; Dako, Glostrup, 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 substrate-chromogen, which results in a red precipitate. Finally, sections were counterstained with Heamatoxyline (VWR International) for 2 minutes, washed extensively in running tap water, and mounted in Aquatex (VWR International).

Immunohistochemistry for pSmad 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 pSmad 2 (Ser 465/467) antibody (Cell Signaling Technology, Beverly, Mass) or control serum (rabbit serum, 1/100 in 2% BSA/TBS; Dako) for 1 hour. After washing in TBS for 10 minutes, a labeled polymer horseradish peroxidase (Dako Envision + System, Peroxidase (AEC) kit) 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 AEC substrate-chromogen, which results in a red precipitate. Finally, sections were extensively washed in running tap water and mounted in Aquatex (VWR International).

Image analysis. Positive intranuclear pSmad 2 staining was analyzed in 6 samples per group. Images from the entire tissue section were obtained with a 40 $\times$ objective lens (final magnification, 400 $\times$) and recorded on a digital camera (Olympus C-5050, Aartselaar, Belgium) with no overlapping zones. Positive intranuclear cell staining was quantified by using ImageJ software (Rasband WS, ImageJ, National Institutes of Health, Bethesda, Md, <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 were then rendered white to

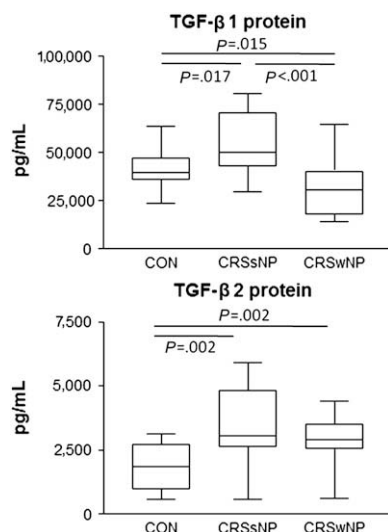


FIG 1. ELISA measurements for TGF-β 1 and 2, expressed as pg/mL. Tissue homogenates were prepared from control tissue (inferior turbinate), CRSsNP, and CRSwNP. CON, Controls.

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 Masson trichrome stainings.⁹ Paraffin sections were prepared as described. Sections were deparaffinized, hydrated, and stained with picrosirius red (direct red 80; Sigma-Aldrich, St Louis, Mo) for 60 minutes. The sections were then washed in 2 changes of acidified water, dehydrated in 3 changes of 100% ethanol, and mounted in Tissue-Tek (Miles Inc, Elkhart, Ind). The sections were analyzed by 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, whereas 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 40× objective lens (final magnification, 400×) 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 ImageJ software. Briefly, the entire section of a slide was captured by consecutive fields under bright-field at a final magnification of 400×, 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 (Schoonjans F, Mariakerke, Belgium). Data are expressed as median and interquartile ranges. When comparisons were made between groups, significant between-group variability was first assessed by using the Kruskal-Wallis test. The Mann Whitney *U* test, 2-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-β 1 and 2

TGF-β 1 protein concentration was significantly lower in CRSwNP (30,767 pg/mL; interquartile range [IQR], 18,669-

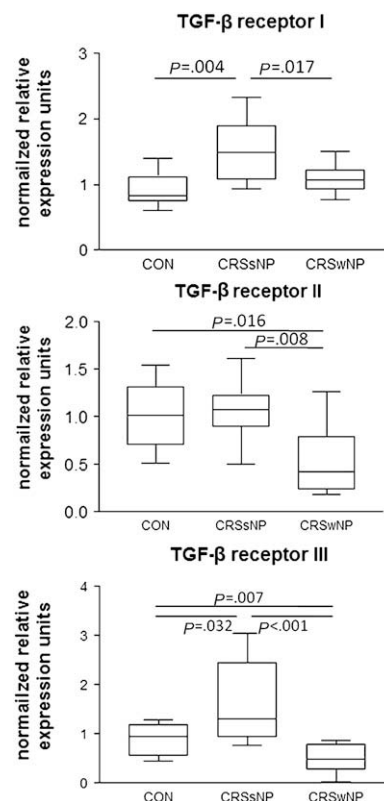


FIG 2. TGF-β RI, II, and III mRNA expression measured by means of RT-PCR in controls, CRSsNP, and CRSwNP. Data are expressed normalized relative expression units/30 ng cDNA. CON, Controls.

