Cytokine Profiles in Nasal Secretions of Patients with Allergic Rhinitis and Chronic Rhinosinusitis

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I Contents

II Abbreviations

AR	allergic rhinitis
CD	cluster of differentiation
CRS	chronic rhinosinusitis
CRSsNP	chronic rhinosinusitis without nasal polyps
CRSwNP	chronic rhinosinusitis with nasal polyps
ECP	eosinophil cationic protein
eotaxin	eosinophil chemotactic protein
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
MCP-1	monocyte chemotactic protein
MIP	macrophage inflammatory protein
PAR	perennial allergic rhinitis
RANTES	regulated on activation, normal T cell expressed and secreted
SAR	seasonal allergic rhinitis
T_H	T-helper cell
TNF	tumour necrosis factor
T_{reg}	regulatory T cells

III Publications

K. Konig et al., Cytokine profiles in nasal fluid of patients with seasonal or persistent allergic rhinitis. Allergy Asthma Clin Immunol 11, 26 (2015); doi: 10.1186/s13223-015-0093-x

K. Konig et al., Cytokine patterns in nasal secretion of non-atopic patients distinguish between chronic rhinosinusitis with or without polyps. Allergy Asthma Clin Immunol 12, 19 (2016); doi: 10.1186/s13223-016-0123-3

1 Introduction

Allergic rhinitis (AR) and chronic rhinosinusitis (CRS) are common nasal inflammatory diseases. Despite differences in pathophysiology, clinical symptoms might resemble each other. Moreover, they are frequently associated (*1*). In recent years, different endotypes of these diseases, differentiated by distinct pathophysiological mechanisms, have been found. New therapeutic approaches aiming at the regulation of the inflammatory process on the level of cytokines and other mediators require endotyping of CRS and AR. Measuring cytokine levels in nasal secretions is a non-invasive and close-to-the-source method to learn more about the inflammatory processes in the individual patient's nose. The present work contributes to the effort on finding biomarkers usable in individualised therapy.

1.1 Disease characteristics

The definition of AR is based on symptoms which include nasal obstruction and rhinorrhoea, nasal itching, and sneezing (*2*). These symptoms are reversible spontaneously or under treatment. The differential diagnoses of AR comprise CRS, infections, mechanical factors such as adenoidal hypertrophy, granulomas and tumours, ciliary defects, and cerebrospinal rhinorrhoea. AR is subdivided into intermittent and persistent disease with symptoms in intermittent disease being less frequent than four days a week or lasting for less than four consecutive weeks (*3*). Several risk factors such as familial history have been identified. However, AR remains a multifactorial burden. Ranking among the atopic syndromes, AR constitutes a risk for new onset asthma and more than one third of the AR patients in school age already suffer from concurrent atopic eczema or asthma (*4*). Besides, AR is often accompanied by asymptomatic bronchial involvement (*5*).

CRS is an inflammation of the nose and the paranasal sinuses. In adults, CRS is clinically defined as having at least two symptoms for more than 12 weeks supported by endoscopic or radiological signs (*1*). These symptoms can be nasal congestion and discharge as well as facial pressure or pain and hyposmia. In primary care, the diagnosis is based on the patient's symptoms. Nasal endoscopy or computed tomography scans demonstrating nasal polyps or mucosal changes may be added. However, these examinations are time-consuming and go

along with exposure to radiation, and thus are left to inexplicit cases. For research purposes, endoscopy is required to distinguish CRS with nasal polyps (CRSwNP) from CRS without nasal polyps (CRSsNP). CRS often occurs together with other diseases such as asthma, aspirin sensitivity, or immunocompromised state (*1*). Moreover, complications such as mucocoele formation or bone involvement may worsen the clinical picture.

AR and CRS are well known to health centres in all parts of Europe as AR affects 23 % and CRS 11 % of the population (*6, 7*). Both significantly deteriorate patients' quality of life, cause sick leaves, and impose notable direct and indirect cost to public health systems and society (*8-10*). Thus, research on effective therapeutic strategies might benefit individual patients as well as society.

1.2 Cytokines

In inflammation, cells and tissues use polypeptides named cytokines for intercellular communication. In 1957, Isaacs and Lindermann discovered interferon which was the first of dozens of cytokines that were found in the following decades (*11*). Cytokines are still subject to a wide variety of studies concerning their function, related and regulating genes, and possible therapeutic uses. Currently, this group of proteins contains interleukins (IL), chemokines, interferons (IFN), growth factors, tumour necrosis factors (TNF), and colony stimulating factors (CSF) (*12*).

Cytokines are a heterogeneous group with some having pro-inflammatory, others anti-inflammatory and modulating qualities. The properties of cytokines often are redundant and cause synergistic effects. Some cytokines have been found to be associated to certain cells of the innate or adaptive immune system. The following instances some of these. Antigen-presenting cells secrete amongst others TNF, IL-1, and IL-6 and thus activate granulocytes and lymphocytes, and induce acute-phase proteins (*13*). Fighting viruses and neoplastic cells, IFN-α and IFN-β are vital to the activity of natural killer cells and cluster of differentiation (CD) 8+ cytotoxic T cells. Besides, they induce pro-apoptotic genes (*11*). IFN-γ, on the other hand, is a signal molecule for T helper lymphocyte (T_H) subclass 1. IFN-γ induces phagocytosis and thus contributes to cellular immunity (*13*). In anti-parasitic

and allergic immune response, eosinophilia, differentiation of naïve CD4+ cells into T_H2, and up-regulated production of immunoglobulin (Ig)-E are seen. Amongst the cytokines made responsible for these processes are IL-4, IL-5, IL-13, and granulocyte-macrophage CSF (GM-CSF) (*13*). Moreover, chemokines are known to be an important factor. Eosinophil chemotactic protein (eotaxin) and others attract eosinophils to the site of inflammation (*14*). In contrast to the aforementioned cytokines, IL-10 has immunoregulatory properties. Primarily produced by regulatory T cells (T_{reg}), IL-10 suppresses secretion of pro-inflammatory cytokines and inhibits T_H differentiation into T_H1 or T_H2 (*15*). Keeping this knowledge on cytokine functions and their sources at the back of our minds, cytokine patterns can be drafted. These patterns are characterised by increased or reduced amounts of specific cytokines. Thus, the activity of particular cells of the immune system can be inferred from the measured amounts of cytokines.

Cytokines mainly act in an autocrine or paracrine way at the site of inflammation. Thus, highest amounts are expected there. However, in high concentrations, some cytokines such as CSF execute endocrine functions.

Several cytokine receptors have been discovered. Binding to its receptor in the cell membrane, a cytokine commences an intracellular biochemical cascade that activates transcription factors. Thus, expression of selected genes is initiated. Most receptors are specific for a certain cytokine. However, others have been found that are shared by different cytokines of the same family, which emphasises the redundant functions of several cytokines.

Ensuring intercellular communication, cytokines contribute to maintaining homoeostasis. Moreover, they are crucial to the immune system when it comes to fighting infections. However, a disequilibrium of cytokines is potentially pathogenic and held responsible for a number of inflammatory diseases and autoimmune disorders, amongst them rheumatoid arthritis, allergy, multiple sclerosis, psoriasis, and sepsis (*15, 16*).

While cytokines remain subject to current research, they are also of relevance concerning patient care. For multiple diagnostic and prognostic purposes, interleukins are quantified in

blood plasma and chemokines in cerebrospinal fluid (*17*). Experimentally, cytokines are measured in different body fluids and in tissue samples to learn more about pathophysiology or establish new diagnostic tools (*18-20*). While colony stimulating factors and interferons have been used as therapy for decades to treat diseases such as neutropenia or viral hepatitis, novel therapeutic approaches with monoclonal antibodies binding cytokines have been developed in recent years. Some are already established in patient care, such as anti-TNF antibodies in rheumatoid arthritis, others are on clinical trials (*21*). To select those patients who might be treated with specific antibodies, diagnostic tools measuring cytokine levels have to be established.

1.3 Pathophysiology of AR

The pathophysiology of AR is that of a type 1 immediate hypersensitivity reaction to aeroantigens.

Prior to the allergic reaction, sensitisation is required. Antigen presenting cells process the antigen to peptides that are presented to naïve CD4+ T lymphocytes. These cells predominantly differentiate into T_H2 lymphocytes which initiate B cell differentiation and induce Ig isotype switching. This results in the production of IgE.

The early phase of allergic reaction is characterised by immediate cell activation. Within minutes, the allergen-IgE interaction activates prevailing IgE-coated mast cells, resulting in degranulation of a number of mediators. These mediators include tryptase, histamine, and neuropeptides, and are held responsible for boosted mucus production, vasodilatation, and increased vascular permeability. This causes the typical nasal symptoms that patients report such as rhinorrhoea or nasal congestion.

In the course of the allergic reaction, released cytokines recruit inflammatory cells such as T lymphocytes, granulocytes, and monocytes. Attracted by chemokines such as eotaxin, regulated on activation, normal T cell expressed and secreted (RANTES), and macrophage inflammatory protein (MIP)-1α, eosinophils migrate into the nasal mucosa. Activated eosinophils release granules containing mediators e.g. eosinophil cationic protein (ECP)

that can cause damage to nasal epithelial cells (*22*). In addition, they produce cytokines inducing migration and activation of inflammatory cells. Thus, a pro-allergic milieu is maintained. In contrast to the early phase, this late reaction generates a persistent inflammation resulting in nasal hyperresponsiveness.

In AR, a disequilibrium of different T cell subsets is held responsible for the pathological reactions. The lymphocyte population is dominated by T_H2, and increased levels of the T_H2 associated cytokines IL-4, IL-5, IL-13, and eotaxin have been measured (*23*). In contrast, normal or even reduced levels of T_H1 associated cytokines have been found, indicating an imbalance of these two types of T helper cells (*23*). A recently found subtype of T helper cells, named T_H17, produces the pro-inflammatory cytokine IL-17. With regard to this subtype, findings are inconsistent and their role in allergy remains to be elucidated (*23, 24*). $T_{reg}s$, another lymphocyte subtype, suppresses both T_H1 and T_H2-type cytokine expression via inhibitory cytokines and has even the ability to inhibit inflammatory cells by direct cell interaction. Concerning $T_{reg}s$, a disequilibrium in comparison to the amount of T_H2 cells has been suspected (*22*).

1.4 Pathophysiology of CRS

Based on endoscopy, CRS is divided into CRSsNP and CRSwNP. Research on pathology and histology of CRS revealed both to be different disease entities and thus confirmed this subdivision.

The aetiology of CRS is not yet entirely understood. Different hypotheses contemplate T cell activation in response to microbial antigens or environmental factors, and a dysregulation of the immune barrier (*1*). In fact, patients suffering from CRSwNP were found to have increased colonisation with Staphylococcus aureus (*25*). However, the impact of microorganisms in CRS remains to be proven. Currently, CRS is described as a multifactorial disease in which misguided interactions between host, pathogens, and exogenous stress contribute (*1*).

In CRSsNP, a T_H1-dominated milieu is found, and levels of the T_H1-associated cytokine

IFN-γ are reported to be elevated. The T_{reg} population is – in contrast to CRSwNP – reported to be normal (*25*). Fibrosis is dominating the histological picture. In addition, basement membrane thickening, subepithelial oedema, and infiltration of inflammatory cells such as monocytes and neutrophils is seen.

On the other hand, CRSwNP presents an eosinophilic inflammation with high amounts of T_H2-associated cytokines amongst these IL-4, IL-5, and IL-13. Mainly produced by lymphocytes, IL-5 is held responsible for the local survival of eosinophils (*26*). Moreover, a diminished population of T_{reg}s is found in polyps, possibly due to attenuated migration (*25*). A dysregulation of epithelial immune barrier function and chemokine production is also suspected (*1*). However, the aforesaid only refers to the Caucasian population. Studies on Asians show a T_H2 negative but T_H1/ T_H17 predominated neutrophilic inflammation in the majority of cases (*25, 27*). Thus, subdivision within the CRSwNP subgroup appears necessary. Macroscopically, in the nasal cavity, the polyps can be easily identified as greyish protuberances. Preparation of tissue samples reveals oedema in stromal tissue as well as pseudocysts, epithelial damage, and inflammatory cell infiltration.

With new therapies emerging, research on the pathophysiology and endotyping of CRS appears more vital than ever.

1.5 Methodological approach

Produced by goblet cells and seromucous glands, nasal secretions moisten and clear the air. In disease, an increased amount of secretions is produced and leaked plasma contents may contribute to the secretions. Different methods are used to investigate pathologies of the nose. For research purposes, cells and tissues as well as nasal secretions are examined. Immunocyto- or -histochemistry and PCR can be performed on cells gained by scraping with cotton swab or cytobrush, and in tissue samples. However, cytology and histology proved to be in an inferior position compared to mediators and cytokines in nasal secretions when differentiation between nasal diseases is required (*28*). Methods to obtain secretions are non-invasive and include nasal lavage with physiological saline and the use of absorptive materials. In contrast to simple nasal blowing and vacuum suction, the

aforementioned methods extract a sufficient amount of secretion (*29*). The cotton wool pieces used in the present studies gain secretions by capillary suction. This method was chosen because it is non-invasive and least uncomfortable for the patient.

The exposure to aeroallergens is not only dependent on the absolute concentration in the air but also to the patient's habits and attempts to abstention. Thus, determining the exact pollution with antigens the patient is exposed to is not feasible. Earlier studies employed allergen or histamine challenge before taking samples (*23, 30, 31*). This results in increasing cytokine release. However, the pace of increase differs in between the biomarkers and for some, peak concentrations are not reached until several hours after provocation (*23*). This requires taking multiple samples and constitutes a time-consuming approach. Thus, this does not well meet the demands that would be made in an actual clinical use. To obtain a true-to-life setting, AR subjects were thus examined irrespective of the season and without prior allergen or histamine challenge.

