Endotype clustering in chronic rhinosinusitis based on the chemokine expression pattern

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May 11, 2020

Abstract

Background: Chronic rhinosinusitis with and without nasal polyps (CRSwNP/ CRSsNP) is an inflammatory disease affecting the nasal and sinus mucosal lining. Here, to further characterize this heterogeneous disease, we performed an extended endotyping of CRS using the nasal tissue from CRS-patients and a new approach of expression profiling of chemokines related to Th2-type cytokine IL-5. Methods: In this case-control study, we included 66 patients with CRS (CRSwNP n=26; CRSsNP n=40) diagnosed according to the EPOS 2020 criteria. The control group (n=25) consisted of CRS-free patients scheduled for inferior turbinate surgery. The concentration of following chemokines and cytokines was determined in tissue samples obtained during routine surgery from all subjects: TARC/ CCL17, PARC/ CCL18, eotaxin/ CCL11, MCP-3/ CCL7, MIP-1α/ CCL3, IP-10/ CXCL10, ENA-78/ CCL5, and IL-5. The analysis was performed by partition-based clustering. Results: In CRS tissues, the concentration of eotaxin, TARC, total IgE, IL-5, and ECP was significantly higher than in control ($p<0.005$). The analysis identified seven clusters. Cluster-1 was IL-5- and inflammatory chemokines-negative (11% CRSwNP). Cluster-2 had low IL-5 concentration and elevated MCP-3/CCL7 (100% CRSsNP). Clusters -3 and -4 expressed IP-10/CXCL10 (type-1-dominated), TARC/CCL17 and eotaxin/CCL11 (both type-2-dominated) (CRSwNP 13-31%). Clusters 5-7 had high concentration of IL-5, TARC/ CCL17, PARC/ CCL18, and eotaxin/CCL11 (type-2-dominated), NP 71-100%, asthma 19-50%, N-ERD 29%. Conclusions: Our chemokine expression-based extended analysis identified distinct CRS endotype clusters, possibly impacting future diagnosis, monitoring, and biologics-based treatment of CRS.

Introduction

Chronic rhinosinusitis (CRS) impairs quality of life as well as work productivity (1) and has a high socioeconomic burden (1). In the Global Allergy and Asthma European Network (GA^{2}LEN) European multicentre study involving 56 000 adult subjects, a prevalence rate of CRS of 10.9% was determined with a range from 6.9 to 27.1% in Europe (2). The CRS comprised heterogeneous diseases and was differentiated by the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2020) in phenotypes with and without nasal polyps (CRSwNP/ CRSsNP) (3). Subtypes are non-steroidal anti-inflammatory drugs (NSAIDs)-exacerbated respiratory disease (N-ERD), cystic fibrosis, and the allergic fungal rhinosinusitis (3). CRS is associated with asthma (4). Evidence-based therapies consist of nasal corticosteroids in CRSsNP and nasal/oral corticosteroids in CRSwNP (3). In cases without improvement under conservative treatment, surgery of nasal sinuses is recommended according to the EPOS 2020 criteria (3). Despite the evidence-based therapy options, an acceptable level of disease control is still not reached in a high proportion of patients (5). There is a particularly high risk of disease recurrence following nasal sinus surgery for patients with CRSwNP (5, 6). New therapy concepts developed for severe CRSwNP consist of modified nasal sinus surgeries as reboot operations that aim to entirely remove all nasal sinus mucosa and allow healthy

reepithelialization from the preserved nasal mucosa (7). Biological treatment options are gaining evidence as an efficient therapy (8-10) in CRSwNP. The CRS can be characterized by a wide range of factors, such as histopathological findings, specific inflammatory and T-cell patterns, tissue remodeling parameters, the concentration of eicosanoids, and IgE production (11). The current phenotyping in CRSwNP and CRSsNP might not sufficiently reflect pathophysiological processes within the CRS disease. Therefore, elucidation of the pathomechanisms underlying CRS, with the aim to develop specific and personalized therapy strategies, might improve specific therapy options. First results of the GA^{2}LEN Sinusitis Cohort study in the framework of the European FP6 research initiative have already been published by Tomassen et al. (12). This multicenter case-controlled study focused on the analysis of immune markers of nasal sinus tissues from CRS patients. This second cluster analysis followed the first analysis, and the CRS patients were assigned to groups CRSsNP, CRSwNP, and asthma comorbidity. The study included parameters regarding tissue remodeling, proinflammatory cytokines, cytokines of type 1-, type 2 inflammation and Th17 lymphocyte pattern, eosinophil and neutrophil activation markers as well as Staphylococcus aureus enterotoxin-specific IgE (SE-IgE). Based on the detected IL-5 concentrations, CRS was divided into ten clusters with low/ undetectable IL-5 and moderate or high concentrations of IL-5. The group of IL-5-negative clusters represented mainly the CRSsNP phenotype, while with increasing IL-5 levels, the proportion of the CRSwNP phenotype raised to 100 %, with high IL-5 and positive SE-IgE. The asthma prevalence increased with the elevation of IL-5 levels and the presence of SE-IgE (12), but the presence and concentration of chemokines were not analyzed so far.