35,096) than controls (39,814 pg/mL; IQR, 36,131-45,750), but significantly higher in CRSsNP (50,135 pg/mL; IQR, 45,397-69,554) than controls and CRSwNP. TGF-β 2 protein was significantly higher in both CRSwNP (3091 pg/mL; IQR, 2662-3845) and CRSsNP (3068 pg/mL; IQR, 2825-4395) than controls (1852 pg/mL; IQR, 1298-2663; Fig 1).

RT-PCR data for TGF-β RI, II, and III

In CRSsNP, transcript levels expressed as normalized relative expression units (NREU) of TGF-β RI (1.52 NREU; IQR, 1.08-1.91) and TGF-β RIII (1.31 NREU; IQR, 0.97-2.39) mRNA were significantly higher than controls (0.84 NREU; IQR, 0.76-1.11; and 0.955 NREU; IQR, 0.55-1.11). In CRSwNP, TGF-β RII (0.43 NREU; IQR, 0.248-0.86) and TGF-β RIII (0.495 NREU; IQR, 0.27-0.79) were significantly lower than controls (1.02 NREU; IQR, 0.86-1.31 and 0.955 NREU; IQR, 0.55-1.11; Fig 2).

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

Immunohistochemistry for TGF-β 1 and 2, and for TGF-β RI and II

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

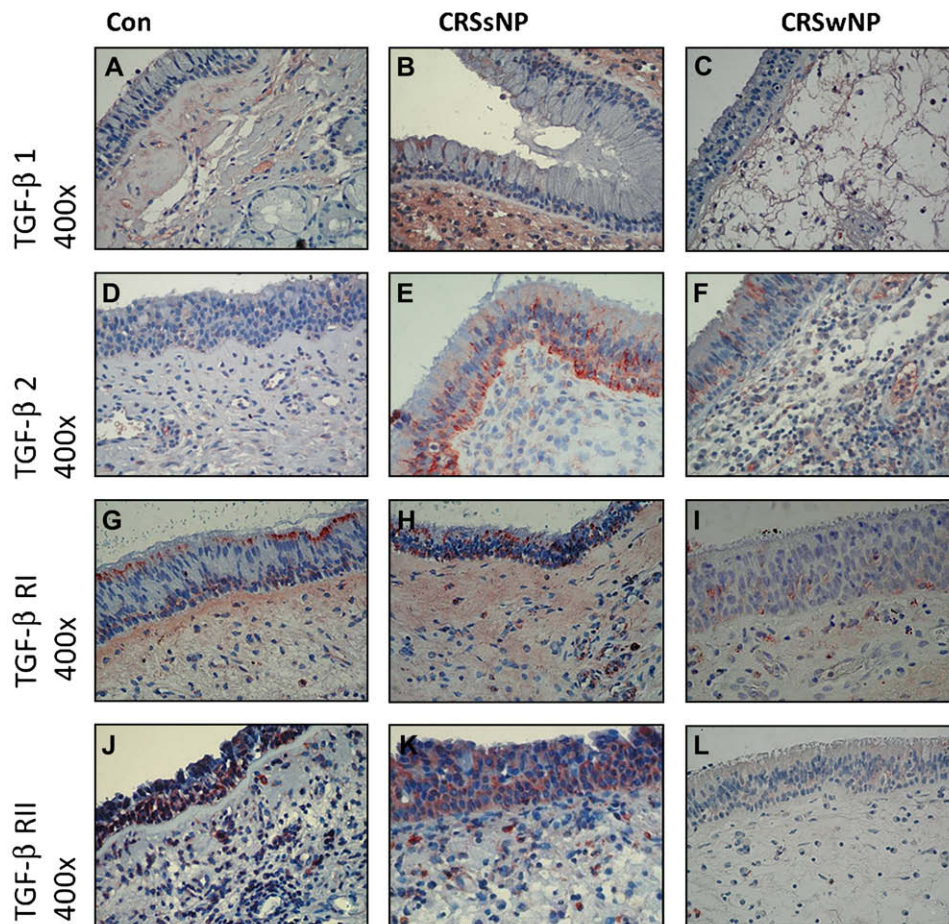


FIG 3. Immunostaining for TGF- β 1 (A-C) and 2 (D-F) and TGF- β RI (G-I) and II (J-L) in controls, CRSsNP, and CRSwNP. Epithelium, basal membrane, and subepithelial region are shown at a final magnification of 400 $\times$. Con, Controls.