This methodological approach unites a technique that is easy to perform, cheap, and small in discomfort for the patient, with a true-to-life setting without demands concerning the time of sampling. These features make it well applicable in clinical routine.

1.6 Current therapy options

Concerning AR, the physician is provided with a bunch of treatment options. Apart from avoiding allergen exposure, current guidelines recommend oral H_1-antihistamines, intranasal application of glucocorticoids, and leukotriene-receptor antagonists (*2*). However, one to two thirds of patients experience only partial relief by medication (*32*). Moreover, for severe AR, immunotherapy is available. New approaches in research of AR therapy focus on biopharmaceuticals such as monoclonal antibodies. Patients suffering from seasonal AR (SAR) are shown to benefit from treatment with omalizumab, a humanised anti-IgE antibody (*2*). Moreover, studies targeting IL-4, IL-5, and IL-13 are conducted (*32*).

Current therapy options in CRS aim to achieve and maintain control of the disease. The

therapeutic standard is topical glucocorticoids (*1*). Besides, saline sprays may be used to appease discomfort. Despite the aforementioned hypothesis of microbial involvement in the aetiology of CRS, studies on antibiotic treatment did not show sweeping success (*1*). Due to unclear effectivity, no recommendation for antibiotics in the therapy of CRS exists. Regrettably, conservative treatment still shows poor success rates, with more than half of the patients not responding adequately to medication (*33*). After abortive medical treatment, sinus surgery preserving normal mucosa is recommended to restore sinus ventilation and drainage (*21*). In recent years, research on biopharmaceuticals made headway. Studies on humanised monoclonal antibodies directed against IgE, IL-5, and the IL-4 receptor α showed auspicious results in small study populations (*21*). Currently, proof of concept studies as well as clinical trials are conducted regarding effectivity and safety of these biopharmaceuticals. However, further research is needed to evaluate the relevance of the particular biopharmaceuticals and to establish biomarkers that predict the response to this treatment.

2 Abstract

2.1 Background

Both AR and CRS rank among the most common nasal diseases. In AR, the inflammatory reaction presents itself with tissue eosinophilia and a disequilibrium of T lymphocytes with T_H2 cells predominating. CRSsNP is characterised by a T_H1 milieu. In contrast, CRSwNP shows a T_H2 driven eosinophilic inflammation. With new therapeutic approaches using biopharmaceutics emerging, determination of biomarkers is required to select patients suitable for these therapies.

2.2 Objective

Aim of the present studies was to describe disease-specific cytokine patterns in nasal secretions. Therefore, levels of mediators and cytokines regulating T lymphocytes or stimulating inflammatory cells were measured in different nasal diseases including SAR, perennial AR (PAR), CRSsNP, and CRSwNP, and in healthy controls.

2.3 Methods

Study population: 230 volunteers participated in both studies, whereof 44 suffered from SAR, 45 from PAR, 48 from CRSsNP, 45 from CRSwNP, and 48 were healthy controls. AR was ascertained by patient history and a positive skin prick test and, according to the allergens tested positive, rated into the SAR or PAR group. CRS was determined by patient history, inspection of the nose and nasal endoscopy revealing the presence or absence of polyps. Thus, subdivision into CRSsNP and CRSwNP was made. Healthy controls presented no history of nasal complaints, normal findings in the endoscopic examination, and a negative in vitro allergy screening test. Medication addressing the nasal disease six weeks prior to the examination, purulent rhinitis, and the presence of a mixed AR-CRS disease pattern constituted exclusion criteria.

Biochemical and immunological methods: Nasal secretions were gained by using cotton wool pieces placed in the middle meatus of the nose for 20 min. Samples were extracted

using centrifugation, and all samples were diluted 1:5 due to the partially small volumes. To determine the amounts of cytokines, a human cytokine 27-plex panel was utilised. This panel uses fluorescently-addressed polystyrene beads with conjugated capture antibodies directed to specific cytokines. The samples were analysed for IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, GM-CSF, granulocyte CSF (G-CSF), IFN-γ, monocyte chemotactic protein (MCP)-1, MIP-1α, MIP-1β, eotaxin, and RANTES. The amounts of ECP and tryptase were quantified by ELISA.

Statistics: For statistical evaluation and graphical presentation, Sigma Plot version 11.0 software was utilised.

2.4 Results

In AR, nasal secretions were examined with regard to T lymphocyte-related cytokine patterns. Concerning the amount of the T_H1-associated biomarkers IL-12 and IFN-γ, a decrease could be demonstrated in nasal secretions of participants suffering from SAR compared to both PAR and controls. With regard to T_H2-associated cytokines, SAR presented elevated levels of IL-5 and decreased amounts of IL-13. The quantity of IL-4 was lowered in PAR. Concerning the other T cell associated cytokine patterns, decreased levels of the T_{reg} related cytokine IL-10, and elevated amounts of the T_H17 indicating cytokine IL-17 were found in SAR while PAR and the controls did not differ. Several degranulation products and cytokines indicating inflammatory cell activation were investigated. In AR, irrespective of seasonal or perennial disease, the levels of MCP-1, MIP-1β, ECP, and tryptase were elevated over the controls. SAR only presented increased amounts of MIP-1α. In SAR, RANTES and eotaxin were elevated over PAR, while no significant difference between either of the AR groups and controls was calculated. The levels of IL-1β, IL-6, GM-CSF, and G-CSF did not differ between the three groups.

In CRS, the T_H2 associated cytokines also showed a heterogeneous picture. IL-4 showed no significant differences between the groups, IL-5 was increased in CRSwNP over CRSsNP, and IL-13 was reduced in both CRS groups. Nasal secretions from participants suffering from CRSwNP showed lower quantities of the T_H1 and T_{reg} associated cytokines

IL-12 and IFN-γ, and IL-10, respectively, compared to both CRSsNP and controls. However, IL-17, indicating T_H17 activity, was elevated in CRSwNP. As markers of inflammatory cell activation, levels of MCP-1, MIP-1α, G-CSF, ECP, and tryptase were elevated in CRSwNP, and the quantity of MIP-1β was increased in both CRS groups. RANTES was found to be increased in CRSsNP over controls. The measurement of IL-8, eotaxin, and GM-CSF did not show any differences among groups.

2.5 Conclusions

In both AR and CRS, the disease can be pictured by cytokines in nasal secretions. Pro-inflammatory cytokines were evidently elevated in AR. ECP and tryptase, as well as MCP-1 and MIP-1β clearly distinguished between healthy subjects and those suffering from AR. The levels of RANTES allowed to differentiate between SAR and PAR. Yet, a more pronounced inflammatory profile could be demonstrated in SAR, indicating a higher degree of inflammatory reaction in this disease entity. Moreover, the data on hand suggests a disequilibrium of T cells in SAR where a downregulation of T_H1 and T_{reg} as well as an upregulation of T_H17 is displayed by their respective cytokines. Furthermore, intensified eosinophil and mast cell activity is displayed.

Similarly, inflammation in CRS presents itself in nasal secretions. Altogether, CRSwNP showed a more distinct cytokine profile than CRSsNP, the latter differed only in two biomarkers from the healthy controls, RANTES and MIP-1β. This study on CRSwNP showed elevated levels of IL-5 and IL-17 combined with low levels of IL-10, IL-12, and IFN-γ in CRSwNP. Thus, it adumbrates an imbalance of T helper cells accompanied by a downregulation of T_{reg}s. Moreover, the elevation of various cytokines illustrated the activity of different inflammatory cells in CRSwNP.

The present work shows that not only the inflammation, but also the T cell disequilibrium can be detected in cytokine profiles in nasal secretions. Moreover, it demonstrates that cytokines differentiate between inflammatory nasal diseases. Thus, examination of cytokine profiles in nasal secretions may constitute a helpful tool in diagnosis and prognosis of sinonasal diseases. Moreover, it constitutes a technique applicable to further research on the pathology of those diseases.

3 Zusammenfassung

3.1 Hintergrund

Sowohl die allergische Rhinitis als auch die chronische Rhinosinusitis gehören zu den häufigsten Erkrankungen der Nase. Die Entzündungsreaktion bei der allergischen Rhinitis ist durch Eosinophilie und ein Ungleichgewicht der T-Lymphozyten gekennzeichnet, wobei hier T_H2-Zellen vorherrschen. Bei der CRSsNP sind T_H1-Lymphozyten der dominierende Zelltyp. Die CRSwNP zeigt hingegen eine Entzündungsreaktion, welche von T_H2-Zellen und Eosinophilen geprägt ist. Gegenstand derzeitiger Forschung zur Behandlung dieser Erkrankungen sind neue Biopharmazeutika wie monoklonale Antikörper. Hieraus ergibt sich die Notwendigkeit der Forschung an Biomarkern, denn mit geeigneten Biomarkern können jene Patienten herausgefiltert werden, die sich für eine solche Therapie eignen.

3.2 Zielsetzung

Ziel der vorliegenden Studien war es daher, krankheitsspezifische Zytokinprofile im Nasensekret zu beschreiben. Hierfür wurden Zytokine und Botenstoffe bestimmt, welche die T-Zell-Aktivität regulieren oder Entzündungszellen stimulieren. Neben gesunden Kontrollen umfassten die Studien Patienten mit SAR, PAR, CRSsNP und CRSwNP.

3.3 Methoden

Studienteilnehmer: An den Studien nahmen 230 Freiwillige teil, die sich in folgende Gruppen einteilen ließen: 44 Teilnehmer litten unter SAR, 45 unter PAR, 48 unter CRSsNP und 45 unter CRSwNP. Die Kontrollgruppe umfasste 48 Personen. Die Diagnose AR wurde nach der Erhebung der Anamnese gestellt und setzte einen positiven Prick-Test voraus. In Abhängigkeit von den positiv getesteten Allergenen wurden die Teilnehmer der SAR- oder PAR-Gruppe zugeteilt. Zur Ermittlung des Vorliegens einer CRS wurden neben der Anamnese eine Inspektion sowie eine Endoskopie der Nase vorgenommen. Hierdurch konnte das Vorliegen von Polypen festgestellt oder ausgeschlossen und die Patienten der

jeweiligen Gruppe zugeordnet werden. Die Teilnehmer in der Kontrollgruppe verneinten nasale Beschwerden in der Krankengeschichte und zeigten Normalbefunde in der Endoskopie der Nase. Ein In-vitro-Screening auf Allergien fiel zudem negativ aus. Teilnehmer mit einer purulenten Rhinitis wurden ebenso von der Studie ausgeschlossen wie Teilnehmer, die in den letzten sechs Wochen vor der Untersuchung Medikamente verwendet hatten, welche eine Erkrankung der Nase oder der Nasennebenhöhlen beeinflussen konnten. Ebenfalls ausgeschlossen wurden Teilnehmer, welche ein gemischtes Krankheitsbild mit AR und CRS aufwiesen.

Biochemische und immunologische Methoden: Das Nasensekret wurde mittels medizinischer Watte aus Baumwollgewebe, die für 20 min in den mittleren Nasengang eingeführt und anschließend zentrifugiert wurden, gewonnen. Da hierbei teilweise nur geringe Volumina erzielt wurden, wurden die Proben 1:5 verdünnt. Mittels eines Human Cytokine 27-Plex Panel wurden die im Nasensekret enthaltenen Zytokine quantifiziert. Bei diesem Verfahren kommen an fluoreszierende Polystyrenkügelchen gebundene Antikörper zum Einsatz, welche sich gegen die jeweiligen Zytokine richten. Für die vorliegenden Studien wurden folgende Zytokine untersucht: IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, GM-CSF, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, Eotaxin und RANTES. Des Weiteren wurden ELISAs zur Messung von ECP und Tryptase verwendet.

Statistik: Zur statistischen Auswertung und zum Erstellen der Grafiken wurde das Programm Sigma Plot Version 11.0 verwendet.

3.4 Ergebnisse

Die Nasensekrete der AR-Patienten wurden in Hinblick auf Zytokinprofile von T-Lymphozyten untersucht. Hinsichtlich der T_H1-zugeordneten Zytokine IL-12 und IFN-γ konnte in der SAR ein gegenüber der PAR und der Kontrollgruppe signifikant erniedrigtes Niveau gezeigt werden. Bei den T_H2-zugeordneten Zytokinen ergaben die Messungen in der SAR-Gruppe erhöhte Werte für IL-5 und erniedrigte für IL-13. IL-4 war in der PAR-Gruppe erniedrigt. Was die anderen T-Zellen anbelangte, zeigte sich eine Erniedrigung des T_{reg}-zugeordneten Zytokins IL-10 in der SAR, während das T_H17-spezifische Molekül IL-

17 erhöht war. Zwischen PAR und der Kontrollgruppe fanden sich für diese Biomarker keine Unterschiede. Neben Zytokinen, die auf eine T-Zell-Aktivität schließen lassen, wurden auch Zytokine und Botenstoffe untersucht, die ein Wirken anderer Entzündungszellen nahe legen. Unabhängig davon, ob eine Sensibilisierung gegen ein saisonal oder ganzjährig auftretendes Allergen vorlag, zeigten sich im Vergleich zur Kontrollgruppe erhöhte Werte für MCP-1, MIP-1β, ECP und Tryptase. Einzig die SAR-Patienten wiesen eine Erhöhung von MIP-1α auf. Zudem waren RANTES und Eotaxin in der SAR höher als bei PAR, es bestand jedoch kein signifikanter Unterschied zur Kontrollgruppe. Für IL-1β, IL-6, GM-CSF und G-CSF ergaben sich keine Unterschiede zwischen den Gruppen.