Chemokines are small peptides (8-14 kDa) that induce chemotactic activity in chemokine-receptor-positive cells. Chemokines influence the activation status of the target cells and selectively regulate the directed migration of specific inflammatory cells. They are also involved in cell recruitment, inflammation, angiogenesis, type 1/ type 2 inflammation, wound healing, and lymphoid trafficking (13). The classification of chemokines is based on the positioning of the first two cysteine residues. Following chemokine families were defined: CXC, CC, C, and CX3C (14). These families differ in function, e.g., CC chemokines activate monocytes, basophils, eosinophils, T-lymphocytes, and natural killer cells, whereas CXC chemokines stimulate mainly neutrophils (15).

The general aim of the present study was analogous to previously published GA2LEN study "Chronic rhinosinusitis and nasal polyposis cohort study", aiming to match the immune patterns with the phenotypes CRSwNP, CRSsNP, and comorbid asthma.

The primary aim of this substudy was to characterize chemokine patterns in the tissue of CRS patients in relation to the levels of the Th2 related parameter IL-5 primarily, and subsequently assign them to the phenotypes CRSwNP and CRSsNP and associated comorbidities asthma and N-ERD. We analyzed the concentration of the CC-chemokines TARC/CCL17, PARC/CCL18, eotaxin/CCL11, MCP-3/CCL7, MIP-1α /CCL3, IP-10 /CXCL10, and the CXC chemokine ENA-78/CCL5.

The secondary aim of the study was to examine the influences of the additional parameters MPO, IL-17 (neutrophilic), IL-22, TNF-α, and IFN-γ (Th1-associated) on chemokine patterns. The use of biologics for the therapy of severe CRS with nasal polyps expands increasingly. Therefore, improving the understanding of pathomechanisms behind CRS, and performing systematic characterization of CRS endotypes could lead to the discovery of new biomarkers, advancing the choice of specific biologics for the treatment of uncontrolled severe CRSwNP.

Patients and Methods

Patient population

The Ethics Committee of the Charité University Hospital approved this study (EA2/009/07). All investigations were conducted according to the principles expressed in the Declaration of Helsinki. The patients diagnosed with CRS according to the EPOS 2012 criteria (16) were recruited to participate in the study. All subjects gave their written informed consent. Control subjects were recruited from patients undergoing inferior turbinate surgery but free of CRS symptoms, as per EPOS 2012 (16). Steroids (oral/ nasal) were

not taken four weeks before surgery. Leukotriene inhibitors were not used two weeks before surgery. Patients were considered allergic according to clinical allergy symptoms (positive skin prick test results or elevated specific IgE-levels to aeroallergens plus present allergy symptoms). Subjects were registered as having asthma upon clinical diagnosis by a pulmonologist. N-ERD diagnosis was made based on the history of reactions to NSAIDs. Of 151 patients from the Berlin cohort, 91 nasal biopsies were obtained from 66 CRS patients and 25 control subjects. The CRS group consisted of 66 patients (mean age 42.3 years [min 25, max 65] 26 females, 40 males). The control group consisted of 25 individuals (mean age 31.4 years [min 18; max 60]; 12 females; 13 males).

In detail, in the CRS group were included 26 CRSwNP patients (mean age 47.2 years [min 28, max 65] 11 females, 15 males) and 40 patients with CRSsNP (mean age 39.8 years [min 25, max 54]; 15 females, 25 males).

Patients' material

Samples from ethmoidal mucosa in the case of CRSsNP and nasal polyps from CRSwNP or mucosal samples from the inferior turbinates in case of controls were collected. The tissues were immediately snap-frozen in the liquid nitrogen and stored at -80°C. Control-tissues were not used in cluster analyses but served as a source for standard values.

Measurement of inflammatory markers

According to the study protocol, 100 mg of tissue was diluted in 1 ml of 0.9% NaCl solution containing protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Next, the tissue was homogenized (1000 rpm, 5 minutes, 4degC) and centrifuged at 1500 g for 10 minutes at 4°C. The protein concentration was measured in each sample using Coomassie Plus (Bradford) assay kit (ThermoFischer Scientific, Darmstadt, Germany).

The chemokines Eotaxin/ CCL11, MIP-1α/ CCL3, MCP-3/ CCL7, ENA-78/ CXCL5, TARC/ CCL17, PARC/ CCL18 and IP-10/ CXCL10 (**Table 1**) were investigated. The chemokine concentrations were measured by using commercially available LEGENDplex Multiple Chemokine Assay (Biolegend San Diego USA).

The IL-5- and TNF-α concentrations were measured with the Luminex 100-system (Luminex, Austin, Texas, USA). The concentration of the additional cytokines (IL-22, IL-17, and IFN-γ) was measured by using commercially available ELISA kits from R&D Systems (Minneapolis, USA). The concentrations of tissue eosinophil cationic protein (ECP) and total IgE and IgE directed against a mixture of enterotoxins from Staphylococcus aureus (S. aureus enterotoxin A, C, and toxic shock syndrome toxin-1) were examined using UniCAP-system (Phadia, Uppsala, Sweden). Myeloperoxidase (MPO) was measured with ELISA (BioCheck, Foster City, Calif., USA). The parameters measured and detection limits are presented in **Table 2** . Values below the limit of detection were considered to be negative.

