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Interleukin 5, IL6, IL12, IFN-γ, RANTES and Fractalkine in human nasal polyps, turbinate mucosa and serum

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Abstract Polyps are considered to develop as an end result of an inflammatory process. Cytokines and chemokines in the respiratory mucosa may be a key to polyp pathophysiology. The main objective was to identify IL-5, IL-6, IL-12, RANTES, IFN-γ and Fractalkine in humans on the protein level in nasal polyps and mucosa from the inferior turbinate (IT). Furthermore, the cytokines and chemokines RANTES and Fractalkine were analyzed in plasma. Tissue homogenates and plasma from 13 patients were analyzed by the ELISA technique. All the patients had longstanding nasal/paranasal polyposis. Fractalkine was detected in polyps and IT in two different patients. IL-5 was expressed in polyps and IT. IL-6 was expressed in all patients with a higher level in polyps than IT. IL-12 was present in plasma, polyps and IT, though at an increased level in polyps. RANTES was present at a higher level in plasma than in polyps and IT. IFN-γ was detectable in polyps and IT. Fractalkine is detected in nasal polyps, which is a new observation. The overall results indicate a mixed T_H1/T_H2 cytokine profile in nasal polyps. RANTES and IL-12 are strongly present in plasma, suggesting an ongoing inflammatory "drive". IL-6 and IL-12 are up-regulated in polyps versus the IT. Up-regulation of IL-6 may be explained by increased fibroblast activity dependant on an ongoing local inflammation possibly initiated by an infection. IL-5, RANTES and IFN-γ are equally represented in polyps and IT, indicating equilibrium between the nasal polyps and surrounding tissue, and that an up-regulation of cytokines in the polyp indicates a potential for polyp growth.

Keywords Nasal polyp · Cytokines · Chemokines · Inflammatory mechanisms

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Introduction

In this study we aimed to identify IL-5, IL-6, IL-12, RANTES, IFN-γ and Fractalkine in nasal polyps, mucosa from the inferior turbinate and serum of human subjects. The etiology of nasal polyps is not known, but one plausible explanation for the development of polyps could be as an end result of a prolonged inflammatory process. In nasal polyps there is an increase in the inflammatory cell population. Eosinophils dominate, but there are also mast cells, different lymphocytes and plasma cells.

Bachert and Gevaert in 1999 stated that the key to understanding polyp pathophysiology was the regulation of the recruitment, activation and survival of eosinophils, as well as their effect on polyp formation and growth [1]. Mediators on the molecular level such as IL-5 and eotaxin are considered to be key factors for eosinophilic accumulation and activation in nasal polyps [2]. Bernstein in 2001 debated the molecular biologic events in the development of nasal polyps. Chemokines such as RANTES and eotaxin are responsible for the movement of eosinophils into the lamina propria of the nasal polyp. The release of major basic protein has an effect on the alteration of the epithelial architecture. These two events, the release of major basic protein from the eosinophil and the alteration of the architecture of the surface epithelium, lead to an increase in sodium absorption and the resulting oedema, the hallmark of

pathology of the nasal polyp [3]. The cytokine pattern in nasal polyps most often assumes neither a T helper 1 (T_H1) nor a T_H2 type predominance, because IL-4, IL-5, IL-12 and IFN-γ all have been shown to be up-regulated in nasal polypoid tissue without the influence of the atopic status [4]. However, the clinical material is in many studies of a heterogeneous nature, and in the present study we used strict clinical selection criteria and a meticulous operative technique.

Our group has previously reported, by the use of reverse transcriptase polymerase chain reaction, a positive identification in situ of the mRNA expression of IL-6, IL-12 and RANTES in patients with polypoid disease and chronic sinusitis with or without known allergy of the upper airways [5].

As a follow-up of our previous study [5], we wanted to identify the pattern of IL-6, IL-12 and RANTES on the protein level in a separate and well-defined clinical material. By including a T_H2 cytokine-IL-5 [6, 7, 8] and a T_H1 cytokine-IFN-γ [9, 10, 11, 12], we wished to focus on a potential shift in the T_H1 and T_H2 profiles. We also wanted to investigate Fractalkine, a CX3C chemokine, not previously identified in inflammatory upper respiratory mucosa or specifically in nasal polyps.