Bei der Untersuchung der CRS wurden ebenfalls die T_H2-zugeordneten Zytokine gemessen. Hier zeigten sich für IL-4 keine Unterschiede zwischen den Gruppen, während sich IL-5 in der CRSwNP im Vergleich zur CRSsNP erhöht zeigte. IL-13 war im Nasensekret beider CRS-Gruppen erniedrigt. Im Vergleich zur Kontrollgruppe und zu CRSsNP zeigten die T_H1- und T_{reg}-zugeordneten Zytokine IL-12, IFN-γ und IL-10 signifikant niedrigere Werte in der Gruppe CRSwNP. IL-17, das eine Aktivität der T_H17-Zellen anzeigt, war in dieser Gruppe hingegen erhöht. Von den Botenstoffen, die eine Aktivierung verschiedener Entzündungszellen anzeigen, waren MCP-1, MIP-1α, G-CSF, ECP und Tryptase bei Patienten, die unter CRSwNP litten, erhöht. In beiden CRS-Gruppen wurden höhere Werte für MIP-1β gemessen als in der Kontrollgruppe. In der CRSsNP war RANTES im Vergleich zur Kontrollgruppe erhöht. Für IL-8, Eotaxin und GM-CSF zeigten sich keine Unterschiede zwischen den Gruppen.

3.5 Fazit

Die vorliegenden Studien konnten zeigen, dass sich die Erkrankungen sowohl im Nasensekret von AR-Patienten als auch in dem von CRS-Patienten darstellen lassen. In beiden AR-Gruppen waren die entzündungsfördernden Zytokine deutlich erhöht. Mittels ECP, Tryptase, MCP-1 sowie MIP-1β konnte eindeutig zwischen der Kontrollgruppe und den AR-Gruppen unterschieden werden. Zudem erlaubten die für RANTES gemessenen Werte eine Unterscheidung von SAR und PAR. Im Nasensekret der SAR-Patienten zeigte

sich die Entzündungsreaktion stärker als in dem der PAR-Probanden. Dies lässt darauf schließen, dass die Entzündungsreaktion in dieser Gruppe deutlich stärker ist. Zudem bilden die vorliegenden Ergebnisse ein gestörtes Gleichgewicht der T-Zellen ab. T_H1- und T_{reg}-Lymphozyten scheinen vermindert aktiv zu sein, während die Erhöhung von IL-17 auf eine gesteigerte Aktivierung der T_H17-Zellen hinweist. Daneben zeigt sich eine verstärkte Aktivität von Mastzellen und Eosinophilen.

Auch bei CRS zeigte sich die Entzündung im Nasensekret. Mit Blick auf die Ergebnisse lässt sich sagen, dass die Entzündungsreaktion bei den Patienten mit CRSwNP deutlicher hervortrat als bei jenen Patienten ohne Polyposis, bei denen sich ein signifikanter Unterschied zur Kontrollgruppe nur für zwei Zytokine, RANTES und MIP-1β, zeigte. Im Nasensekret der CRSwNP-Patienten ließen sich erhöhte Werte für IL-5 und IL-17 nachweisen. Gemeinsam mit den erniedrigten Werten für IL-10, IL-12, und IFN-γ weist dieses Zytokinprofil auf ein unausgeglichenes Verhältnis der T-Helferzellen und eine verminderte Aktivität der T_{reg}s bei CRSwNP hin. Auch legt die Erhöhung unterschiedlicher Botenstoffe nahe, dass verschiedene Immunzellen die Entzündung unterhalten.

Zusammenfassend kann gesagt werden, dass sowohl die Entzündung selbst als auch ein gestörtes Gleichgewicht der T-Lymphozyten anhand des Zytokinprofils im Nasensekret dargestellt werden können. Zudem kann mittels Zytokinprofilen zwischen verschiedenen Erkrankungen der Nase unterschieden werden. Daher könnte sich die Untersuchung von Zytokinen in Bezug auf AR und CRS als diagnostisch und prognostisch wichtiges Werkzeug erweisen. Zudem ist die hier verwendete Methode ein geeignetes Mittel für weitere Forschung über die Pathologie nasaler Krankheiten.

4 Prospects

Currently, new therapies targeting cytokines are emerging. This generates need for reliable and uncomplicated methods to identify patients who might benefit from these therapies. One of these methods suitable for clinical practice is the cotton-wool method to gain nasal secretions as it is easy to handle, non-invasive, and cheap. Moreover, analysing cytokines by multiplex assays provides data rapidly and reproducibly. The conducted studies provide evidence that inflammation is depicted in nasal secretions. Hence, the utilised methods constitute techniques which also meet the needs of researchers. On the one hand, further research is required for clinical purposes as biomarkers are needed for differential diagnosis, prognosis, and matching anti-cytokine therapies to the individual patient. On the other hand, research on cytokines in nasal secretions provides the opportunity to learn more about the pathophysiology of nasal diseases. Understanding the underlying mechanisms, new therapies can be developed, and possibly even prevention can be initiated. Two key approaches should be adopted. Firstly, healthy individuals have to be examined to establish norm values. According to the findings of the present studies, these norm values need to include data on the interleukins 5, 10, 12, and 17, on IFN-γ, RANTES, MCP-1, MIP-1α, MIP-1β, ECP, and tryptase. Yet, this is easily done in an automated fashion using cytokine assays, once the samples are collected using the highly convenient cotton-wool method. In addition, cytokine profiles should be measured in other nasal diseases such as acute rhinitis, cystic fibrosis, or neoplastic diseases. The next step would be to study cytokine profiles in individuals suffering from overlapping disease. Thereby, conclusions could be drawn about the impact of each disease in the individual patient. Thus, therapies tailored to the individual patient's needs could be initiated.

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7 Appendix

A Publications

Katrin König, Christine Klemens, Katharina Eder, Marion San Nicoló, Sven Becker, Matthias F. Kramer and Moritz Gröger. Cytokine profiles in nasal fluid of patients with seasonal or persistent allergic rhinitis. Allergy Asthma Clin Immunol 11, 26 (2015); doi: 10.1186/s13223-015-0093-x

Katrin König, Christine Klemens, Mareike Haack, Marion San Nicoló, Sven Becker, Matthias F. Kramer and Moritz Gröger. Cytokine patterns in nasal secretion of non-atopic patients distinguish between chronic rhinosinusitis with or without polyps. Allergy Asthma Clin Immunol 12, 19 (2016); doi: 10.1186/s13223-016-0123-3

König et al. Allergy Asthma Clin Immunol (2015) 11:26
DOI 10.1186/s13223-015-0093-x

ALLERGY, ASTHMA & CLINICAL IMMUNOLOGY

RESEARCH Open Access

Cytokine profiles in nasal fluid of patients with seasonal or persistent allergic rhinitis

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Abstract

Background: New therapeutic approaches with biologic agents such as anti-cytokine antibodies are currently on trial for the treatment of asthma, rhinosinusitis or allergic diseases necessitating patient selection by biomarkers. Allergic rhinitis (AR), affecting about 20 % of the Canadian population, is an inflammatory disease characterised by a disequilibrium of T-lymphocytes and tissue eosinophilia. Aim of the present study was to describe distinct cytokine patterns in nasal secretion between seasonal and perennial AR (SAR/PAR), and healthy controls by comparing cytokines regulating T-cells or stimulating inflammatory cells, and chemokines.

Methods: Nasal secretions of 44 participants suffering from SAR, 45 participants with PAR and 48 healthy controls were gained using the cotton wool method, and analysed for IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, eotaxin, and RANTES by Bio-Plex Cytokine Assay as well as for ECP and tryptase by UniCAP-FEIA.

Results: Participants with SAR or PAR presented elevated levels of tryptase, ECP, MCP-1, and MIP-1β, while values of GM-CSF, G-CSF, IL-1β, and IL-6 did not differ from the controls. Increased levels of IL-5, eotaxin, MIP-1α, and IL-17 and decreased levels of IFN-γ, IL-12 and IL-10 were found in SAR only. RANTES was elevated in SAR in comparison to PAR. Interestingly, we found reduced levels of IL-4 in PAR and of IL-13 in SAR.

Conclusions: Elevated levels of proinflammatory cytokines were seen in both disease entities. They were, however, more pronounced in SAR, indicating a higher degree of inflammation. This study suggests a downregulation of T_H1 and T_{reg}-lymphocytes and an upregulation of T_H17 in SAR. Moreover, the results display a prominent role of eosinophils and mast cells in AR. The observed distinct cytokine profiles in nasal secretion may prove useful as a diagnostic tool helping to match patients to antibody therapies.

Keywords: Allergic rhinitis, Nasal secretion, Mediators, Cytokines, Chemokines, Interleukins

Background

Allergic rhinitis (AR) is a common disorder of the nose. Patients' symptoms include nasal obstruction, rhinorrhoea, sneezing and nasal itching. All of them are reversible spontaneously or under treatment. AR is subdivided into intermittent and persistent disease. Intermittent disease is defined by the patient having symptoms for less than 4 days a week or for less than 4 weeks [1]. It is estimated that 400 million people worldwide are affected, with a prevalence of AR of about 20 % in Canada and 23 % in Europe [2–4]. Todo-Bom et al. [5] found that intermittent and persistent disease are equally frequent in adults. AR is often associated with asthma, sinusitis, otitis media or nasal polyps and has a significant impact on patients' quality of life [1, 6]. In addition, the disease imposes a substantial economic burden for society [7].

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The underlying pathology of AR is known to be a type 1 immediate hypersensitivity reaction. During the period of sensitisation, the allergen is presented to CD4+ T-lymphocytes inducing differentiation to the T-helper cell (T_H) 2 phenotype. T_H2-lymphocytes secrete cytokines which promote the differentiation of B cells as well as induce immunoglobulin (Ig) synthesis and regulate Ig isotype switching. This results in increased levels of specific IgE, both local and systemic [8]. In the early-phase of allergic reaction, mast cells, coated with specific IgE, recognise the allergen and release several mediators such as histamine and tryptase. In contrast, the late-phase is characterised by the secretion of chemokines like eosinophil chemotactic protein (eotaxin), "regulated on activation, normal T cell expressed and secreted" (RANTES), and macrophage inflammatory protein-1α (MIP-1α) [9], which induce the recruitment of eosinophils and other inflammatory cells. Activated eosinophils release granules containing amongst others eosinophil cationic protein (ECP) and major basic protein (MBP) [10]. In addition, eosinophils synthesise and secrete cytokines, e.g. interleukin (IL)-5 or granulocyte–macrophage colony-stimulating factor (GM-CSF). Whereas the early-phase response to allergen exposure leads to acute symptoms, the late-phase reaction is held responsible for persisting inflammation.

AR is determined by a disequilibrium of T-helper cells with a predominance of T_H2-type cytokines but normal levels of T_H1-type cytokines. Another subtype of T-cells, regulatory T-cells (T_{reg}), suppresses both T_H1 and T_H2-type cytokine expression [11]. Thus, it has been suggested that in AR, an imbalance between T_H2 and T_{reg}-cells exists as well [10]. Concerning T_H17-lymphocytes, some authors found elevated levels of IL-17. However, the findings on IL-17 are ambiguous and the role of T_H17-cells in AR remains unclear [12, 13].

Aim of the present study was to investigate whether in AR caused by a seasonal (SAR) or a perennial (PAR) allergen, representative cytokines and mediators in nasal discharge show distinct patterns picturing the pathophysiology. Therefore, we analysed the levels of cytokines and other inflammatory mediators in the nasal fluid of participants suffering from SAR or PAR, focusing on three main topics: cytokines (1) regulating T_H1 (interferon-γ (IFN-γ), IL-12), T_H2 (IL-4, IL-13), T_{reg} (IL-10), and T_H17 (IL-17) cells, or (2) stimulating and activating inflammatory cells like granulocytes and mast cells (granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-1β, IL-5, and IL-6), and (3) chemokines such as eotaxin, RANTES, monocyte chemotactic protein-1 (MCP-1), or MIP-1α/β.

Methods

Study population

Clinical history was taken by one of the investigators. Patients presenting a history of chronic rhinosinusitis, nasal polyposis or aspirin sensitivity were excluded from the study (Table 1). Any medication concerning the nasal disease during 6 weeks prior to the examination constituted an exclusion criterion, especially anti-inflammatory medication such as nasal steroids or antihistamines. Also, nasal endoscopy was performed in all participants in order to assess clinical signs of rhinitis and to exclude patients with signs of purulent rhinitis or polyposis. After exclusion, 137 volunteers (73 males, 64 females, mean age 38 ± 16 years) participated in this study.

AR was determined by the participant's history and by a positive skin prick test (SPT) (ALK-Abelló, Wedel, Germany) for the following allergens: timothy grass, rye, birch, hazel, alder, beech, mugwort, ribwort, nettle, dandelion, house dust mite, storage mite, dog, cat and horse epithelial dander, alternaria, aspergillus, cladosporium, and penicillium; histamine dihydrochloride solution at 1 mg/ml as positive control and allergen-free saline solution as negative control were used. The SPT was constituted positive if the diameter of the wheal was >3 mm. Thereafter, specific IgE to allergens tested positive in skin prick test was measured in serum (UniCAP-FEIA, Phadia, Freiburg, Germany).

SAR (n = 44) was determined by sensitisation to at least one seasonal allergen with a positive skin prick test and a compatible positive specific IgE measurement (≥0.8 kU/l) as well as typical seasonal complaints in participant's history. If patient's history did not allow a definite rating of the seen sensitisation with respect to its clinical relevance, a intranasal challenge to the suspected allergen was performed. Participants additionally sensitised to a perennial allergen were excluded.

PAR (n = 45) was determined by participant's history, a sensitisation to house dust mite, animal dander, or perennial mold like aspergillus with a positive skin prick test and a specific IgE ≥ 0.8 kU/l. Moreover, an intranasal allergen challenge was performed in case of a sensitisation to house dust mite or perennial mold, or a

Table 1 Exclusion criteria

All groups	Chronic rhinosinusitis Nasal polyposis Aspirin sensitivity Purulent rhinitis Specific medication during the last 6 weeks
SAR	Sensitisation to perineal allergen
PAR	Sensitisation to seasonal allergen

sensitisation to animal dander whose clinical relevance could not be clearly rated by patient's history. Participants additionally sensitised to a seasonal allergen were excluded.