In agreement with the general study protocol (12), the IL-5 levels were retrospectively defined as follows: negative IL-5 level *when* under the concentration of 12.98 pg/mL (assay detection limit), low IL-5 level (IL-5 < 100 pg/mL), moderate IL-5 level (IL-5 100 to 151 pg/mL) or high IL-5 level (IL-5 > 151 pg/mL).

Statistical methods

Component analysis and cluster analysis were performed following the first study (12). The optimal number of clusters was based on the scree plot, and additional for visualization a "cluspot" was drawn, plotting subjects in two dimensions after multidimensional scaling. The variables SAE, TNFα, IL-17, and IFNg, were omitted from the cluster analysis because they were not or barely detected in the samples. We calculated differences between patients and controls and clusters and controls using the Mann-Whitney U test. Between-cluster differences of all parameters were tested by using the Kruskal-Wallis test with multiple group-comparisons. Overall, the p-values of [?]0.05 were set to be significant.

Results

Comparison of CRS and control subjects

The control subjects (n=25) were significantly younger (p=0.001; 31 yrs vs. 40.0 yrs) than all CRS subjects (n=66). The proportion of gender, smoking status, allergies, asthma, or N-ERD did not significantly differ between CRS cases and controls. Eotaxin/ CCL11, TARC/ CCL17, total IgE, IL-5, and ECP concentrations were significantly higher in CRS compared to the control group (all p<0.005). The other parameters were not significantly different in CRS cases vs. controls (**Table 3**).

Cluster analysis reveals 7 clusters of the chemokine expression pattern.

Cluster analysis of the data obtained from CRS patients, based on chemokine measurements only (independent of clinical phenotype) resulted in the identification of 7 distinct clusters (**Figure 1**). The clusters were well separated from each other. Means and ratios of chemokines/ cytokines and phenotype data were calculated and tabulated as a heat map (**Table 4 and Table 5**) to characterize the clusters. From the examined CC- and CXC chemokines, ENA-78/CCL5 revealed no significant difference in the cluster analysis.

Characterization of chemokines, other parameters, and clinical phenotypes and association with the severity of IL-5 values as the main parameter (see Tables 4 and 5):

Cluster group with negative/ low IL-5 levels consisted of clusters 1-4:

The first cluster group was categorized by undetectable IL-5 in cluster-1 and low IL-5 levels in clusters 2-4. IL-5 revealed no significant differences in cluster-1-3. Also, the type 2-associated parameters ECP and total IgE did not differ significantly in clusters 1 and 2, ECP raised and was significantly elevated in clusters 3 and 4, IL-5, and total IgE were significantly elevated in cluster-4.

Characterization of clusters:

In cluster-1 (9 CRS cases) , no significant differences were detectable between the chemokine levels. Regarding the additional parameters, MPO was significantly elevated compared to two or more clusters, IL-22 was significantly higher than in three or more clusters, and IFN-γ (not included in the cluster analysis) was present in cluster-1 (*neutrophilic type 1-biased pattern).*

- **In cluster-2 (11 CRS cases)** , the MCP-3/CCL7 (expressed in type1/ type 2) level was significantly higher than in three or more other clusters. The neutrophilic additional parameter IL-17 (not included in the cluster analysis) was present *(mixed type 1 -> type 2-inflammatory pattern).*
- **In cluster-3 (8 CRS cases)** , the IP-10/CXCL10 (expressed by type 1), MCP-3/CCL7, and MIP-1α/CCL3 (type 1/ type 2) levels were significantly higher than 5 or more other clusters. The TARC/CCL17) and eotaxin/CCL11 (type 2) levels were significantly higher than in two or more other clusters *(mixed type 1- > type 2-inflammatory pattern).*
- **In cluster-4 (13 CRS cases)** , the levels of type 2-related chemokines TARC/CCL17 and eotaxin/CCL11 were significantly higher than in two or more other clusters. Other chemokines revealed no significant differences. The neutrophilic type 1-biased pattern of the additional parameters was significantly elevated (MPO > 3 other clusters; IL-22 > 2 other clusters), and IL-17, IFN-γ , and TNFα (not included in the cluster analysis) were present in cluster-4 (*type 1- > type 2-inflammatory pattern*).

Association of endotypes to phenotypes in the negative/ low IL-5 levels cluster group:

Nasal polyps were present in clusters 1-4 between 0% in cluster-2 to 31% in cluster-4. Asthma, but not N-ERD, was reported in 0 to 23% in clusters 1 to 4.

Cluster group with high levels of IL-5 consisted of clusters 5-7 :

The concentration of IL-5 and also total IgE values were significantly elevated in more than in three other clusters in cluster-5 and more than in five other clusters in clusters 6 and 7, ECP was significantly elevated in

> 5 other clusters in cluster-5 and 7 and significantly elevated in > than in three other clusters in cluster-6.