Anacuta et al. [13] showed that Fractalkine preferentially mediates the arrest and migration of CD16+ monocytes and suggest that recruitment of this proinflammatory monocyte subset to vessel walls occurs and thus may contribute to vascular and tissue injury during pathological conditions. Anacuta et al. [13] did not differentiate between monocytes and natural killer (NK) cells, on which the CD16 antigen might be primarily expressed. In our present study, we did not look into NK cells in particular. In addition, Fractalkine has been reported to be a chemoattractant to leukocytes in vitro and to act as a chemoattractant in the CNS in vivo [14]. By examining mucosa of the inferior turbinate from each patient we intended to reveal any potential limitation of the inflammatory response. Detailed knowledge of cytokines and chemokines in respiratory mucosa in inflammatory conditions such as nasal polyps is of considerable interest in developing new therapeutic approaches [9].

Materials and methods

Materials

Nasal polypoid tissue was obtained from 13 patients with bilateral nasal/paranasal polyposis (12 patients) and unilateral polyposis (1 patient). The patient group included three females and ten males aged 30 to 72 years (mean 54.8 years). Our primary inclusion criteria were: (1) visible polyps in the middle meatus or free nasal cavity and (2) no previous major surgery of the nose and sinuses. Our exclusion criteria were: (1) previous major surgery of the nose and sinuses, (2) severe lung disease treated with systemic steroids, (3) multi-allergies, (4) systemic disorders such as rheumatism, diabetes and hypo-/hyperthyroidism, (5) diffuse polypoid rhino-sinopathy (DPRS), (6) antero-choanal polyp, (7) unilateral polyposis with malignancy not excluded and (8) unilateral polypoid formation secondary to infection, e.g., dental sinusitis. One patient (no. 1) had unilateral polypoid formation. Specific infection or malignancy was excluded and the patient was included in the study.

Methods

Blood sampling

At the primary consultation a blood sample (20 ml EDTA blood) was taken from each patient included (sample 1) to be examined both by standard laboratory methods and by ELISA (IL5, IL6, IL12, RANTES, IFN-γ and Fractalkine). Until the time of surgery the included patients were instructed to regularly wash their nasal cavities by means of a ''wash-out'' (isotonic saline solution). Topical steroids or systemic medication other than what was known on the primary examination was not allowed. One month later, a secondary examination prior to surgery was agreed upon. The included patients were then re-examined and a new blood sample (20 ml EDTA blood) was taken (sample 2) for standard testing and ELISA. All patients primarily included were thereafter remitted to surgery.

Surgical procedure

The aim of the surgery was: (1) to collect polypoid tissue, (2) to obtain a biopsy (1.0–1.5 cm) from the posterior part of the inferior turbinate and (3) to complete a normal surgical procedure. An endoscopic endonasal approach, performed by one surgeon (AD), was used in all 13 cases. Meticulous surgical care was taken to avoid leakage from the polyps and to minimize bleeding.

Laboratory techniques

Sample1 For the first sample, 20 ml EDTA blood collected in tubes (BD Vacutainer Systems, Plymouth, PL6, UK) was separated on Lymphoprep (Axis-Shield PoC As Oslo, Norway), then 5 ml blood diluted 1/2 in PBS was placed on 10 ml Lymphoprep. After washing of the lymphocytes, they were counted and subsequently stored at –70°C. Plasma was stored at –70°C until analysis.

Sample 2 For the second sample, 20 ml EDTA blood was treated as in the first sample. When thawed, distilled water was added to the lymphocytes so that each specimen contained 6 million lysed lymphocytes. Nasal polyps and specimens from the inferior turbinate were weighed and stored at –70°C until analysis. When

thawed, 1 ml of 0.9% NaCl was added per every 0.1 g of the nasal polyp and the specimens from the inferior turbinates. The tissue was then homogenized with a mechanical homogenizer (Heidolph Diax 900, Germany) 4–5 min at 3,000 rpm on ice. After homogenization the suspensions were centrifuged for 10 min at 1,400 g and the supernatants were stored at –70 °C until analysis. Supernatants from lymphocytes, supernatants from nasal polyps, conchae specimens and plasma were all assayed for IL-5, IL-6, IL-12 (p40), IFN-γ and RANTES by means of Elisa with commercially available kits from Biosource International Inc., Camarillo, Calif., following the manufacturer's instructions. Supernatants and plasma were also assayed for Fractalkine. Polyclonal anti-human Fractalkine, biotinylated anti-human Fractalkine and recombinant human Fractalkine were purchased from BioSite, Täby, Sweden, and the following Elisa was performed.