Healthy controls (n = 48) presented no history of inflammatory nasal complaints and a negative in vitro allergy screening test Sx1 (Phadia, Freiburg, Germany).

Samples were collected during as well as outside pollen season. Collection was not done in relation to actual allergen exposure or actual complaints.

The study was approved by the ethics committee of the medical faculty of Ludwig-Maximilians-University and written informed consent was obtained from all participants.

Biochemical and immunological methods

For sampling of nasal fluids, the cotton wool method was performed with minor modifications as invented by Rasp and coworkers [14]. Nasal secretions were gained as previously described using small cone-shaped cotton wool pieces (absorbent cotton, Hartmann, Heidenheim/Brenz, Germany) with a length of about 3 cm and a diameter of about 6 mm [15]. Introduced into the middle meatus of the nose, the cotton wool pieces were left in place for 20 min and were subsequently centrifuged (+4 °C, 2000*g*) on a sieve for 10 min [16].

Because of partially small volumes, all samples were diluted 1:5 and were analysed for IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, eotaxin, and RANTES using a human cytokine 17-plex panel (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, California). The cytokine assay uses fluorescently-addressed polystyrene beads with conjugated capture antibodies directed to the above-mentioned cytokines. After washing, a fluorescently marked detection antibody builds an immunoassay with the cytokine. For analysis, two lasers excite the fluorochromes: one for classifying each bead, the other for quantifying the amount of analyte bound [17]. Detection levels were 0.5 pg/ml.

ECP and tryptase were measured by ELISA (UniCAP-FEIA, Phadia, Freiburg, Germany). Thresholds for detection were 10 ng/ml for ECP and 5 ng/ml for tryptase.

Statistics

SigmaPlot for Windows version 11.0 software (Systat Software, San José, California, USA) was used for statistical evaluation and graphical presentation. All data failed normality testing (Shapiro–Wilk). Therefore, the Kruskal–Wallis One Way Analysis of Variance (ANOVA) on Ranks was used for testing a statistically significant difference in the median values among the three groups. To isolate the group or groups that differ from the others, the All Pairwise Multiple Comparison Procedures (Dunn's Method) was used in the following step. p values <0.05 were regarded as significant. For graphic presentation of results, data is given in a box plot with the median (horizontal line within the box), the 25th and 75th percentile (boundary of the box), and the 10th and 90th percentile (whiskers above and below the box). Significances are graphically represented between the corresponding plots: * indicates p value <0.05, ** p value <0.01, and *** p value <0.001.

Results

44 participants suffering from SAR, 45 participants suffering from PAR and 48 healthy subjects were included in this study. Demographics and sensitisation profiles are depicted in Table 2. The mean age varied from 36 to 40 years. The highest percentage of subjects suffering from asthma was found in the SAR group, followed by the PAR group and the controls. Participants suffering from SAR were frequently sensitised to grass and birch while house dust mite and animal dander were the main antigens in PAR. In SAR as well as in PAR one participant (2 %) was sensitised to mold with alternaria (seasonal) or aspergillus (perennial) being the relevant allergen.

AR is a T_H2 dominated disease. Therefore, an increase of T_H2 cytokines and possibly a decrease of T_H1 and T_{reg} cytokines could be expected. Concerning the markers of T_H2 induced B cell stimulation, we did not find elevated levels of either IL-4 nor IL-13. As shown in Fig. 1a, similar levels of IL-4 were found in SAR (median 7 pg/ml, range 2–17 pg/ml) and controls (median 7 pg/ml, range 0–32 pg/ml), but significantly lower levels in PAR (median 4 pg/ml, range 0–38 pg/ml) compared to controls as well as to SAR ($p < 0.001$ vs. controls/SAR). The quantity of IL-13 was decreased in SAR (median 11 pg/ml, range 6–137 pg/ml) compared to both the controls (median 19 pg/ml, range 10–32 pg/ml; $p < 0.001$) and

Table 2 Demographic data and results of specific IgE

	Controls	SAR	PAR
Participants (N)	48	44	45
Mean age (years)	40	37	36
Gender ♀/♂ (%)	62/38	34/66	42/58
Asthma (%)	9	24	18
Poaceae (%)	n.d.	83	0
Betulaceae (%)	n.d.	52	0
Asteraceae (%)	n.d.	12	0
House dust mite (%)	n.d.	0	82
Mold (%)	n.d.	2	2
Animal dander (%)	n.d.	0	27

n.d. not determined

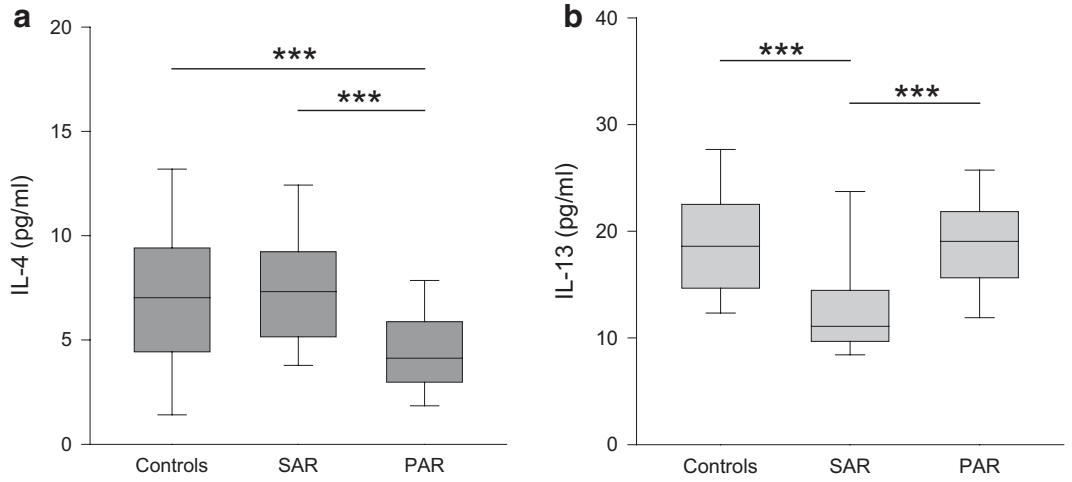

Fig. 1 Levels of IL-4 and IL-13 in nasal fluid in controls, SAR and PAR: *box plots* of the levels of IL-4 (**a** *dark grey*) and IL-13 (**b** *light grey*) in nasal secretion are shown. IL-4 is significantly decreased in PAR compared to the controls as well as the SAR group. IL-13 is significantly decreased in SAR compared to both the controls and PAR. ***$p < 0.001$

PAR (median 19 pg/ml, range 7–48 pg/ml; $p < 0.001$) (Fig. 1b).

As pictured in Fig. 2a, b, a decrease of the T_H1 marker cytokines IFN-γ and IL-12 was found in SAR (IFN-γ: median 85 pg/ml, range 5–299 pg/ml; $p < 0.01$ vs. control, $p < 0.001$ vs. PAR; and IL-12: median 111 pg/ml, range 45–299 pg/ml; $p < 0.001$ vs. control/PAR) compared to PAR (IFN-γ: median 118 pg/ml, range 18–822 pg/ml; and IL-12: median 180 pg/ml, range 71–348 pg/ml) and the controls (IFN-γ: 107 pg/ml, range 34–551 pg/ml; and IL-12: median 200 pg/ml, range 59–358 pg/ml).

Moreover, the quantity of the mainly T_{reg} cell released cytokine IL-10 was lower in SAR (median 47 pg/ml, range 21–139 pg/ml) than in the controls (median 73 pg/ml, range 31–158 pg/ml; $p < 0.001$) and PAR (median 61 pg/ml, range 21–118 pg/ml; $p < 0.01$) (Fig. 3).

IL-17 levels, representing T_H17 activity, were significantly elevated in the SAR group (median 20 pg/ml, range 0–90 pg/ml; $p < 0.001$ vs. control/PAR) while the PAR group and the controls showed similar low levels (PAR: median 0 pg/ml, range 0–147 pg/ml; controls: median 2 pg/ml, range 0–320 pg/ml) (Fig. 4).

Investigating the stimulation and activation of inflammatory cells, several degranulation products and cytokines were measured. Depicted in Fig. 5a, a comparison of the levels of ECP as a marker of eosinophil

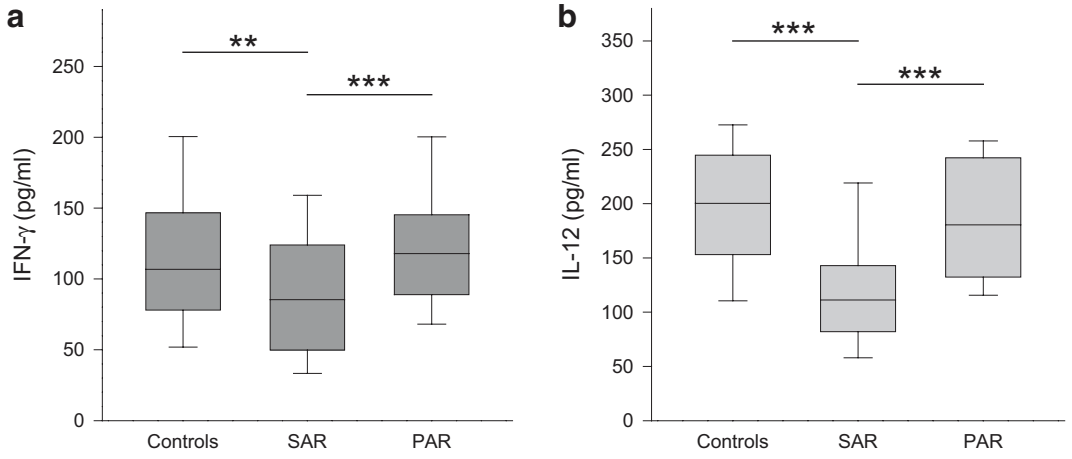

Fig. 2 Levels of IFN-γ and IL-12 in nasal fluid in controls, SAR and PAR: *box plots* of the levels of IFN-γ (**a** *dark grey*) and IL-12 (**b** *light grey*) in nasal secretion are shown. IFN-γ is significantly decreased in SAR compared to the controls or PAR. IL-12 is significantly decreased in SAR compared to the controls as well as to PAR. **$p < 0.01$; ***$p < 0.001$

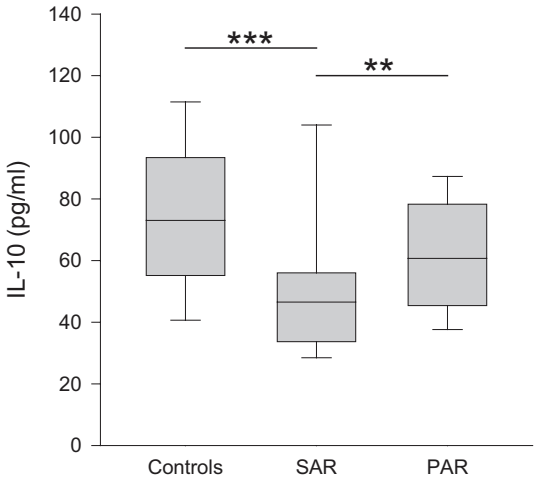

Fig. 3 Levels IL-10 in nasal fluid in controls, SAR and PAR: *box plot* of IL-10 levels in nasal secretion is shown. IL-10 is significantly decreased in SAR compared to the controls as well as to PAR. $^{**}p < 0.01$; $^{***}p < 0.001$

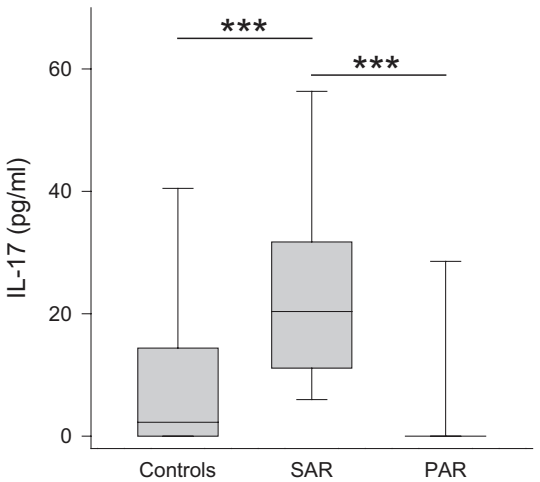

Fig. 4 Levels IL-17 in nasal fluid in controls, SAR and PAR: *box plot* of IL-17 levels in nasal secretion is shown. IL-17 is significantly increased in SAR compared to both the controls and PAR. $^{***}p < 0.001$

activation in nasal mucosa revealed an increase in SAR (median 116 ng/ml, range 0–1000 ng/ml; $p < 0.001$) and PAR (median 43 ng/ml, range 0–1000 ng/ml; $p < 0.01$) compared to the controls (median 20 ng/ml, range 0–467 ng/ml). Likewise, tryptase levels displaying mast cell activation were significantly elevated in the nasal secretions of the SAR (median 20 ng/ml, range 0–452 ng/ml; $p < 0.001$) and the PAR group (median 9 ng/ml, range 0–1000 ng/ml, $p < 0.001$) compared to controls (median 0 ng/ml, range 0–94 ng/ml) (Fig. 5b). As shown in Table 3, for G-CSF and GM-CSF, no significant differences among the three groups were found. Also, the amount of IL-1β in the nasal secretions was rather similar in all groups. Levels of IL-5 in SAR were significantly increased over the controls. However, no statistically significant difference between the controls and PAR was seen. The measurement of IL-6 revealed no differences among the three groups.