In cluster-5 (7 CRS cases) , the levels of type-2 related chemokines eotaxin/ CCL11, TARC/ CCL17, and PARC/ CCL18 were significantly higher than in > 5 other clusters. Also, the Th1/ Th2 chemokines MCP-3/ CCL7 and MIP-1α/ CCL3 were significantly higher than > 5 other clusters. IP-10/CXCL10 and ENA-78/CXCL5 did not differ between the clusters. MPO and IL-22 were significantly elevated (> 5 other clusters), and IFNγ (not included in the cluster analysis) was present in cluster-5 *(strong Th1/Th2 pattern).*

- **In cluster-6 (16 CRS cases)** , the eotaxin/ CCL11 (>than five other clusters), PARC/CCL18, and TARC/CCL17 levels (> 3 other clusters) were significantly elevated. SE-IgE was also significantly elevated (> 5 other clusters). MPO was significantly elevated (> 2 other clusters), and IFN-γ (not included in the cluster analysis) was present in cluster-6 (*type 2-inflammatory pattern*).
- **In cluster-7 (2 CRS cases)** , the chemokines TARC/CCL17, PARC/CCL18 were > 5 times and eotaxin/CCL11 > 3 times significantly higher than other clusters. All other chemokines revealed no significant differences. SE-IgE was also significantly elevated in >5 other clusters. MPO (included in the cluster analysis) was significantly elevated, and TNFα was present in cluster-7 (*type 2-inflammatory pattern*).

Association of endotypes to phenotypes in the high IL-5 levels cluster group:

CRS with polyp formation ranged from 71 to 100%, and asthma was present in 43% to 50% of subjects. N-ERD was diagnosed in clusters 5 and 6 only, with a proportion of 19 to 29%.

Discussion

Previous research (12) demonstrated that CRS could be subdivided into endotypes based on inflammatory markers. The division of CRS into endotypes is useful to understand the natural course of the disease and to make a treatment choice. Here, we examined the chemokine expression in the CRS tissues to cluster and match with the CRS phenotypes CRSwNP/ CRSsNP and the comorbidities asthma and N-ERD. To the best of our knowledge, this is the first study analyzing this panel of chemokines in the context of CRS. The results of the current study allow associating chemokines signatures to the endotypes mentioned above, with type 1-related biomarkers such as IP-10/ CXCL10 and TARC/ CCL17 as well as eotaxin/ CCL11 as early-type 2-related biomarkers. Additional neutrophilic and type 1-related inflammatory parameters were detected in IL-5 negative/ low and also in IL-5 high cluster groups, respectively.

Based on the chemokine expression pattern, we have identified seven CRS clusters in the dataset of the multicenter cooperation GA2LEN "Chronic rhinosinusitis and nasal polyposis cohort study"(12). As analytical targets, we selected type-2-associated CC-chemokines and a neutrophil CXC-chemokine (**Table 1**). Previous studies revealed that the concentration of MIP-1α/ CCL3 (17), eotaxin/ CCL11 (18), TARC/CCL12 (19), and PARC/CCL18 (20) were higher in tissue samples obtained from CRSwNP patients, as opposed to the patients with CRSsNP. In addition, MCP-3/CCL7 was found to be increased in CRS patients but not providing distinction into CRSwNP and CRSsNP (21). Similarly, the concentration of IP-10/CXCL10 was reported to increase in response to viral infection in CRSwNP tissue (22). There was no difference reported in the levels of ENA-78/CCL5 between CRS patients and healthy subjects (23). However, none of these studies provided the classification of CRS endotypes, which we performed here using the expression of the type 2-cytokine IL-5 as basis for differentiation.

In the clusters 1-4, IL-5 levels were negative or low in the low*inflammatory* or low mixed Th1/ Th2 *inflammatory* endotype groups. Corroborating the results of Tomassen et al. (12), we detected a low inflammatory pattern in cluster 1 with negative IL-5 levels. In cluster 2 with low IL-5 levels, we found elevated concentrations of a single chemokine (MCP-3/CCL7) in all patients who suffered from CRSsNP. The MCP-3/CCL7 receptor CCR1 is known to be expressed during Th1- and Th2 inflammatory responses, pointing to Th1 associated effects of MCP-3/CCL7 in cluster 2. In cluster 3 with mixed Th1/Th2 inflammatory patterns, eotaxin/CCL11 - a typical Th2-chemokine - and IP-10/ CXCL10 - a typical Th1- - were significantly elevated. Interestingly, in cluster-3, the levels of eotaxin/ CCL11 and TARC/CCL17 were significantly higher than in

other clusters. At the same time, IL-5 and IgE concentrations showed no significant difference, suggesting a possibility for using eotaxin/ CCL11 and TARC/ CCL17 as early biomarkers of type-2 related inflammation. Eotaxin/ CCL11 serves as eosinophilic chemokine (24) for CRSwNP patients compared to controls (18, 25). TARC/ CCL17 is associated with a type-2-profile (19) and was found to be elevated in fibroblasts isolated from CRSwNP patients, following stimulation with IL-4 (26). In addition, its concentration was higher in nasal secretions from the CRSwNP compared to CRSsNP patients (27).