Since the tissues (polyp and IT) were homogenized, we had relative protein concentrations that were measured according to Bio-Rad Protein Assay techniques (Bio Rad Laboratories, US). Standards have been used to estimate the protein concentration (standard 1: bovine gamma globulin; standard 2: bovine serum albumin).

Elisa

Costar high-binding plates (Costar, Cambridge, Mass.) were coated with polyclonal anti-human Fractalkine 1 μg/ml PBS and left at room temperature overnight. After washing with PBS/0.05%Tween, the plates were blocked with PBS/1%BSA for 1 h. Specimens and standard (in twofold dilution ranging from 2,000 pg/ml diluent buffer) were added, after washing, and the plate was left at room temperature for 2 h. After washing, biotinylated anti-human Fractalkine 0.3 μg/ml diluent buffer was added and the plates left at room temperature for 2 h.

Streptavidin-HRP (R&D, Minneapolis, Minn.) diluted 1/200 in diluent buffer was added, after washing, and the plates left at room temperature for 30 min. After washing, substrate [3.3,, 5,5,-tetramethyl-benzidine (TMB), Sigma-Aldrich Corporation, St. Louis, Mo.] was added. The reaction was stopped after 30 min with 1 M H_2SO_4. PBS with 0.05% Tween 20 and 0.1% BSA was used as diluent buffer. All steps were carried out with 100 μl volume. All specimens and standards were performed in duplicates. The optical density (OD) was read at 450 nm with the E-max precision microplate reader (Molecular Devices Corporation, Sunnyvale, Calif.).The software SOFTmax (Molecular Devices Corporation) was used to calculate the values.

Statistical analysis

Cytokine levels from blood, the inferior turbinate and polypoid tissue in 13 patients were quantified. Based on these data we calculated the mean value for each tissue, e.g., the mean value of IL-6 in blood for the whole group. This gave us three means, one each for plasma, polyp and IT, which we subsequently compared. We found the observations to be independent and normally distributed (no ''dangerous'' skewness). We therefore chose Student's t -test. The significance level was set at five percent, and double-sided significance was used.

Results

The protein concentration in plasma, polyps and the inferior turbinate (IT) was measured and is considered to be an indicator of cytokine production in these tissues (Figs. 1 and 2). Statistical analysis showed a non-significant difference in protein concentration between plasma 1 vs. plasma 2 and polyp vs. IT. In one patient (no. 4) there was an especially high concentration of protein in the IT. There was a significant (higher) concentration of protein in both polyps vs. plasma and IT vs. plasma. Detectable values were not found by ELISA for IL-5, IL-6, IFN-γ or Fractalkine in either plasma 1 or plasma 2, while IL-12 was measured between 35.1–128.4 pg/ml and RANTES between 1,033–40,000 pg/ml in plasma.

Fractalkine was detected in the polyp from one patient (no. 11) and at a high level in the IT from one patient (no. 10). Otherwise no detectable values of Fractalkine were observed. IL-5 was expressed both in the polyp and IT. There was no significant difference in the group of patients between polyp and IT. IL-6 was also expressed in all patients both in polyp and IT. Here, a significantly higher mean level was revealed in polyp (Fig. 3). IL-12 was present both in plasma, polyp and IT. Between plasma 1 and plasma 2 there was no statistical difference. However, there was a significantly higher level of IL-12 in polyps than in IT (Fig. 4). RANTES was detected in plasma 1 and plasma 2 with no significant difference (Fig. 5).

There was a significant gap between the mean levels of RANTES in plasma versus in polyp/IT, where the highest levels were measured in plasma. There was no significant difference between polyp and IT concerning mean levels of RANTES, even though RANTES was detected at a higher level in IT in three patients (nos. 4, 8, 10) (Fig. 6).

IFN-γ was only detectable in polyp and IT, even though in low values (measured in pg/ml). Although there was a huge gap between values of IFN-γ in polyp and in IT in patient no. 6, there was no significant difference in mean values for the whole group (Fig. 7).