Also displayed in Table 3 are the levels of chemokines in nasal discharge of AR participants and controls. An elevation of eotaxin was found in SAR compared to PAR. Concerning RANTES, higher levels were detected in SAR than in PAR whereas no significant difference could be seen between the control group and either of the AR groups. In comparison to the controls, elevated levels of MCP-1 were found in both AR groups. MIP-1α showed a significantly elevated level in the SAR group compared to control as to PAR. For MIP-1β, compared to control (median 103 pg/ml, range 0–2049 pg/ml), an increase was found in SAR (median 226 pg/ml, range 16–1769 pg/ml; $p < 0.001$) as well as in PAR (median 161 pg/ml, range 0–2138 pg/ml; $p < 0.05$) (Fig. 6).

Discussion

Nasal secretion is easily accessible and Bio-Plex Cytokine Assay is simple to perform. Thus, it constitutes a methodological approach possibly applicable in clinical routine. Cytokines in the nasal fluid of participants suffering from SAR or PAR were analysed in a true-to-life clinical setting. Aim of the present study was to investigate whether in AR, representative cytokines in nasal discharge show distinct patterns proving the used methodology helpful for endotyping of inflammatory nasal diseases.

For a lifelike approach, we chose to collect the samples neither during specific seasons of the year nor after allergen provocation. In SAR, the participants' exposition to aeroallergens depends not only on the absolute amount of antigens in the air but also on the habitation, profession and habits of the individual participant as well as his efforts of abstention. Likewise, it is difficult to find objective measurements for the individual pollination in PAR participants' everyday life which also varies in the course of the year [18]. We thus refrained from determining the exact pollution with antigens. Moreover, not using subjective or objective measures of AR, we did not know if participants were actually suffering from AR at the time of sample collection. The magnitude of the allergic response is associated with the preseasonal values of IgE [8] and the levels of cytokines were found to differ between atopic and non-atopic subjects during as well as outside the pollen season [19]. Addressing the important question of trends in cytokine levels over time, longitudinal studies instead of the presented cross-sectional study are mandatory.

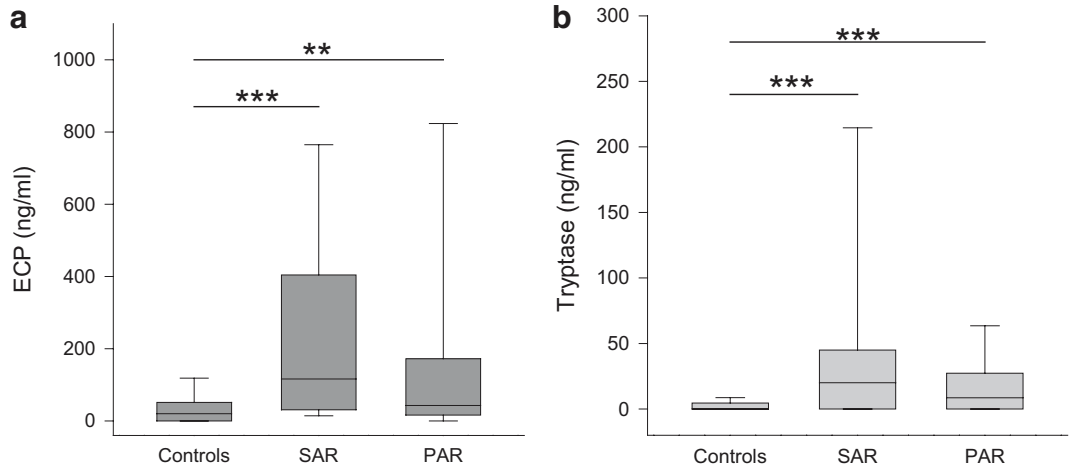

Fig. 5 Levels of ECP and tryptase in nasal fluid in controls, SAR and PAR: *box plots* of the levels of ECP (**a** *dark grey*) and tryptase (**b** *light grey*) in nasal secretion are shown. ECP is significantly elevated in SAR and PAR compared to controls. Tryptase is significantly elevated in SAR and PAR compared to controls. $^{**}p < 0.01$; $^{***}p < 0.001$

Table 3 Cytokine levels in nasal fluid in healthy controls, SAR and PAR patients

	IL-1β	IL-5	IL-6	G-CSF	GM-CSF	Eotaxin	RANTES	MCP-1	MIP-1α
Controls	20	5	25	90	32	45	9	66	0
	4–1000	1–238	0–3036	9–7962	0–137	0–154	0–259	17–401	0–113
SAR	33	13	39	165	28	67	16	94	8
	2–1677	0–829	5–443	10–10,681	0–115	0–503	0–766	30–600	0–66
PAR	31	6	32	146	27	30	0	93	0
	5–7894	1–761	0–397	0–17,211	0–149	0–220	0–509	0–866	0–119
p values									
SAR-Con	n.s.	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	<0.01	<0.001
PAR-Con	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05	n.s.
SAR-PAR	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	<0.01	n.s.	<0.001

Concentrations are given in pg/ml. Data are presented as median (upper line) and range (lower line)

n.s. not significant

IL-4 and IL-13 are produced by T_H2-cells and other inflammatory cells such as mast cells, eosinophils or basophils [20]. In the pathology of allergy, similar responses to these cytokines are known. They act in concert or alone to induce differentiation of T_H-cells, migration of T-cells and eosinophils, Ig class switching or mucus secretion [20, 21]. In the present study, we surprisingly found normal or decreased levels of these T_H2 characterising cytokines, contradicting an expected upregulation, which would lead to stimulation of IgE production. Previous studies on IL-4 and IL-13 revealed normal or elevated levels in nasal secretions under natural allergen exposure, while increases were reported after provocation tests [9, 15, 19, 22]. One group found decreased levels of IL-4 in SAR patients [23]. We measured the cytokine levels without prior nasal allergen challenge, which might explain the missing elevations in our study. On the one hand it might be concluded that the amount of allergens in natural environment is not high enough to provoke profuse production of IL-4 and IL-13 but on the other hand this cannot explain decrease. No definite explanation can be given to the normal or even decreased values of IL-4 and IL-13, a methodological cause cannot be ruled out.

Although allergy is known to be a T_H2-dominated disease, the role of other T-cell subsets was also of interest in the presented work. IL-12 and IFN-γ are well-known indicators of T_H1-type inflammation and crucial to induction and maintenance of T_H1 response, activating preferably phagocytic and cytotoxic immune cells [24,

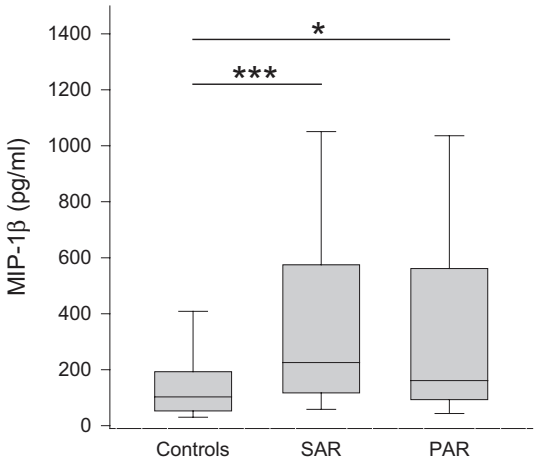

Fig. 6 Levels of MIP-1β in nasal fluid in controls, SAR and PAR: *box plot* of MIP-1β levels in nasal secretion. MIP-1β is significantly elevated in SAR as well as in PAR compared to controls. *$p < 0.05$; ***$p < 0.001$

25]. Moreover, these cytokines counteract the differentiation of T_H2 and T_H17 [24]. In our study, the levels of IL-12 and IFN-γ were both decreased in SAR but not in PAR. This points to a downregulation of T_H1-lymphocytes in SAR.

Having the ability to reduce Ig production and tissue eosinophilia as well as T_H2- and T_H17-dependent reactions, regulatory T-cells are essential in maintaining peripheral tolerance. Allergen-specific T_{reg}-cells have been reported to be diminished in PAR and have decreased suppressive capacity in SAR [26]. IL-10 is an immunomodulatory cytokine which, together with tumor growth factor (TGF)-β, is important for T_{reg} operability. Previous studies on the levels of IL-10 revealed discordant data. Unchanged or increased levels were found in naïve nasal secretions of SAR patients, while IL-10 was elevated after allergen provocation and specific immunotherapy [12, 22, 26]. Our results might suggest a diminished influence of T_{reg}-cells in SAR, illustrating the impaired peripheral tolerance in AR. However, no final conclusion on T_{reg} can be drawn based on our results as IL-10 is produced by other cell types like T_H2 cells as well.

IL-17 is a cytokine with proinflammatory properties influencing diverse cells. IL-17 producing cells, named T_H17, were discovered in the beginning of this century. Though T_H17-lymphocytes were a subject of interest in recent years, their role in AR remains unclear. Scadding suspects elevated levels of this cytokine, predominantly in PAR [12]. Our study does not support this thesis, showing an elevation of IL-17 in SAR but undetectable levels in the majority of the PAR samples. This is in line with a previous study reporting no elevation of IL-17 in nasal discharge of PAR patients [15].

Concerning the role of the discussed T-cell subsets in AR, our results suggest a downregulation of T_H1 and T_{reg}-lymphocytes especially in SAR. This indicates an imbalance between the different T-cell subsets resulting in an impaired tolerance to allergens. Furthermore, increased markers of T_H17 activity were found in AR leaving this T-cell subset as a field of future research.

Mast cells, activated by antigen and IgE, immediately release preformed mediators such as histamine, serotonin, and tryptase. Moreover, stimulated mast cells newly produce a number of other mediators, which are released during the late-phase of allergic reaction [27]. We utilised tryptase as a marker of mast cell activation and detected elevated levels in both SAR and PAR. This is concordant with previous reports of elevated levels of tryptase caused by natural or artificial allergen exposure [28, 29] and emphasises the importance of mast cells in AR.

As the eosinophil is one of the predominant cell types in AR, we measured two indicators of eosinophil activation, ECP and IL-5. The level of ECP, which is secreted by eosinophils and important in the defence of pathogens, correlates positively with the number of nasal eosinophils [14, 30]. Consistent with previous reports, our study found significantly elevated levels of ECP [14]. The amount of nasal ECP was sixfold higher in SAR and doubled in PAR in comparison to the controls. IL-5, primarily produced by mast cells and T_H2-lymphocytes, is thought to be responsible for eosinophil survival, chemotaxis, and activation [31]. This makes this cytokine a second suitable indicator of eosinophil activation. Just as for ECP, we found elevated levels in SAR, highlighting the importance of eosinophils in this disease entity. However, the concentration of IL-5 in PAR was in normal range. We conclude that the role of eosinophils might be less in PAR than in SAR, and other factors are more important in maintaining the more chronic inflammation. The elevated level of IL-5 in SAR might also be a possible therapeutic target. Pavord et al. [32] found reduced numbers of eosinophils in blood samples of asthmatics treated with an monoclonal antibody against IL-5. In conclusion, we found elevated levels of markers of eosinophil activation in both AR groups. However, the effect was more pronounced in SAR, suggesting a greater influence of eosinophils in SAR than in PAR.

Concerning the next group of cytokines, the colony-stimulating factors, surprisingly no increases could be shown. GM-CSF is a multifunctional proinflammatory cytokine produced by a host of different cells, amongst them epithelial cells, mononuclear cells or eosinophils. It acts locally in the nose stimulating dendritic cells as well as neutrophils and eosinophils. Moreover,

GM-CSF induces proliferation and differentiation of stem cells [33]. An increase of GM-CSF in AR patients was reported [9]. However, we could not reproduce this finding, which might be due to the fact that our samples were taken without prior allergen provocation. For G-CSF, we did not find a statistically significant increase in either of the groups as well. G-CSF is known to regulate proliferation of haematopoietic progenitor cells and to influence neutrophil function. As most groups did not examine the amount of G-CSF in AR, little is known about its impact on AR. Pelikan [34] found elevated levels in tears of SAR patients after allergen provocation, providing evidence for an influence of G-CSF in this disease entity. But further research is needed to define its role in allergy.

We also measured the amount of two well-established proinflammatory cytokines, IL-1β and IL-6. For both cytokines, no statistically significant difference between the three groups was found. IL-1β and IL-6 are rather unspecific markers of inflammation. Various inflammatory cells are able to produce these pleiotropic cytokines. Physiologically, IL-1β and IL-6 influence the growth and maturation of immune cells as well as haematopoiesis. Furthermore, they are involved in auto-inflammatory diseases and oncogenesis [35, 36]. Data on these two general markers of inflammation in nasal fluids are inconsistent [12]. Pelikan [34] did not find elevated levels of IL-6 in tears of allergic subjects. This is consistent with our results, but disagrees with an elevation of IL-1β and IL-6 found by others [9]. An explanation could be that we examined nasal secretions under natural allergen exposure while elevated levels were described after experimental allergen challenge.

The late-response of allergic reaction is characterised by the influx of inflammatory cells into the site of inflammation. In this process, chemokines play a crucial role. To measure the recruitment of eosinophils, we examined three chemokines potently attracting these cells: eotaxin, RANTES, and MIP-1α. For all three, the concentration in nasal secretions of SAR participants was increased. Eotaxin, a specific eosinophil attractant, was elevated in SAR over the PAR group. Our results affirm the findings of Chawes et al. [22], who found elevated levels of eotaxin in nasal secretions of SAR patients under natural allergen exposure. Moreover, an increase of eotaxin positive cells and eosinophils in nasal biopsies was reported after allergen provocation [37]. Concerning RANTES, there was a significant elevation in SAR over PAR, while the differences between either of the AR groups and the controls were not significant. RANTES is not only known to attract eosinophils but also to cause activation of eosinophils and basophils resulting in inflammatory mediator release [21]. Further, elevated levels were reported after nasal allergen challenge [9]. The levels of MIP-1α were significantly increased in SAR participants. MIP-1α is produced by a number of inflammatory cells and is able to attract granulocytes as well as to activate eosinophils, to stimulate T-cells and to regulate Ig production [21, 38]. It is reported to be elevated after nasal allergen challenge [9, 12]. Interestingly, this chemokine was not detectable in the majority of our controls or PAR participants, while in SAR, most participants had detectable levels of MIP-1α. In summary, our results show an increase of eosinophil attractants in SAR. This is in line with the elevated levels of ECP and IL-5, emphasising the prominent role of eosinophils in SAR, while the normal levels of IL-5 and just slightly elevated levels of ECP in PAR indicate a minor role of eosinophils in the chronic inflammation of PAR.