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

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

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

Immunohistochemistry for pSmad 2

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

Picrosirius red stainings for collagen

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

Sections were additionally examined through crossed polars (Fig 4, J-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

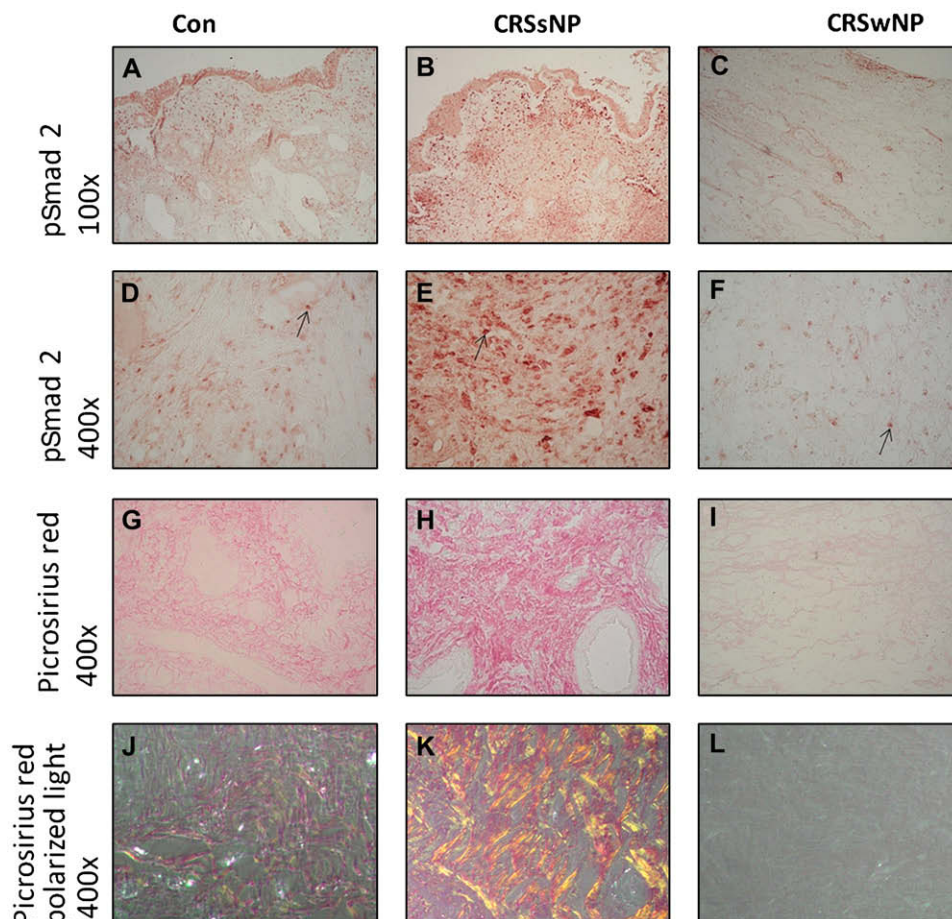


FIG 4. Immunostaining for pSmad 2 in controls (**A and D**), CRSsNP (**B and E**), and CRSwNP (**C and F**). Positive intranuclear staining is indicated by an arrow. Picrosirius red stainings for collagen in controls, CRSsNP, and CRSwNP, first viewed in bright-field microscopy (**G–I**), and viewed under polarized light (**J–L**) to assess fiber thickness. Con, Controls.

characteristic for thick collagen fibers. In contrast, almost no thick orange fibers could be detected in CRSwNP (Fig 4, J–L).

DISCUSSION

TGF-β is a multifunctional and ubiquitously expressed growth factor, of major interest in airway disease. TGF-β 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-β 1 between CRSsNP and CRSwNP, confirming previous studies.^{1,4,15} Low TGF-β 1 protein levels appear to be a constant finding in nasal polyp disease (CRSwNP), whereas TGF-β 1 upregulation is characteristic for CRSsNP. However, posttranslational modifications might complicate the interpretation of TGF-β measurements at the tissue level. The regulation of TGF-β is a complex mechanism. TGF-β is secreted in an inactive form to prevent binding to ubiquitously expressed receptors. Moreover, TGF-β can be associated to ECM proteins to assure an extracellular reservoir of TGF-β, which can be activated on demand. This association to the extracellular matrix was observed on immunohistochemical stainings in CRSsNP, demonstrating intense ECM staining for TGF-β 1. In addition, the TGF-β protein findings were linked to receptor expression and intracellular signaling.