Discussion

In this study we chose to use tissue from the inferior turbinate in the same patient as the reference and also to see whether or not the inflammatory process was limited

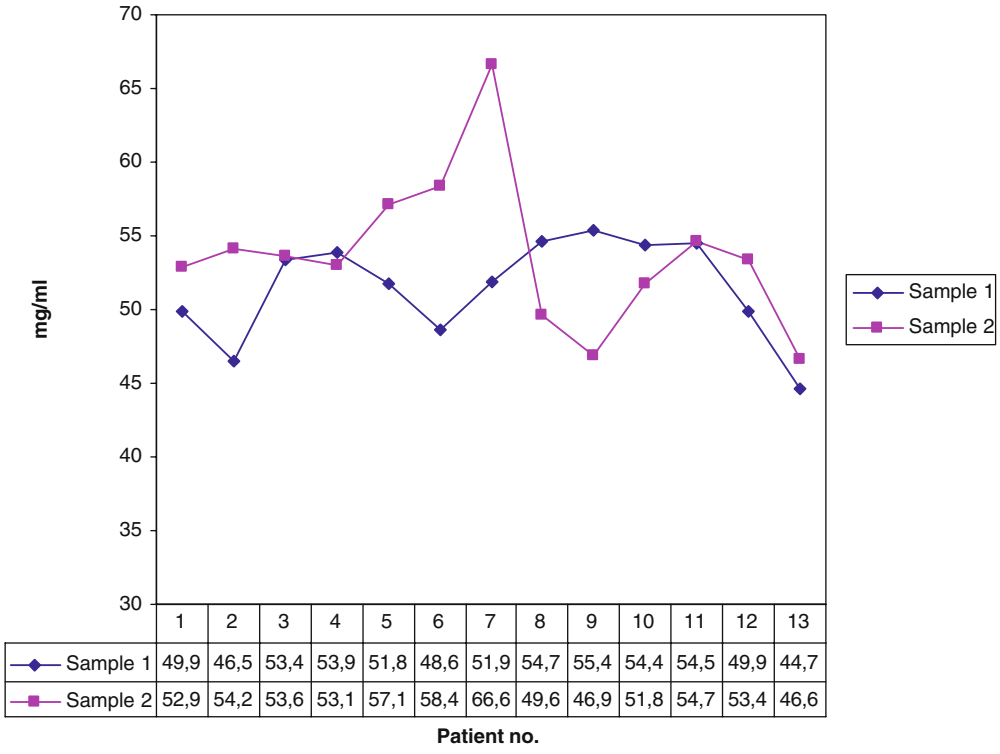


Fig. 1 Protein concentration in plasma: sample 1 and sample 2

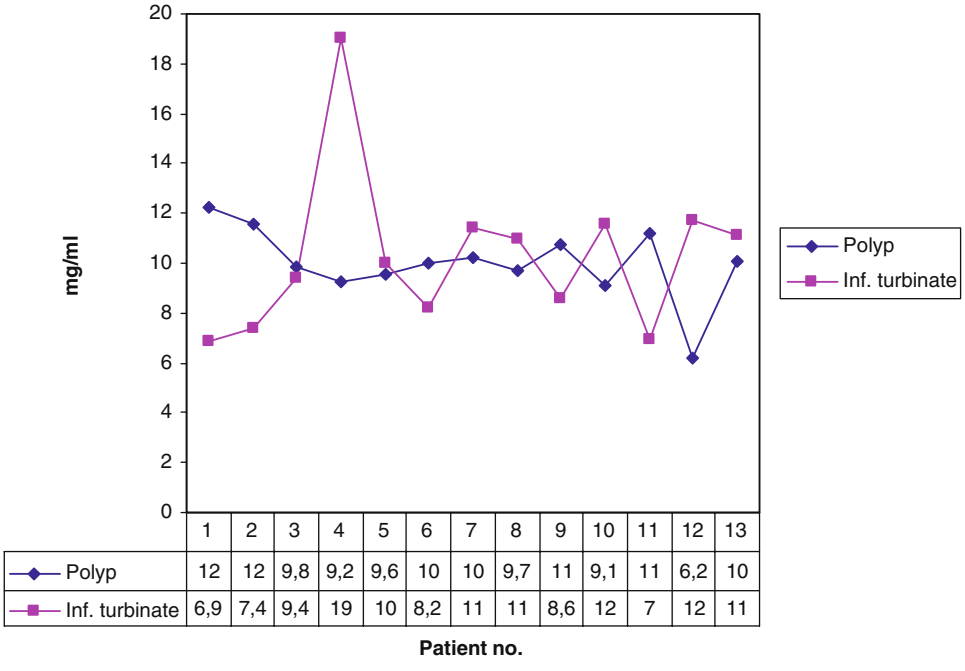


Fig. 2 Protein concentration in polyp and inferior turbinate

to the polyp itself. IL-5 was up-regulated both in polyp and IT without significant differences in concentration. It corresponds to the results of previous studies that IL-5 is up-regulated in nasal polyps [4, 5]. Activation and survival of eosinophils in nasal polyps are thought to be regulated by autocrine stimulation by IL-5, and it was postulated that IL-5 represented the main target for future therapy in nasal polyposis [9, 15]. IL-5 and eotaxin were found to be key factors for eosinophilic accumulation and activation in NP [2].

IL-6 is a multifunctional cytokine that functions in both innate and adaptive immunity. It is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to microbes and to