The levels of MCP-1 and MIP-1β were elevated in either of the AR groups. Increased MCP-1 and MIP-1β release has been reported under natural exposure as well as after allergen provocation in SAR subjects [12, 22]. MCP-1 potently attracts and activates monocytes and basophils, and recruits macrophages and neutrophils [38, 39]. Secreted by monocytes, natural killer cells and activated lymphocytes, MIP-1β recruits lymphocytes, natural killer cells and immature dendritic cells [40]. The elevation of these two chemokines clearly shows that in both SAR and PAR, a bunch of diverse inflammatory cells is recruited. Our results thus support the concept of minimal persistent inflammation in PAR [41]. This concept states a persistent infiltration of neutrophils under continuous low allergen exposure while eosinophils and mast cells have minor influence.

Conclusions

Aim of our study was to find distinct cytokine profiles in nasal discharge of AR participants in a lifelike approach, which might be useful for diagnostic purposes. Evaluating our results, ECP, tryptase, MCP-1, and MIP-1β are suitable markers to differentiate AR participants from healthy subjects. Furthermore, in SAR eotaxin, MIP-1α, and IL-17 are elevated in comparison to both PAR participants as well as controls. In addition, reduced levels of IFN-γ and IL-10 are found. Moreover, SAR and PAR can be distinguished by the levels of RANTES. Even though some questions remain unanswered, we have demonstrated that the methodology used in this study could be developed into a diagnostic tool for “endotyping” of patients in daily clinical routine. If such an “endotyping” is feasible in nasal discharge, this method is superior to immunohistochemical analysis of nasal biopsy specimen because nasal discharge is easily accessible and collection is harmless to the patient. Further research is needed

to describe the cytokine patterns in nasal fluid of pure CRS with or without nasal polyps followed by examinations of mixed forms of CRS and AR. In the long term, easily accessible biomarkers could help to match patients with innovative therapeutic approaches like anti-cytokine antibodies. Uncovering specific endotypes out of clinically similar phenotypes might result in a more targeted, individualised therapy beneficial to the patient.

Authors' contributions

SB, MK and MG contributed to the study design, and to interpretation and analysis of the data. MG additionally coordinated the study and helped to draft the manuscript. KK performed the Pharmacia CAP System ECP/Tryptase FEIA as well as the Bio-Rad Cytokine Multi-Plex assays and drafted the manuscript. CK participated in the Bio-Rad Cytokine Multi-Plex assays and contributed—together with KE and MSN—to the sample collection and data acquisition. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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RESEARCH Open Access

Cytokine patterns in nasal secretion of non-atopic patients distinguish between chronic rhinosinusitis with or without nasal polys

Katrin König[1], Christine Klemens[1], Mareike Haack[1], Marion San Nicoló[1], Sven Becker[1,2], Matthias F. Kramer[1] and Moritz Gröger[1*]

Abstract

Background: Being one of the most common nasal diseases, chronic rhinosinusitis (CRS) is subdivided into CRS with nasal polyps (NP) and CRS without nasal polyps (CRSsNP). CRSsNP presents itself with a T_H1 milieu and neutrophil infiltration, while NP is characterised by a mixed T_H1/T_H2 profile and an influx of predominantly eosinophils, plasma cells and mast cells. For the purpose of discovering disease-specific cytokine profiles, the present study compares levels of mediators and cytokines in nasal secretions between CRSsNP, NP, and healthy controls.

Methods: The study included 45 participants suffering from NP, 48 suffering from CRSsNP and 48 healthy controls. Allergic rhinitis constituted an exclusion criterion. Nasal secretions, sampled using the cotton wool method, were analysed for IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IL-8, GM-CSF, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, eotaxin, and RANTES, and for ECP and tryptase, using Bio-Plex Cytokine assay or ELISA, respectively.

Results: Elevated levels of IL-5, IL-17, G-CSF, MCP-1, MIP-1α, MIP-1β, ECP, and tryptase, as well as decreased levels of IL-10, IL-12, IL-13, and IFN-γ were detected in NP. CRSsNP presented increased levels of RANTES and MIP-1β while IL-13 was decreased. No differences between the three groups were found for IL-4, IL-8, GM-CSF, and eotaxin.

Conclusions: The present work suggests a disequilibrium of T_H1 and T_H2, together with a down-regulation of regulatory T lymphocytes and up-regulated T_H17 in NP. Moreover, elevated levels of diverse mediators represent the activation of various inflammatory cells in this disease entity. The inflammation in CRSsNP, however, is only weakly depicted in nasal secretions. Therefore, cytokines in nasal secretions may provide helpful information for differential diagnosis.

Keywords: Chronic rhinosinusitis, Nasal polyps, Nasal discharge, Mediators, Cytokines, Chemokines

Background

Chronic rhinosinusitis (CRS) is one of the most common nasal diseases, affecting 5 % of the Canadian population and 11 % of Europeans [1, 2]. Deteriorating both physical and mental health, CRS significantly impairs patients' quality of life and imposes immense costs on the public health system [3, 4]. CRS is characterised by an inflammation of the nose and paranasal sinuses for more than 12 weeks, causing nasal obstruction and discharge, facial pain, and reduction of smell [5]. By nasal endoscopy, this disease is subdivided into CRS with nasal polyps (NP) and CRS without nasal polyps (CRSsNP) [5].

Nasal polyps manifest themselves macroscopically as grey masses, prolapsing into the nasal cavity. In histological sections, oedema, pseudocysts, and a colourful infiltrate of inflammatory cells are seen. In contrast, CRSsNP is characterised by fibrosis and basement membrane thickening [6].

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The pathophysiology of CRS is not yet well understood. Although CRS may be associated to genetic or systemic diseases such as cystic fibrosis or sarcoidosis, the majority of the CRS patients seems to suffer from idiopathic disease [7]. Concerning the aetiopathology, local and systemic host factors as well as environmental factors have been discussed [8]. However, hypotheses about impaired innate immunity, fungi, or superantigens remain to be verified. Atopic diseases are more frequent in CRS patients than in the general population, and allergy as an associated or deteriorating factor has also been discussed [9]. Yet, a definitive answer is owing.

According to reported cell and cytokine patterns, CRSsNP and NP seem to be different disease entities. CRSsNP is characterised by a T_H1 milieu and neutrophils. NP, on the other hand, shows a mixed T_H1/T_H2 profile with increased numbers of eosinophils, plasma cells and mast cells [6, 10]. However, this only applies to the majority of the Caucasian NP patients; Asian NP patients have been reported to show a T_H1/T_H17 polarisation, while the T cell patterns of CRSsNP were similar in both races [6, 11, 12].

The present work compares cytokines in nasal secretions of NP and CRSsNP patients to those of healthy subjects. In the present study, we wanted to study CRS in pure form. As interference between the pathophysiological processes of allergic rhinitis and CRS is conceivable, allergy testing was performed to exclude allergic patients from the study. Levels of cytokines were investigated in order to determine whether the pathophysiology of CRS is depicted in nasal secretions. Our study focusses on two major aspects: the regulation of the T cell subsets T_H1, T_H2, T_H17, and regulatory T cells (T_{reg}), represented by levels of interleukin (IL)-4, IL-5, IL-10, IL-12, IL-13, IL-17, and Interferon (IFN)–γ, and the regulation and activation of inflammatory cells such as granulocytes and mast cells by levels of IL-5, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), eotaxin, "regulated on activation, normal T cell expressed and secreted" (RANTES) protein, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein-1 (MCP-1), eosinophil cationic protein (ECP), and tryptase.

Methods

Study population

141 volunteers (64 males, 77 females, mean age 41 ± 15 years) participated in the present study. Clinical history was taken by one of the investigators. All subjects were tested for allergy to aeroallergens with the in vitro allergy screening test Sx1 (Phadia, Freiburg, Germany). Based on a fluorescence-enzyme-immunoassay (FEIA) this method tests for IgE to inhalant allergens in participants' sera. Volunteers presenting a history of allergy or a positive Sx1 were excluded from the study.

Any medication concerning the nasal disease during 6 weeks prior to the examination constituted an exclusion criterion, particularly anti-inflammatory medication such as topical nasal steroids. To detect nasal polyps and exclude patients with signs of purulent rhinitis, nasal endoscopy was performed in all volunteers. For ethical reasons, X-ray computed tomography (CT) scanning was only performed if indicated for medical care, but not for the purpose of this study.

NP (n = 45) was determined by the patient's history and the presence of endoscopically visible polyps in the nasal cavity, the paranasal sinuses, or both.

CRSsNP (n = 48) was determined clinically by typical complaints in the patient's history such as midfacial pain or pressure, postnasal drip, nasal obstruction, or reduction of smell. Inspection of the nose and nasal endoscopy revealed the picture of a chronic mucosal inflammation in the absence of polyps.

Healthy controls (n = 48) presented no history of inflammatory nasal complaints and normal findings in the endoscopic examination.

The study was approved by the ethics committee of the medical faculty of Ludwig-Maximilians-University in Munich, Germany, and written informed consent was obtained from all participants.

Biochemical and immunological methods

Nasal secretions were gained and processed with minor modifications as described by Rasp and co-workers [13]: For the sampling of nasal secretions, small cone-shaped cotton wool pieces (absorbent cotton, Hartmann, Heidenheim/Brenz, Germany) with a length of about 3 cm and a diameter of about 6 mm were used. After positioning the cotton wool pieces in the middle meatus of the nose, they were left in place for 20 min and subsequently centrifuged (+4 °C, 2000*g*) on a sieve for 10 min [14].

Diluted 1:5, all samples were analysed for IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IL-8, GM-CSF, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, eotaxin, and RANTES using a human cytokine 17-plex panel (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, California). This cytokine assay uses fluorescently-addressed polystyrene beads with conjugated capture antibodies directed to the aforesaid cytokines. After washing, a fluorescently marked detection antibody builds an immunoassay with the cytokine. For analysis, two lasers excite the fluorochromes: one for classifying each bead, the other for quantifying the amount of analyte bound [15]. The detection threshold was 0.5 pg/ml.

ECP and tryptase were measured by ELISA (UniCAP-FEIA, Phadia, Freiburg, Germany). Detection levels were 10 ng/ml for ECP, and 5 ng/ml for tryptase.

Statistics

SigmaPlot for Windows version 11.0 software (Systat Software, San José, California, USA) was utilised for statistical evaluation and graphical presentation. As all data failed normality testing (Shapiro–Wilk), the Kruskal–Wallis one way analysis of variance (ANOVA) on Ranks was used, testing for statistically significant difference in the median values among the three groups. To isolate the group or groups that differ from the others, the all pairwise multiple comparison procedures (Dunn's Method) was used in the following step. To reduce the false discovery rate, the method of Benjamini and Yekutieli was used [16]. Data are given as median and range. For graphic presentation, data are displayed in a box plot with the median (horizontal line within the box), the 25th and 75th percentile (boundary of the box), and the 10th and 90th percentile (whiskers above and below the box). Significances are graphically represented between the corresponding plots: * indicates p value <0.05, ** p value <0.01, and *** p value <0.001.

Results

In total, 141 participants were included in this study, 45 people suffering from NP (28 males, 17 females; mean age 42 ± 15 years), 48 suffering from CRSsNP (18 males, 30 females; mean age 42 ± 15 years) and 48 healthy subjects (18 males, 30 females; mean age 40 ± 16 years).

The levels of T_H2 related cytokines presented an inhomogeneous picture (Table 1). For IL-4, the three groups showed no significant differences. The level of IL-5 was increased in NP in comparison to CRSsNP, while a comparison between either of both groups of chronic rhinosinusitis versus controls revealed no differences. As shown in Fig. 1, CRSsNP (median 15 pg/ml, range 2–92 pg/ml; p < 0.01 vs. controls and vs. NP) as well as NP (median 10 pg/ml, range 4–62 pg/ml; p < 0.001 vs. controls) presented reduced amounts of IL-13 (controls: median 19 pg/ml, range 10–32 pg/ml).

Compared to the controls and CRSsNP, the quantities of T_H1 associated cytokines IL-12 (Fig. 2a), as well as IFN-γ (Fig. 2b) were decreased in NP (IL-12: median 108 pg/ml, range 17–211 pg/ml, p < 0.001 vs. controls and vs. CRSsNP; INF-γ median 63 pg/ml, range 0–308 pg/ml, p < 0.001 vs. controls and p < 0.01 vs. CRSsNP). CRSsNP (IL-12: median 158 pg/ml, range 60–318 pg/ml; INF-γ median 102 pg/ml, range 0–683 pg/ml) did not differ from the controls (IL-12: median 200 pg/ml, range 59–358 pg/ml; INF-γ median 107 pg/ml, range 34–551 pg/ml).

Likewise, IL-10 (Fig. 3), a T_{reg} related cytokine, was decreased in NP (median 41 pg/ml, range 8–72 pg/ml) compared to controls (median 73 pg/ml, range 31–158 pg/ml; p < 0.001) as well as to CRSsNP (median 74 pg/ml, range 20–118 pg/ml; p < 0.001).