In clusters 5-7, we observed elevated concentrations of IL-5 in association with the type-2 inflammatory profile. In addition to TARC/ CCL17 and eotaxin/ CCL11, also the concentration of PARC/ CCL18 was significantly higher in these clusters. Type-2 cytokines upregulate PARC/ CCL18 in the sputum of asthma patients, and PARC/ CCL18 markedly correlates with the number of eosinophils in sputum (28). Elevated PARC levels were found in the CRSwNP tissues (20). PARC/ CCL18 and TARC/ CCL17 are considered to be typical chemokines produced by dendritic cells and are essential for the regulation of type-2 immune response as well as for trafficking of memory T cells, as described in atopic dermatitis (29). An increased number of dendritic cells were described in nasal polyps (30). In a study of Jonstam et al. the levels of PARC/ CCL18 and the eotaxin-group eotaxin-2 and -3 (CCL24/ CCL26) decreased significantly in nasal secretions following 16 weeks of therapy with dupilumab (31) and also reduced in serum in recent phase 3 studies in dupilumab *versus* placebo-treated CRSwNP patients (8).

Further, the concentration of MCP-3/ CCL7 and MIP-1α/ CCL3 (32) was significantly greater in the "high IL-5 group", pointing at additional type-2 inflammatory effects of these chemokines. Both CC chemokines are known to chemoattract eosinophils in the type-2 inflammatory environment (33, 34). MCP-3/ CCL7 and MIP-1α/ CCL3 predominantly attract type-1/type-2 cells with MCP-3/ CCL7 attracting monocytes, dendritic cells, lymphocytes, NK cells, basophils, neutrophils, and eosinophils via CCR1 and CCR3 (35). In contrast, MIP-1α/ CCL3 attracts IFN-γ-activated neutrophils as well as a small subpopulation of CCR1-expressing eosinophils (33, 34).

Of interest, a typical marker of neutrophilic inflammation – MPO - was significantly elevated in all clusters, stressing the impact of neutrophiles in the not-Th2 CRSwNP with comorbid neutrophilic asthma, and also in the Th2 CRSwNP with eosinophilic asthma (35, 36). Similarly, the type-1 related cytokines IL-22and IFN-γ were overexpressed in low- and high-IL-5 clusters (clusters 1, 4, and 5; IFN-γ also in cluster 6). These results are comparable to the results of Tomassen et al. (12); however, in contrast to Tomassen et al., IL-17 and TNFα were only present in the low IL-5 cluster group. Yet, the relatively small number of patients per identified cluster in our study has to be considered a shortcoming. More studies should follow to confirm our results and add statistical weight to the findings.

Corroborating previous results (12), also in our study, the phenotype of CRSwNP correlated positively with the IL-5 concentrations, increasing from 11% in cluster-1 to 100% in cluster-7. This observation confirms recent data about CRSwNP subjects with a negative IL-5 profile, which is of importance to consider before making the therapeutic decision regarding use of type 2-related biologicals. Additionally, also non-type-2 asthma exists in CRSwNP subjects (9% in cluster-2), in contrast to the type-2-biased inflammatory patterns with an asthma prevalence of 50% in cluster-7. We diagnosed N-ERD only in type-2 endotypes, consistent with the results of Tomassen et al. The relevance of IFN-γ being present in N-ERD-patients was described before (37).

In conclusion, the endotype clustering of chronic rhinosinusitis based on chemokine expression pattern has confirmed and extended previous findings of CRS being a heterogeneous inflammatory disease (12). We identified detailed CRS endotypes characterized by a wide diversity of inflammatory markers. Increased levels of TARC/ CCL17, eotaxin/ CCL11, and PARC/ CCL18 associated with type-2 biomarkers in CRSwNP, and TARC and eotaxin may serve as early markers in patients without upregulation of IL-5 and IgE. Additional neutrophilic biomarkers contribute to endotype characterization in CRSsNP and CRSwNP. Our observations should be further expanded to increase the general knowledge about CRS, to augment the diagnostic and monitoring possibilities, and tune-up the therapeutic approach. The recent expansion of biologics drugs used to treat upper respiratory tract conditions fully justifies the individual, patient-directed diagnostic approach

to warrant a successful treatment and monitoring of CRS.

References:

1. Rudmik L, Soler ZM, Mace JC, Schlosser RJ, Smith TL. Economic evaluation of endoscopic sinus surgery versus continued medical therapy for refractory chronic rhinosinusitis. Laryngoscope. 2015;125(1):25-32.

2. Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A, et al. Chronic rhinosinusitis in Europe–an underestimated disease. A GA(2)LEN study. Allergy. 2011;66(9):1216-23.

3. Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. Executive summary of EPOS 2020 including integrated care pathways. Rhinology. 2020.