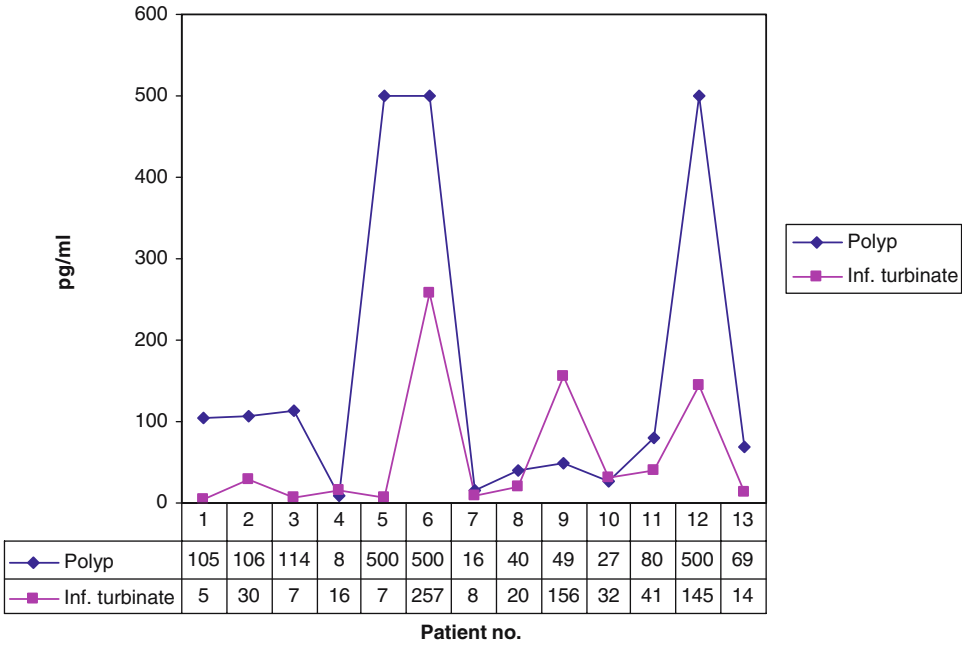


Fig. 3 IL-6 in polyp and inferior turbinate

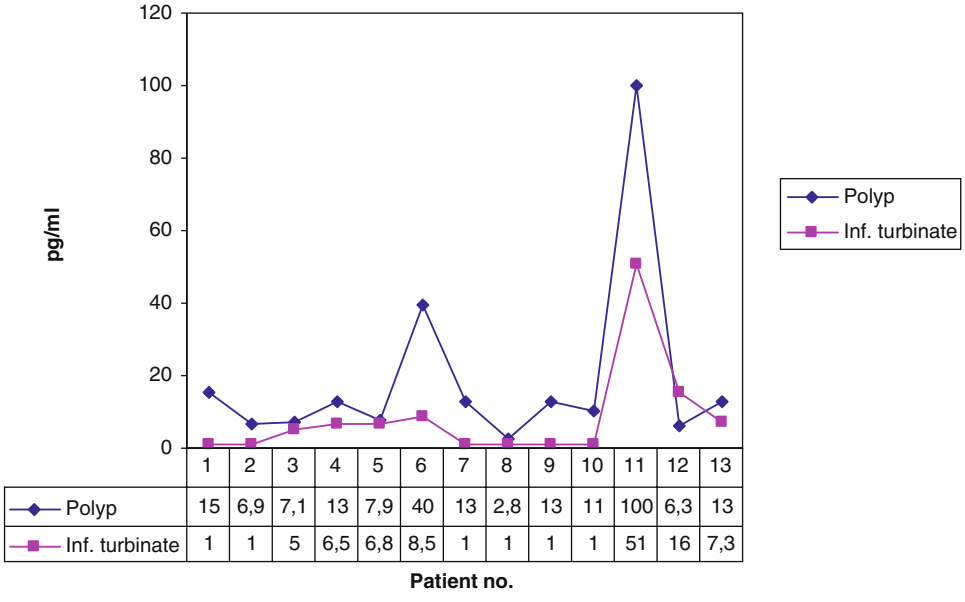


Fig. 4 IL-12 in polyp and inferior turbinate

other cytokines, notably IL-1 and TNF. In a previous study [5] IL-6 mRNA expression was found in 6 of 49 patients, 2 of whom had chronic sinusitis and 4 nasal polyposis. In this study, IL-6 was expressed on a protein level in all patients and statistically up-regulated in polyp versus IT. In the literature few have commented on IL-6 and nasal polyps. The pro-inflammatory cytokines IL-1, IL-6 and IL-8 have been found to play a dominant role in acute sinusitis [16]. The pathogenesis of nasal polyposis involves fibroblasts that synthesize IL-6 to modulate the activation of immune responses (plasma cell formation) and synthesis of stroma. Inducible cyclooxygenase also contributes to nasal polyp development by promoting vasodilatation and modulating the cytokine-induced IL-6 gene expression in nasal polyp fibroblasts [17]. Our present finding may be explained by increased fibroblast activity partly dependent on an ongoing local inflammation. Perhaps an infection is the initiating factor. This may also have implications for the remodelling processes in the respiratory mucosa. IL-12 is the major inducer of T_H1 cells by stimulating the differentiation of $CD4^+$ helper T lymphocytes into IFN-γ-producing T_H1 cells. IL-12 is mainly produced by activated mononuclear phagocytes and dendritic cells.

In our previous study [5] IL-12 was expressed on a mRNA level in a majority of the patients. In this study, on a protein level, IL-12 was expressed in all patients