In contrast to these diminished cytokine levels, the T_H17 respective cytokine IL-17 (Fig. 4) was elevated in nasal secretions of NP patients (median 15 pg/ml, range 0–105 pg/ml) in comparison to controls (median 2 pg/ml, range 0–320 pg/ml; p < 0.001) and to CRSsNP (median 2 pg/ml, range 0–146 pg/ml; p < 0.001).

Mast cell activation was seen in NP patients by elevated levels of tryptase in nasal secretion, as indicated in Fig. 5a (NP median 11 pg/ml, range 0–75 pg/ml; controls: median 0 pg/ml, range 0–94 pg/ml; CRSsNP median 0 pg/ml, range 0–75 pg/ml; p < 0.001 vs. controls). Additionally, ECP (Fig. 5b), a marker of eosinophil activation, was increased in NP (NP median 56 pg/ml, range 0–1000 pg/ml; controls: median 20 pg/ml,

Table 1 Cytokine levels in nasal fluid in healthy controls, NP and CRSsNP participants

	IL-4	IL-5	IL-8	IL-13	Eotaxin	GM-CSF	RANTES	MCP-1	MIP-1α
Controls	7 0–32	5 1–238	1310 189–42,868	19 10–32	45 0–154	32 0–137	9 0–259	66 17–401	0 0–113
NP	7 0–17	10 0–500	1851 0–265,037	10 4–62	75 0–422	27 0–112	14 0–563	117 13–3867	6 0–60
CRSsNP	5 0–30	3 1–1831	1877 0–1,384,113	15 2–92	49 0–339	42 0–132	35 0–426	89 0–1676	0 0–673
P values									
NP—Con	n.s. (0.822)	n.s. (0.070)	n.s. (0.562)	*s. (< 0.001)*	n.s. (0.114)	n.s. (0.286)	n.s. (0.447)	*s. (< 0.001)*	*s. (< 0.001)*
CRSsNP—Con	n.s. (0.133)	n.s. (0.025)*	n.s. (0.291)	*s. (< 0.01)*	n.s. (0.361)	n.s. (0.456)	*s. (< 0.050)*	n.s. (0.028)*	n.s. (0.028)*
NP—CRSsNP	n.s. (0.213)	*s. (<0.001)*	n.s. (0.887)	*s. (< 0.01)*	n.s. (0.315)	n.s. (0.148)	n.s. (0.079)	n.s. (0.024)*	n.s. (0.037)*

Concentrations are given in pg/ml. Data are presented as median (upper line) and range (lower line). To control the false discovery rate, we used the method of Benjamini and Yukatieli. Thus, values marked with '*' are regarded as non-significant despite p < 0.05

n.s. not significant; *s.* significant

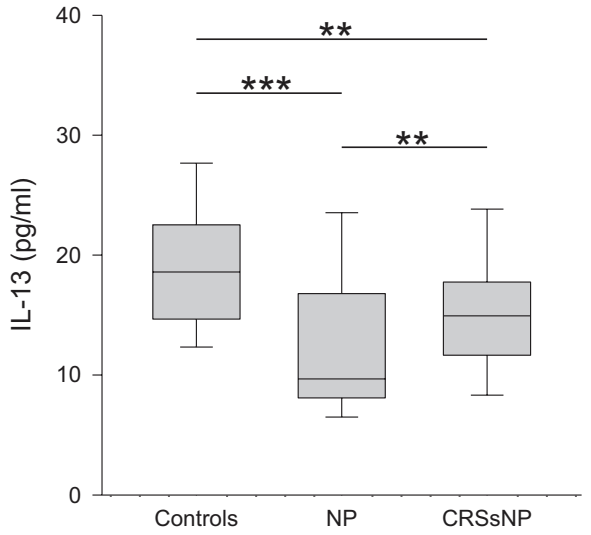

Fig. 1 Levels of IL-13 in nasal fluid in controls, NP and CRSsNP: *box plots* of the levels of IL-13 in nasal secretion is shown. IL-13 is significantly decreased in NP compared to both CRSsNP and the controls. Moreover, IL-13 is decreased in CRSsNP compared to the controls. **p < 0.01, ***p < 0.001

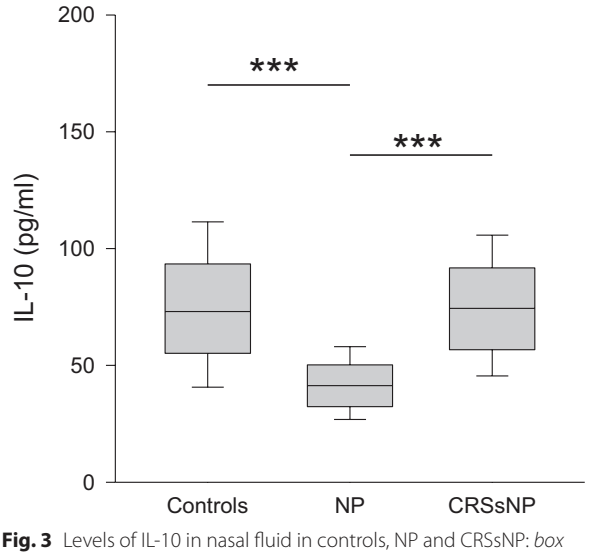

Fig. 3 Levels of IL-10 in nasal fluid in controls, NP and CRSsNP: *box plot* of IL-10 levels in nasal secretion is shown. IL-10 is significantly decreased in NP compared to the controls as well as to CRSsNP. ***p < 0.001

range 0–467 pg/ml; CRSsNP median 45 pg/ml, range 0–1000 pg/ml; p < 0.001) while the quantity of eotaxin in nasal discharge showed no statistically significant differences among groups (Table 1).

Neutrophil associated factors such as IL-8 (Table 1) partially showed a non-significant elevation in nasal secretion from patients with chronic rhinosinusitis either with or without nasal polyps. While in NP G-CSF (Fig. 6) was increased threefold over the controls (NP median 277 pg/ml, range 0–9802 pg/ml; controls: median 90 pg/ml, range 9–7962 pg/ml; CRSsNP: median 155 pg/ml, range 0–8611 pg/ml; p < 0.01), levels of GM-CSF (Table 1) were not different among groups.

Quantities of chemoattractant proteins were increased in chronic rhinosinusitis. MCP-1 and MIP-1α were significantly elevated in NP only (Table 1). Irrespective of the existence of nasal polyps, levels of MIP-1β (Fig. 7) were significantly increased in NP (median 251 pg/ml, range 12–2088 pg/ml; p < 0.001) as well as in CRSsNP (median

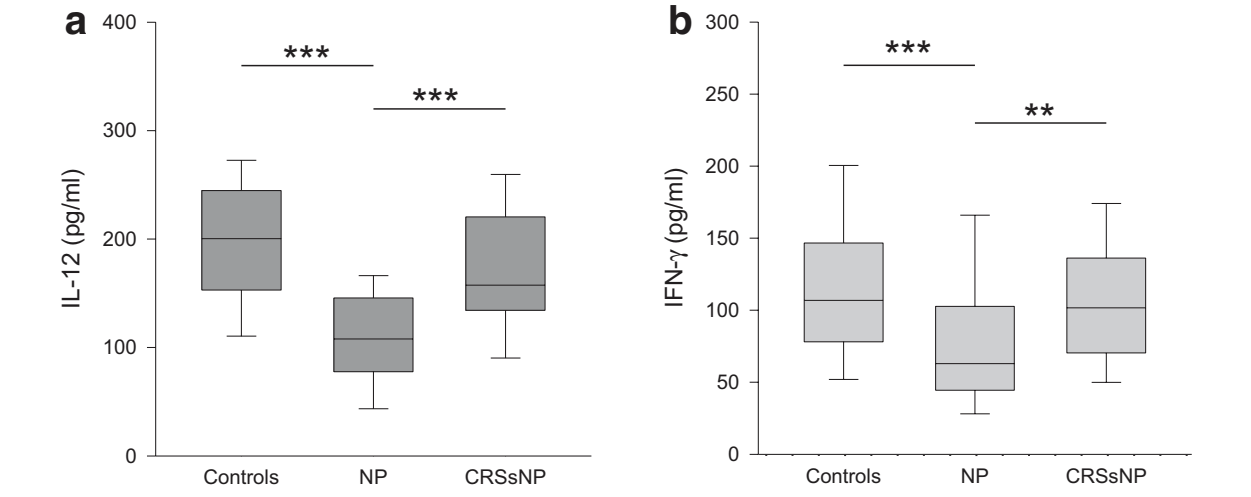

Fig. 2 Levels of IL-12 and IFN-γ in nasal fluid in controls, NP and CRSsNP: *box plots* of the levels of IL-12 (**a** *dark grey*) and IFN-γ (**b** *light grey*) in nasal secretion are shown. IL-12 as well as IFN-γ are significantly decreased in NP compared to both CRSsNP and the controls. **p < 0.01, ***p < 0.001

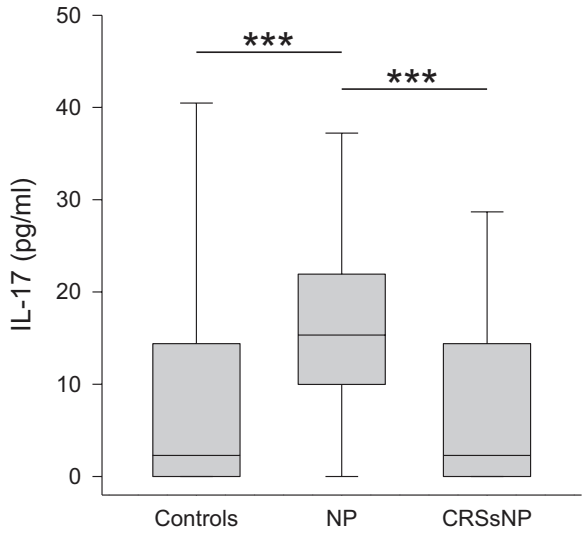

Fig. 4 Levels of IL-17 in nasal fluid in controls, NP and CRSsNP: *box plot* of IL-17 levels in nasal secretion is shown. IL-17 is significantly increased in NP compared to both the controls and CRSsNP. ***p < 0.001

182 pg/ml, range 0–5296 pg/ml; p < 0.01) over controls (median 103 pg/ml, range 0–2049 pg/ml). Concerning RANTES, a statistically significant increase was found only in CRSsNP compared to the controls, whereas levels in NP did not differ from the other groups (Table 1).

Discussion

This study is part of an extensive project, aiming for distinct cytokine patterns in chronic nasal diseases. CRS seems to be a heterogeneous group of diseases presenting not only different phenotypes like CRS with or without nasal polyps but also consisting of diverse endotypes. New therapeutic approaches with biologic agents are currently in development [17]. These new approaches necessitate patient selection by biomarkers. To determine this reason, there is demand for tools helping to define endotypes as well as to select suitable patients for therapies with anti-cytokine antibodies. Bio-Plex Cytokine Assay in nasal secretion could be such a tool as collection of nasal discharge is an easy procedure harmless to the patient, and the assay is simple to perform. Thus, it constitutes a methodological approach possibly applicable in clinical routine. We therefore have already analysed cytokines in nasal secretions of patients with allergic rhinitis in a true-to-life clinical setting as a first step [18]. In the present study, we measured the amount of cytokines in nasal fluid of participants suffering from NP or CRSsNP as well as healthy controls. The aim of the current study was to investigate whether in CRS with or without nasal polyps, representative cytokines in nasal discharge show distinct patterns proving the used methodology helpful for endotyping inflammatory nasal diseases. In the long term, we aim for providing easily accessible biomarkers allocating patients to specific endotypes and therapies.

IL-4, IL-5, and IL-13 are usually regarded as T_H2 cytokines, being predominantly involved in the humoral immune response. These cytokines are not only produced by T_H2 lymphocytes but also by other cells involved in this response pattern, such as plasma cells, mast cells, and eosinophils [10, 19]. For IL-4, we did not find any differences between the three groups, which is in accordance with previous findings [20]. However, other authors observed an elevation in nasal secretions in NP,

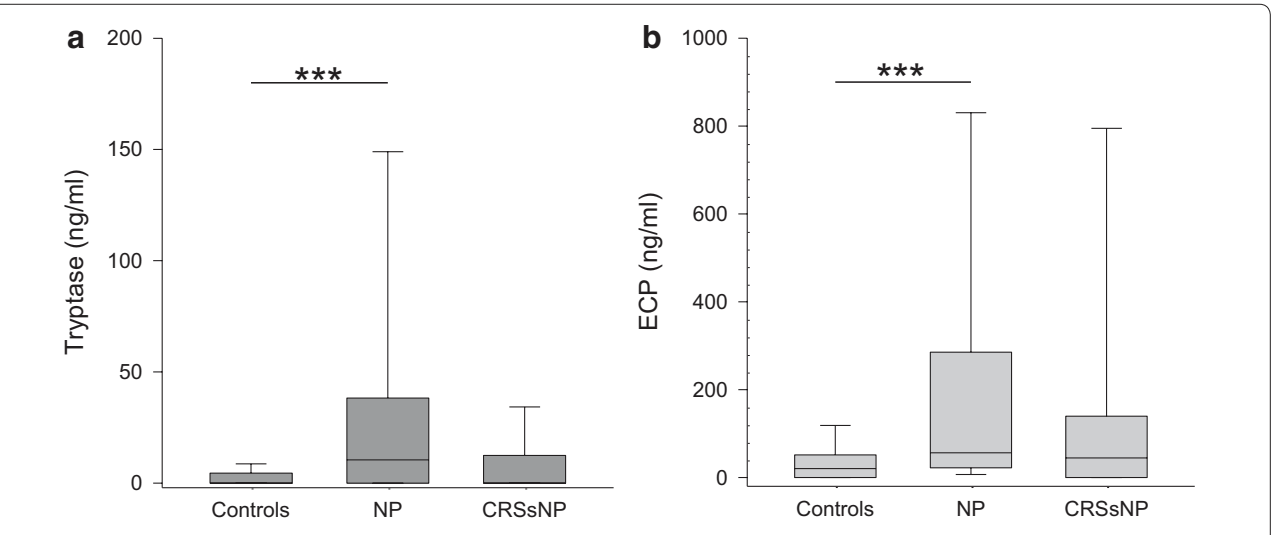

Fig. 5 Levels of tryptase and ECP in nasal fluid in controls, NP and CRSsNP: *box plots* of the levels of tryptase (**a** *dark grey*) and ECP (**b** *light grey*) in nasal secretion are shown. Tryptase and ECP are significantly elevated in NP compared to controls. ***p < 0.001