4. Jarvis D, Newson R, Lotvall J, Hastan D, Tomassen P, Keil T, et al. Asthma in adults and its association with chronic rhinosinusitis: the GA2LEN survey in Europe. Allergy. 2012;67(1):91-8.

5. van der Veen J, Seys SF, Timmermans M, Levie P, Jorissen M, Fokkens WJ, et al. Real-life study showing uncontrolled rhinosinusitis after sinus surgery in a tertiary referral centre. Allergy. 2017;72(2):282-90.

6. Van Zele T, Holtappels G, Gevaert P, Bachert C. Differences in initial immunoprofiles between recurrent and nonrecurrent chronic rhinosinusitis with nasal polyps. Am J Rhinol Allergy. 2014;28(3):192-8.

7. Alsharif S, Jonstam K, van Zele T, Gevaert P, Holtappels G, Bachert C. Endoscopic Sinus Surgery for Type-2 CRS wNP: An Endotype-Based Retrospective Study. Laryngoscope. 2019;129(6):1286-92.

8. Bachert C, Han JK, Desrosiers M, Hellings PW, Amin N, Lee SE, et al. Efficacy and safety of dupilumab in patients with severe chronic rhinosinusitis with nasal polyps (LIBERTY NP SINUS-24 and LIBERTY NP SINUS-52): results from two multicentre, randomised, double-blind, placebo-controlled, parallel-group phase 3 trials. Lancet. 2019.

9. Bachert C, Sousa AR, Lund VJ, Scadding GK, Gevaert P, Nasser S, et al. Reduced need for surgery in severe nasal polyposis with mepolizumab: Randomized trial. J Allergy Clin Immunol. 2017;140(4):1024-31 e14.

10. Gevaert P, Calus L, Van Zele T, Blomme K, De Ruyck N, Bauters W, et al. Omalizumab is effective in allergic and nonallergic patients with nasal polyps and asthma. J Allergy Clin Immunol. 2013;131(1):110-6 e1.

11. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy. 2006;61(11):1280-9.

12. Tomassen P, Vandeplas G, Van Zele T, Cardell LO, Arebro J, Olze H, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. J Allergy Clin Immunol. 2016;137(5):1449-56 e4.

13. Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol. 2000;18:217-42.

14. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12(2):121-7.

15. Palomino DC, Marti LC. Chemokines and immunity. Einstein (Sao Paulo). 2015;13(3):469-73.

16. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology. 2012;50(1):1-12.

17. Konig K, Klemens C, Haack M, Nicolo MS, Becker S, Kramer MF, et al. Cytokine patterns in nasal secretion of non-atopic patients distinguish between chronic rhinosinusitis with or without nasal polys. Allergy Asthma Clin Immunol. 2016;12:19.

18. Olze H, Forster U, Zuberbier T, Morawietz L, Luger EO. Eosinophilic nasal polyps are a rich source of eotaxin, eotaxin-2 and eotaxin-3. Rhinology. 2006;44(2):145-50.

19. Teplyakov A, Obmolova G, Gilliland GL. Structural insights into chemokine CCL17 recognition by antibody M116. Biochem Biophys Rep. 2018;13:27-31.

20. Peterson S, Poposki JA, Nagarkar DR, Chustz RT, Peters AT, Suh LA, et al. Increased expression of CC chemokine ligand 18 in patients with chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol. 2012;129(1):119-27 e1-9.

21. Wright ED, Frenkiel S, Ghaffar O, al-Ghamdi K, Luster A, Miotto D, et al. Monocyte chemotactic protein expression in allergy and non-allergy-associated chronic sinusitis. J Otolaryngol. 1998;27(5):281-7.

22. Yoshikawa M, Wada K, Yoshimura T, Asaka D, Okada N, Matsumoto K, et al. Increased CXCL10 expression in nasal fibroblasts from patients with refractory chronic rhinosinusitis and asthma. Allergol Int. 2013;62(4):495-502.

23. Rudack C, Sachse F, Alberty J. Primary role of growth-related oncogene-alpha and granulocyte chemotactic protein-2 as neutrophil chemoattractants in chronic rhinosinusitis. Clin Exp Allergy. 2006;36(6):748-59.

24. Elsner J, Hochstetter R, Kimmig D, Kapp A. Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. Eur J Immunol. 1996;26(8):1919-25.

25. Yao T, Kojima Y, Koyanagi A, Yokoi H, Saito T, Kawano K, et al. Eotaxin-1, -2, and -3 immunoreactivity and protein concentration in the nasal polyps of eosinophilic chronic rhinosinusitis patients. Laryngoscope. 2009;119(6):1053-9.

26. Kim B, Lee HJ, Im NR, Lee DY, Kim HK, Kang CY, et al. Decreased expression of CCL17 in the disrupted nasal polyp epithelium and its regulation by IL-4 and IL-5. PLoS One. 2018;13(5):e0197355.

27. Tsybikov NN, Egorova EV, Kuznik BI, Fefelova EV, Magen E. Biomarker assessment in chronic rhinitis and chronic rhinosinusitis: Endothelin-1, TARC/CCL17, neopterin, and alpha-defensins. Allergy Asthma Proc. 2016;37(1):35-42.