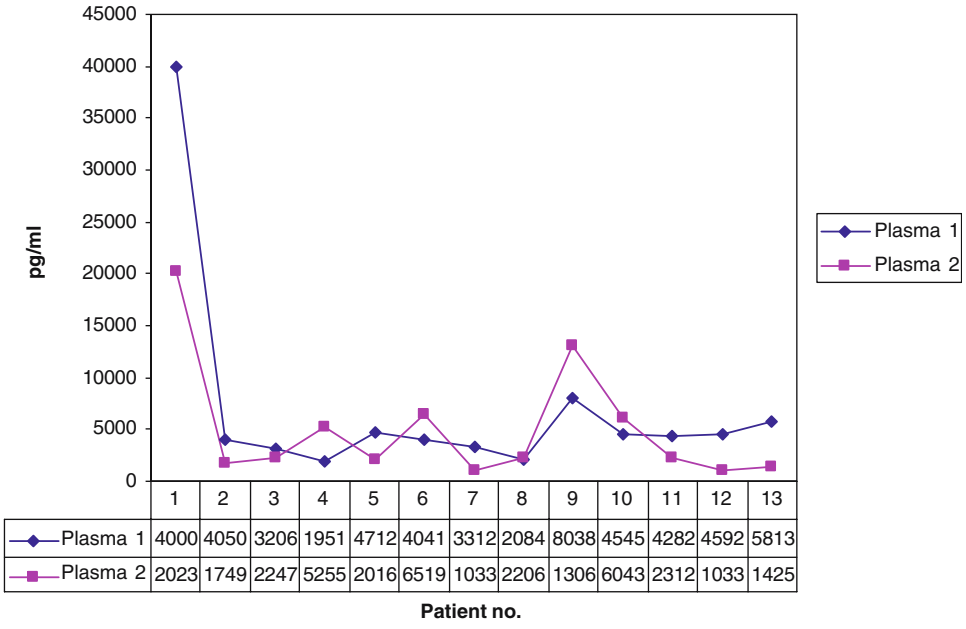


Fig. 5 RANTES in plasma 1 and plasma 2

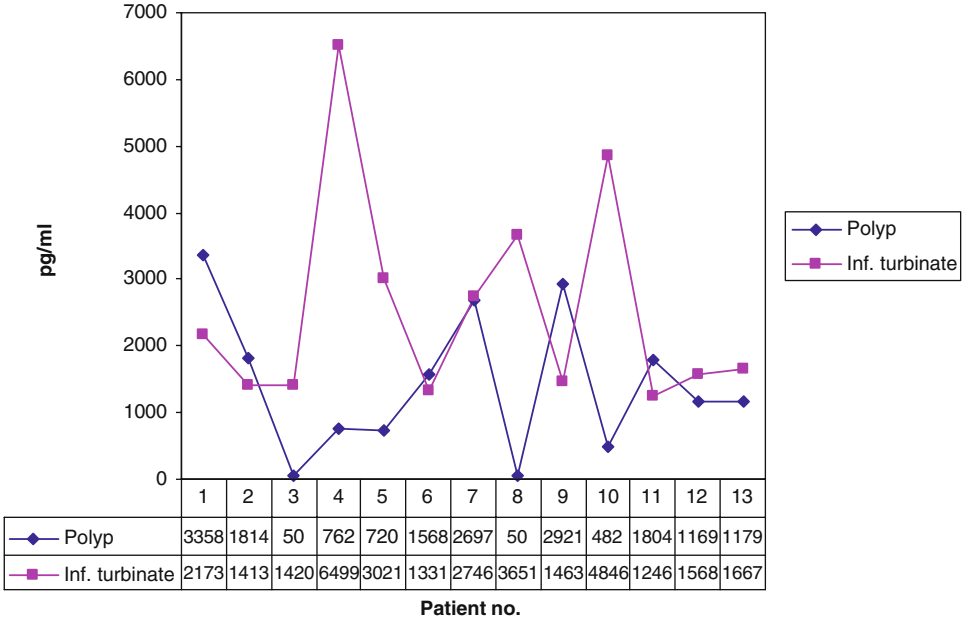
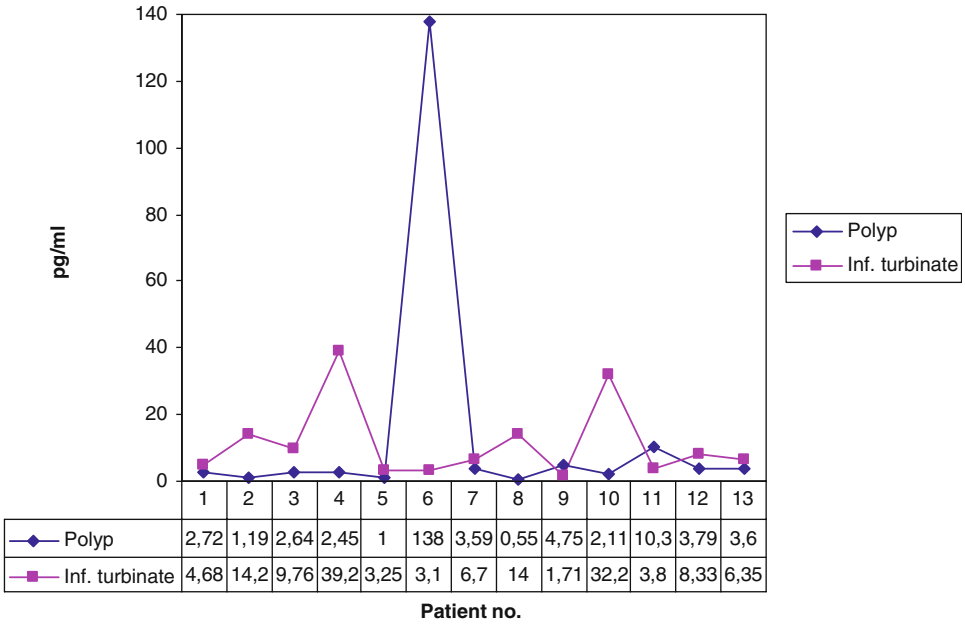


Fig. 6 RANTES in polyp and inferior turbinate

and significantly up-regulated in polyps. A high concentration is notable in the plasma in all patients. Due to the up-regulation of IL-12 in polyps, this could lead to an up-regulation of INF-γ in these patients with longstanding polyposis.