TGF-β 1 first binds to TGF-β RII. This complex then recruits TGF-β RI. TGF-β RIII acts as a facilitator to the binding of TGF-β RI and TGF-β RII, and thus has no direct signaling role.¹⁶ After binding to TGF-β, TGF-β RII recruits and phosphorylates TGF-β RI, leading to phosphorylation 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 coactivators, corepressors, and other transcription factors, the Smad complex regulates gene expression of TGF-β target genes.⁸ Because TGF-β RI and RII are both necessary for TGF-β signaling, a decreased expression of TGF-β RII observed in CRSwNP together with low TGF-β 1 protein concentration suggests suppressed TGF-β signaling. In strong contrast, in CRSsNP, the increased TGF-β RI expression was observed together with high TGF-β 1 protein expression. TGF-β RIII, which acts as an enhancer for TGF-β RI and RII binding, was found decreased in CRSwNP and higher in CRSsNP compared with controls, further supporting the decreased TGF-β receptiveness in CRSwNP and increased TGF-β susceptibility in CRSsNP.

As a downstream signal of active TGF-β, the expression of pSmad 2 was analyzed. Smad 2 proteins are a family of transcription factors and are the only TGF-β 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

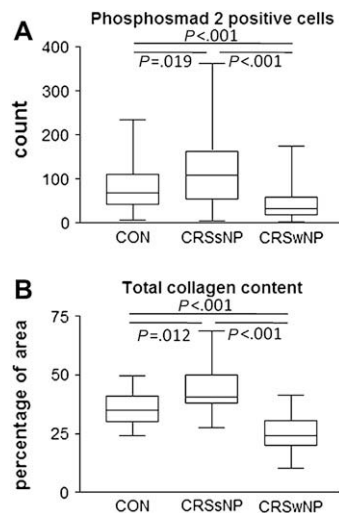


FIG 5. **A**, Quantification by means of image analysis of the number of pSmad 2-positive cells in controls, CRSsNP, and CRSwNP. **B**, Quantification by means of image analysis of total collagen content in controls, CRSsNP, and CRSwNP. CON, Controls.

number of positive cells was higher in CRSsNP and lower in CRSwNP compared with controls, pointing toward increased active TGF- β signaling in CRSsNP and suppressed signaling in CRSwNP.

Remarkably, little is known about the presence of TGF- β 2 in sinonasal tissue. In contrast with previous findings at mRNA level,¹⁹ we here show a differential expression of TGF- β 1 and 2 at protein level in CRSwNP. Recent studies in lower airway disease suggest a distinctive role for TGF- β 2, predominantly expressed in severe asthma and mainly related to eosinophils.⁷ Similarly, CRSwNP, a disease characterized by severe eosinophilic inflammation, revealed a higher expression of TGF- β 2 compared with controls. However, no correlation could be found in biopsies of CRSwNP between eosinophilic cationic protein, an important end product of eosinophils, and TGF- β 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- β 2 levels. On the basis of our immunohistochemical findings, we could detect TGF- β 2-positive eosinophils; however, epithelial cells and other inflammatory cell types also stained positive for TGF- β 2. Moreover, total TGF- β 2 protein levels were also increased in CRSsNP, a disease lacking tissue eosinophilia.

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

We here show a low amount of collagen present in the ECM of CRSwNP compared with controls, and an absence of thick collagen fibers when viewed under polarized light. The lack of TGF- β 1 in CRSwNP can be interpreted as a lack of tissue repair, reflected by loose connective tissue and edema formation in a severely inflamed tissue. In contrast, TGF- β 1 levels are higher in CRSsNP compared with controls, together with TGF- β RI and

RIII, and with a higher number of pSmad 2-positive cells. A higher collagen content was present in CRSsNP compared with controls. This is indicative of excessive tissue repair and fibrosis formation in CRSsNP.