28. Kim HB, Kim CK, Iijima K, Kobayashi T, Kita H. Protein microarray analysis in patients with asthma: elevation of the chemokine PARC/CCL18 in sputum. Chest. 2009;135(2):295-302.

29. Kapitany A, Beke G, Nagy G, Doan-Xuan QM, Bacso Z, Gaspar K, et al. CD1c+ Blood Dendritic Cells in Atopic Dermatitis are Premature and Can Produce Disease-specific Chemokines. Acta Derm Venereol. 2017;97(3):325-31.

30. Pezato R, Perez-Novo CA, Holtappels G, De Ruyck N, Van Crombruggen K, De Vos G, et al. The expression of dendritic cell subsets in severe chronic rhinosinusitis with nasal polyps is altered. Immunobiology. 2014;219(9):729-36.

31. Jonstam K, Swanson BN, Mannent LP, Cardell LO, Tian N, Wang Y, et al. Dupilumab reduces local type 2 pro-inflammatory biomarkers in chronic rhinosinusitis with nasal polyposis. Allergy. 2019;74(4):743-52.

32. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med. 1998;187(1):129-34.

33. Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. J Exp Med. 1992;176(6):1489-95.

34. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36(10):1882-6.

35. Zhang N, Van Zele T, Perez-Novo C, Van Bruaene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. J Allergy Clin Immunol. 2008;122(5):961-8.

36. Thomson NC. Novel approaches to the management of noneosinophilic asthma. Ther Adv Respir Dis. 2016;10(3):211-34.

37. Steinke JW, Liu L, Huyett P, Negri J, Payne SC, Borish L. Prominent role of IFN-gamma in patients with aspirin-exacerbated respiratory disease. J Allergy Clin Immunol. 2013;132(4):856-65 e1-3.

38. Islam SA, Ling MF, Leung J, Shreffler WG, Luster AD. Identification of human CCR8 as a CCL18 receptor. J Exp Med. 2013;210(10):1889-98.

39. Cai M, Bonella F, He X, Sixt SU, Sarria R, Guzman J, et al. CCL18 in serum, BAL fluid and alveolar macrophage culture supernatant in interstitial lung diseases. Respir Med. 2013;107(9):1444-52.

40. Bellinghausen I, Reuter S, Martin H, Maxeiner J, Luxemburger U, Tureci O, et al. Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. J Allergy Clin Immunol. 2012;130(6):1384-93.

41. Wu D, Zhou J, Bi H, Li L, Gao W, Huang M, et al. CCL11 as a potential diagnostic marker for asthma? J Asthma. 2014;51(8):847-54.

42. Elsner J, Escher SE, Forssmann U. Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. Allergy. 2004;59(12):1243-58.

43. Van Coillie E, Van Damme J, Opdenakker G. The MCP/eotaxin subfamily of CC chemokines. Cytokine Growth Factor Rev. 1999;10(1):61-86.

44. Proost P, Van Leuven P, Wuyts A, Ebberink R, Opdenakker G, Van Damme J. Chemical synthesis, purification and folding of the human monocyte chemotactic proteins MCP-2 and MCP-3 into biologically active chemokines. Cytokine. 1995;7(2):97-104.

45. Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. J Leukoc Biol. 1997;62(5):604-11.

46. Lukacs NW, Hogaboam CM, Kunkel SL, Chensue SW, Burdick MD, Evanoff HL, et al. Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. J Leukoc Biol. 1998;63(6):746-51.

47. Strieter RM, Kunkel SL, Burdick MD, Lincoln PM, Walz A. The detection of a novel neutrophil-activating peptide (ENA-78) using a sensitive ELISA. Immunol Invest. 1992;21(6):589-96.

48. Mazzi V, Fallahi P. Allergic rhinitis and CXCR3 chemokines. Clin Ter. 2017;168(1):e54-e8.

49. Medoff BD, Sauty A, Tager AM, Maclean JA, Smith RN, Mathew A, et al. IFN-gamma-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. J Immunol. 2002;168(10):5278-86.

Table 1 Origin and functions of analyzed chemokines.

Chemokines	Origin	Function
TARC/CCL17 (**CC chemokine**) thymus and activation-regulated chemokine CCR4, CCR8 receptor	secreted from monocytes, dendritic and epithelial cells (19)	attracts T lymphocytes (Th2)