RANTES is a CC chemokine. It acts as a chemoattractant for numerous inflammatory cells, including eosinophils, unlike CXC chemokines like IL-8, which act on neutrophils. In this study, it was up-regulated both in nasal polyps and IT without a significant difference in concentration. An especially high level in plasma is remarkable. Our previous study was the first to demonstrate RANTES on the mRNA level in human nasal polyp tissue in situ. Since then several studies have confirmed the presence of RANTES in respiratory mucosa [18, 19, 20, 21]. In polyps RANTES is supposed to play its most important role together with eotaxin and IL-5 as an attractant to eosinophils and to contribute to eosinophil migration and survival [22].

IFN-γ is a major macrophage-activating cytokine. It serves critical functions in innate immunity and in specific cell-mediated immunity. It is produced by NK (natural killer) cells, $CD4^+$ T_H1 cells and $CD8^+$ T cells. IFN-γ promotes the differentiation of native $CD4^+$ T cells to the T_H1 subset and inhibits the proliferation of T_H2 cells. In this study IFN-γ was not detectable in



	1	2	3	4	5	6	7	8	9	10	11	12	13
Polyp	2,72	1,19	2,64	2,45	1	138	3,59	0,55	4,75	2,11	10,3	3,79	3,6
Inf. turbinate	4,68	14,2	9,76	39,2	3,25	3,1	6,7	14	1,71	32,2	3,8	8,33	6,35

Fig. 7 IFN-gamma in polyp and inferior turbinate

plasma. It was detected in both nasal polyps and IT in all patients even though in low values with no significant difference for polyps versus IT.

Fractalkine is a CX3C chemokine and acts as a chemoattractant to leucocytes. In this material, it was not detectable in plasma, but to our knowledge, this is the first report to describe the identification of Fractalkine in nasal polyps and in mucosa of the inferior turbinate. Even though this was shown in only two of the patients, it shows that Fractalkine is present in respiratory mucosa. From our data it is obviously not of importance in longstanding polyposis. Nevertheless, it remains to be seen whether or not Fractalkine might play a role in the initiation and formation of polyps. This can be an aim for further studies.

In order to demonstrate a potential shift between type 1 subset cells and type 2 subset cells, we chose to include IFN-γ as a T_H1 inducer and IL-5 favouring a differentiation towards T_H2 cells. However, in this study we found that the results indicate a mixed profile in nasal polyps. This is in line with both previous and recent studies [4, 5, 6, 7, 8, 10], but in contrast to Li et al., who in 2001 suggested that Th2-typed cytokines can be viewed as a target of treatment for nasal polyps [23]. It is notable that RANTES and IL-12 are strongly present in plasma in these patients, all with a longstanding nasal/paranasal polyp condition, suggesting a constant inflammatory "drive".

Only IL-6 and IL-12 are significantly up-regulated in polyps versus mucosa of the inferior turbinate. IL-5, RANTES and IFN-γ are more equally represented in both polyps and the inferior turbinate. This might indicate a state of equilibrium between the nasal polyp itself and the surrounding tissue—where both the duration of the disease and the activity of inflammation are of importance—and that an up-regulation of cytokines in the polyp indicates potential polyp growth.

Conclusions

Treatment of nasal/paranasal polyposis is a great challenge. Long ago clinicians realized that this was not a surgical condition per se. A combination of medical and surgical treatment has so far been the recommended treatment modality. Nevertheless, we are a still far from a causal therapy. An understanding of the complicated inflammatory mechanisms causing nasal/paranasal development is crucial for therapeutic success and the development of new treatment strategies. For further advances within this field collaboration between immunologists, molecular biologists and clinicians is of utmost importance.

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