Corticosteroids are currently the recommended treatment for both chronic rhinosinusitis with and without nasal polyps. In this study, almost all patients were treated with topical corticosteroids for 3 months. Treatment failure of corticosteroids indicated the surgery. A washout period of 4 weeks was maintained before the actual surgery. To our knowledge, there are no data available on the effect of the previous use of topical steroids on TGF- β 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- β expression, as observed earlier in severe lower airway disease.^{25,26}

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

Conclusion

Clear differences in the TGF- β signaling cascade are observed between CRSsNP and CRSwNP. This supports the hypothesis that CRSsNP and CRSwNP are 2 distinct disease entities. A low TGF- β 1 protein concentration, a decreased expression of TGF- β RII, and a low number of pSmad 2-positive cells in CRSwNP all indicate a low level of TGF- β signaling in nasal polyp disease. In strong contrast, in CRSsNP, high TGF- β 1 protein expression, increased TGF- β RI expression, and a high number of pSmad 2-positive cells all indicate an enhanced TGF- β signaling in CRSsNP. This is reflected by the typical ECM 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.

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

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METHODS

ELISA for TGF- β 1 and 2

Samples were snap-frozen in liquid nitrogen and stored at -80°C until homogenization.

For homogenization, the tissue was thawed and weighed, and 1 mL 0.9% NaCl with protease inhibitor Complete (Roche, Mannheim, Germany) was added per every 0.1 g tissue. The tissue was then homogenized using a B. Braun homogenizer (B. Braun, Melsungen, Germany) for 5 minutes. The homogenates were centrifuged at 3000g at 4°C for 10 minutes. After centrifugation, 250- μL aliquots were made and stored at -80°C until needed for ELISA. To release latent TGF- β from the ECM, samples were treated with acid before ELISA. TGF- β 1 and 2 levels were determined by using commercially available ELISA kits from R&D Systems (Minneapolis, Minn). All data were expressed as nanograms per milliliter.

mRNA Gene expression analysis

RNA preparation and cDNA synthesis. Clinical tissue samples (30 mg) were homogenized in Lysis Buffer (Bio-Rad Laboratories, Hercules, Calif). Total RNA isolation was performed by using the Aurum Total RNA kit (Bio-Rad Laboratories) following the manufacturer's instructions. RNA quality and percent of degradation were assessed with the Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, Calif). cDNA was synthesized from 2 μg RNA with the

iScript cDNA synthesis kit (Bio-Rad Laboratories) following the manufacturer's instructions.

Quantitative real-time PCR. mRNA levels of TGF- β RI, II, and III were determined by real-time PCR. Amplification reactions were performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using specific primer sequences (Table E1). PCR reactions contained 30 ng cDNA (total RNA equivalent) of unknown samples, 1X SYBR Green I Master mix (Bio-Rad Laboratories), and 250 nmol/L 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 3 housekeeping genes, β -actin, hydroxymethyl-bilane synthase, and elongation factor 1, was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software.^{E1, E2} The relative expression units of each gene per 30 ng cDNA sample was determined by using the qBase program (version 1.3.5; Ghent University, Belgium).

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TABLE E1. Primer sequences used for real-time PCR amplifications*

	Forward (5' → 3')	Reverse (5' → 3')	Amplicom size (bp)	Accession number
TGF-β R1	CTTGTAAGCCAAGTTTTTCACCC	CTCCACATGCTTAGGGGTGT	123	NM_004612
TGF-β R2	GCAGGTGGGAAGTGAAGAT	GAAGGACTCAACATTCTCCAAATTC	74	NM_003242
TGF-β R3	ACATGGATAAGAAGCGATTCAGC	AACGCAATGCCCATCACGGTTAG	331	NM_003243

TGFb1R: *Reference:* Electronic PCR, Genbank (<http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=40540>).

TGFb2R: *Reference:* Sakaguchi J, Kyo S, Kanaya T, Maida Y, Hashimoto M, Nakamura M, Yamada K, Inoue M. Aberrant expression and mutations of TGF-β receptor type II gene in endometrial cancer. *Gynecol Oncol* 2005;98:427-33.

TGFb3R: RTPrimerDB ID: 2677 (<http://medgen.ugent.be/rtpimerdb/>).

*Sequences were obtained from the Real-Time PCR primer and probe database (<http://medgen.ugent.be/rtpimerdb/>).