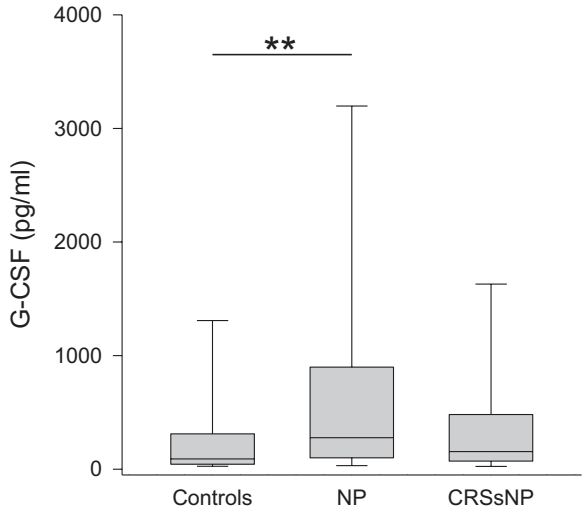

Fig. 6 Levels of G-CSF in nasal fluid in controls, NP and CRSsNP: *box plot* of G-CSF levels in nasal secretion. G-CSF is significantly elevated in NP compared to controls. **p < 0.01

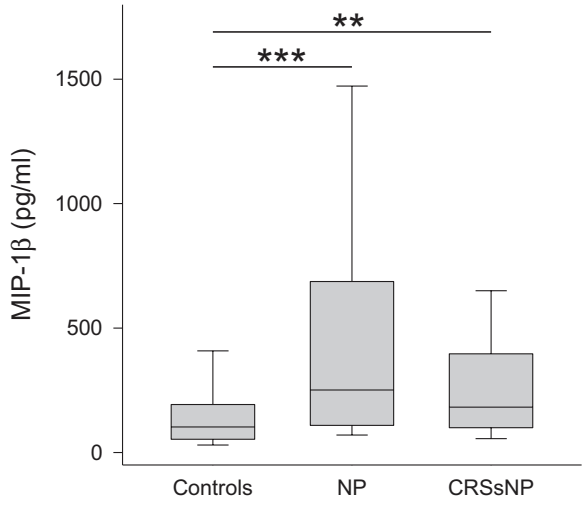

Fig. 7 Levels of MIP-1β in nasal fluid in controls, NP and CRSsNP: *box plot* of MIP-1β levels in nasal secretion. MIP-1β is significantly elevated in NP and CRSsNP compared to controls. **p < 0.01, ***p < 0.001

and a correlation between IL-4 levels and the patients' CT scores was described [21, 22]. IL-4, as well as IL-13, supports the expression of a T_H2 inflammatory pattern by modulating lymphocyte differentiation, inducing IgE production, and facilitating eosinophil infiltration by the up-regulation of chemoattractants and adhesion molecules [19, 23]. Moreover, in vitro studies revealed a negative influence of IL-4 on the epithelial integrity in NP [24]. We surprisingly found decreased levels of IL-13 in both CRS groups, contradicting previous reports of an up-regulation of IL-13 mRNA in NP [22, 25]. Using the same control group, we unexpectedly detected decreased levels in allergic rhinitis patients in a previous study [18]. This might imply a methodical error forming the basis of the decreased amounts of IL-13. We are not able to offer a suitable concept for this unexpected result. Concerning IL-5, detected levels in CRSsNP and NP were not significantly different from controls. However, the amount of IL-5 in NP secretions was significantly higher than in CRSsNP. Several authors found elevated levels of this cytokine [25–27]. IL-5 is a hematopoietic growth factor and crucial for the survival and maturation of eosinophils at the site of inflammation [19, 23]. Therefore, it is discussed as a possible therapeutic target in NP and studies with anti-IL-5 monoclonal antibodies show auspicious results [28]. In conclusion, our results indicate a down-regulation of T_H2 lymphocytes in CRSsNP. Furthermore, they rebut an expected up-regulation in NP and are opposed to the general assumption that the majority of Caucasian NP patients show a T_H2 pattern of inflammation with elevations of T_H2-type cytokines.

IL-12 and IFN-γ are indicators of T_H1 lymphocyte activity. Both cytokines were decreased in NP compared to both the controls and CRSsNP, indicating a down-regulation of T_H1 cells in nasal polyposis. Others found up-regulated or unchanged levels of IFN-γ and IL-12 in NP and CRSsNP [27, 29]. However, these studies used tissue samples instead of nasal secretions. Both IL-12 and IFN-γ induce a predominantly cellular immune response, involving cytotoxic cells and macrophages. They promote T_H1 differentiation and counteract T_H2 and T_H17 development [30]. Moreover, they influence neutrophil survival as well as epithelial integrity [24, 31]. In a study on mice, IFN-γ expression was shown to be associated with deteriorated olfactory function [32]. Accordingly, this cytokine might be considered a therapeutic target for treating the burdensome reduction of smell in patients suffering from CRS.

IL-10 was used as a reference to the role of T_{reg} in CRS. A decrease was detected in NP which fits the findings of *Kim* et al. who detected impaired migration of regulatory T cells in NPs [33]. This points to a derogated immunomodulation in the mucosa of NPs. Furthermore, the level of IL-17 was sevenfold higher in NP than in the controls or CRSsNP. IL-17 is characteristic for T_H17 lymphocytes and a proinflammatory cytokine affecting neutrophils and eosinophils [34, 35]. Data on IL-17 is still ambiguous. While elevated levels have been described in Chinese NP patients, studies on Caucasians reveal conflicting results, ranging from elevated to reduced amounts [12, 25, 36]. Thus, further research on this topic might be needed. In conjunction with the aforesaid results, we state that a relative ascendancy of T_H2

over T_H1 as well as an up-regulation of T_H17 was seen in NP while an impaired function of T_{reg} suggests itself in this disease entity. CRSsNP, however, showed normal quantities of all cytokines except for decreased levels of the T_H2 cytokine IL-13. Our results argue for a more severe inflammation in NP, whereas the inflammation in CRSsNP was only weakly depicted in nasal secretions.

Eosinophilic inflammation has frequently been described in the nasal mucosa of patients suffering from NP. As mentioned, IL-5, a cytokine inducing survival and activation of eosinophils was elevated in NP compared to CRSsNP. Another major factor in eosinophilic inflammation is eotaxin. It is up-regulated preferably by T_H2 and potently attracts eosinophils [10, 37, 38]. Elevated levels were found in the sinunasal mucosa of CRS patients as well as in nasal secretions of NP patients [27, 39]. In our study highest levels were also seen in NP, however differences between the three groups did not reach statistical significance. The levels of ECP, on the other hand, were significantly elevated in NP but not in CRSsNP. ECP is a protein holding antimicrobial as well as modulatory properties [40]. Plenty of reports of elevations of ECP levels in different nasal diseases exist, indicating that ECP is rather a general marker of inflammation than disease-specific [14, 29, 41]. Our results suggest an infiltration of eosinophils into the mucosa of nasal polyps but not into the mucosa of CRSsNP. In allergic rhinitis, mast cells have frequently been investigated, and much is known about their role in the early-phase of allergic reaction [42]. We detected elevated levels of tryptase in nasal secretions of the NP patients. This is in conformity with findings from others describing an increased amount of mast cells and tryptase in mucosal tissue and nasal secretions of NP patients. Further, the level of tryptase in nasal secretions correlated with nasal obstruction and rhinorrhoea [14, 43]. This might suggest a benefit from mast cell targeting medication in NP.

Di Lorenzo and colleagues reported that the levels of tryptase and ECP in NP exceeded those in allergic rhinitis [44]. We compared the levels of these two mediators in NP with the previously reported levels in allergic rhinitis (AR) [18]. For ECP and tryptase, the levels in seasonal AR were twice as high as in NP, while the values in perineal AR were slightly lower than in NP. However, in contrast to the findings of Di Lorenzo and co-workers, in our study, the differences between the levels in NP and AR did not reach statistically significance. Di Lorenzo et al. gained their samples by nasal lavage while we used the cotton wool method. ECP release was found to be higher in polyps than in the lower turbinate of NP patients [45]. Probably, the amount of ECP and tryptase would be higher under assured placement of the cotton wool pieces on the polyp. This might explain the difference to Di Lorenzo's results.

Neutrophil infiltration has been seen in both CRSsNP and NP [46]. In order to get indication of neutrophil attraction, we measured the levels of IL-8 and detected elevated amounts in both CRS groups but not reaching a level of significance. Others report a more pronounced increase of IL-8 in NP [47, 48].

The colony-stimulating factors delay neutrophil death [31]. While G-CSF influences proliferation and differentiation of neutrophil progenitor cells as well as the function of mature neutrophils, GM-CSF often appears in the context of recruitment, activation, and survival of eosinophils [38, 49]. Concerning G-CSF, we found levels threefold higher in NP than in controls. GM-CSF was in a normal range in the nasal secretions in CRS, irrespective of nasal polyps, as opposed to elevations described in tissue samples of NP patients [50]. In summary, we saw no definite evidence of increased neutrophil attraction by IL-8, but elevated levels of G-CSF in NP might indicate a role of this type of granulocyte in polyposis.

In addition, different chemokines were examined. RANTES was elevated in CRSsNP but not in NP, others reported increased levels of RANTES in tissue samples of polyps [50, 51]. RANTES is known to attract eosinophils, basophils and mast cells, and is present in nasal secretions during ongoing infection [38, 52, 53]. Plasma levels of RANTES have been found to correlate with disease severity [54]. In contrast to RANTES, we found MCP-1 to be elevated in NP. MCP-1 attracts different inflammatory cells, among them monocytes and T cells. In CRS, increased amounts of MCP-1 have been reported in nasal secretions as well as in nasal mucosa biopsies [29, 55].

Two other chemokines, MIP-1α and MIP-1β, are structurally related proteins, with 68 % of their amino acids being identical [56]. Produced by a host of inflammatory cells, they both have a number of cellular targets, such as monocytes and dendritic cells. However, only MIP-1α is ascribed to attract granulocytes [56, 57]. In our study, increased amounts of MIP-1α were detected in polyposis patients while being undetectable in the majority of the CRSsNP patients and controls. MIP-1β, on the other hand, showed elevated levels in both CRSsNP and NP. Peric and co-workers found a correlation between MIP-1α levels in nasal secretions and endoscopic and CT scores in NP [57]. Moreover, MIP-1α gene expression was elevated in patients with early recurrence of polyps after surgery over those being treatment-responsive [50]. Further research is needed to evaluate the diagnostic and prognostic utility of this chemokine in CRS.

In conclusion, the evaluation of the chemokines and growth factors in the present study revealed an elevation of G-CSF, MCP-1, MIP-1α, and MIP-1β in NP, while CRSsNP shows increased levels of RANTES and MIP-1β only. We conclude that a number of different

inflammatory cells are involved in NP and inflammation is more pronounced in NP than in CRSsNP.

Conclusions

Colleagues from Belgium recently emphasised in their review "Emerging biologics for the treatment of chronic sinusitis": "The greatest challenge for the future is to define the different endotypes of CRSwNP using easily accessible biomarkers to select the patients who have the best chance of a positive therapeutic response to innovative approaches." [58]. With the present study, we tried to take a closer look exactly on this topic evaluating cytokine profiles in participants suffering from CRS with or without nasal polyps.

Overall, we found a more pronounced inflammatory profile in NP than in CRSsNP. IL-5, IL-10, IL-12, IL-17, and IFN-γ represent a disequilibrium of T cells in NP, and ECP, tryptase, G-CSF, MCP-1, MIP-1α, and MIP-1β depict the activation of various inflammatory cells in this disease entity. CRSsNP participants, on the other hand, did not differ much from healthy individuals. Merely RANTES and MIP-1β seem to be suitable mediators to distinguish between CRSsNP and healthy individuals. As we did not detect any significant differences between the three groups for IL-4, IL-8, GM-CSF, and eotaxin, we conclude that these mediators are not of distinctive function in chronic rhinosinusitis.

In the long term, we aim to evaluate multiplex-analyses of cytokines in nasal discharge being a suitable diagnostic tool for the "endotyping" of patients with chronic sinonasal diseases. To us, this is a crucial step for selection of patients with regard to a therapy with biologic agents, especially anti-cytokine antibodies. The sampling of nasal secretions is an easily performable and non-invasive method and could benefit many patients if established as a diagnostic and prognostic tool. However, further research regarding suitable indicators of different nasal diseases and the establishment of norm values is needed to attain this goal. Thus, therapies tailored to the individual patient's needs should become accessible in the future.

Authors' contributions

SB, MK and MG contributed to the study design, and to interpretation and analysis of the data. MG additionally coordinated the study and helped to draft the manuscript. KK performed the Pharmacia CAP System ECP/Tryptase FEIA as well as the Bio-Rad Cytokine Multi-Plex assays and drafted the manuscript. CK participated in the Bio-Rad Cytokine Multi-Plex assays and contributed—together with MH and MSN—to the sample collection and data acquisition. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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B Eidesstattliche Versicherung

König, Katrin Elisabeth

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

Cytokine Profiles in Nasal Secretions of Patients with Allergic Rhinitis and Chronic Rhinosinusitis

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München, den 29.10.2017 Katrin Elisabeth König