PARC/CCL18 (**CC chemokine**) pulmonary and activation- regulated chemokine CCR8 receptor (38)	secreted from antigen-presenting cells, mast cells, eosinophils and neutrophils (39)	attracts naive $CD4^+$ and $CD8^+$ T cells, B cells, and immature dendritic cells (38, 40)
Eotaxin-1/CCL11 (**CC chemokine)** eosinophil chemotactic protein CCR3 receptor	secreted from eosinophils (41)	attracts eosinophils (42), selective on CCR3 receptor (24)
MCP-3/CCL7 (**CC chemokine)** monocyte chemotactic protein-3 CCR1, CCR3 receptor	secreted from mononuclear cells (43)	attracts monocytes, dendritic cells, lymphocytes, natural killer cells, eosinophils, basophils and neutrophils (44)
MIΠ1α/ˆ˜Λ3 (**CC chemokine)** macrophage inflammatory protein-1α CCR1, CCR5 receptors	secreted from T- and B-lymphocytes, neutrophils, dendritic cells, mast cells and NK cells (34)	attracts IFNγ-activated neutrophils and only small subpopulations of CCR1 expressing eosinophils (34)
ENA-78/CCL5 (**CXC chemokine)** the epithelial cell-derived neutrophil-activating peptide CXCR2 receptor	secreted from monocytes, endothelial cells, mast cells, keratinocytes, fibroblasts (45, 46)	attracts neutrophils (47)
IP-10 /CXCL10 (**CC chemokine)** IFNγ-inducible protein 10 CXCR3 receptor	secreted from monocytes, T-lymphocytes, fibroblasts, keratinocytes (48) in response to the Th1 cytokine IFNγ	attracts activated Th1 lymphocytes (49)

Table 2 Markers used for the component and cluster analysis, the cut-off value for markers that were analyzed as categorical.

Marker	**Cutoff values***	**Interpretation of increased concentrations**
Eotaxin/ CCL11	NA	Type 2 activity
MCP-3/ CCL7	NA	Type 1 and type 2 activity
MIP-1 α/CCL3	NA	Type 1 and type 2 activity
ENA-78/CXCL5	NA	Neutrophilic inflammation
TARC/CCL17	NA	Type 2 activity
PARC/CCL18	NA	Type 2 activity
IP-10/ CXCL10	NA	Type 2 activity
ECP	NA	Type 2 activity
IgE	NA	Adaptive immunity marker
SE-IgE	3.85 kU_A/L*	Marker or superantigen effect on local mucosa
IL-5	12.98 pg/ml*	Type 2 activity
MPO	NA	Neutrophilic activity
IL-22		Type 1activity
TNF-α	38.94 pg/ml*	Type 1activity
IL-17	25.06 pg/ml*	TH17 activity
IFN-g	85.8 pg/ml*	Type 1 activity

*Cutoff values according to Tomassen et al. 2016 (12)

Table 3 Descriptive statistics of CRS patients and control subjects.

		Patients	Controls	Significance
Clinic	Number	66	25	
	Age MV	MV 40.04	MV 30.96	**p=0.001**
	Gender male %	65.3%	56.0%	p=0.474
	Smoker former %	69.4%	64.0%	p=0.626
	Smoker current %	33.3%	48.0%	p=0.232
	Allergy %	26.4%	16.0%	p=0.415
	Asthma %	2%	26.4%	p=0,.088
	N-ERD %	8.3%	0%	p=0.334
Laboratory Findings	Eotaxin/ CCL11 pg/ml	MV 37.98	MV 11.68	**p=0.002**
	MCP-3/ CCL7 pg/ml	MV 3.98	MV 5.73	p=0.226
	MIP-1α/ CCL3pg/ ml	MV 4.18	MV 5.94	p=0.334
	ENA-78/ CXCL5pg/ml	MV 43.97	MV 113.19	p=0.063
	TARC/ CCL17 pg/ml	MV 8.42	MV 5.86	**p=0.000**
	PARC/ CCL18pg/ml	MV 383.61	MV 119.03	p=0.038
	IP-10/CXCL10 pg/ml	MV 424.12	MV 274.25	p=0.287
	Total IgE kU/l	MV 337.34	MV 30.00	**p=0.002**
	ECP pg/ml	MV 5122.77	MV 194.37	**p=0.000**
	IL-5 pg/ml	MV 412.83	MV 2.91	**p=0.000**
	SAE kU/l	MV 8.34	MV 0.00	p=0.073
	IL-22 pg/ml	MV 629.97	MV 484.64	p=0.283
	TNF-α pg/ml	MV 13.29	MV 10.06	p=0.715
	IL-17 pg/ml	MV 14.56	MV 14.49	p=0.067
	IFN-γ pg/ml	MV 55.23	MV 163.51	p=0.227

Table 4 Endotype profiles: Modified heat map of clustering of individual cases. Rows define clusters of patients with CRS. Variables used in cluster analysis are given in columns. Some parameters were used in a categorical manner according to the high rate of measurements under the detection limit (SAE, TNF-alpha, IL-17 and IFN-gamma). Significantly elevated values between the CRS cluster differences are underlined.

		Variables analyzed in cluster analysis	Variables analyzed in cluster analysis	Variables analy
		ENA_78 / CXCL5	**MPO**	**IP-10 / CXCL1**
		MV	MV	MV
Cluster	**1**	32.91463	2866.261	332.7771
	2	53.37202	1982.33	346.3294
	3	82.2664	2426.655	1110.102
	4	45.04413	4034.732	264.4073
	5	74.70387	5507.627	173.8526
	6	10.73267	2795.157	87.71971
	7	7.239	35603.54	33.57911

Table 5 Association of the endotypes with the clinical phenotypes: Summary graph.

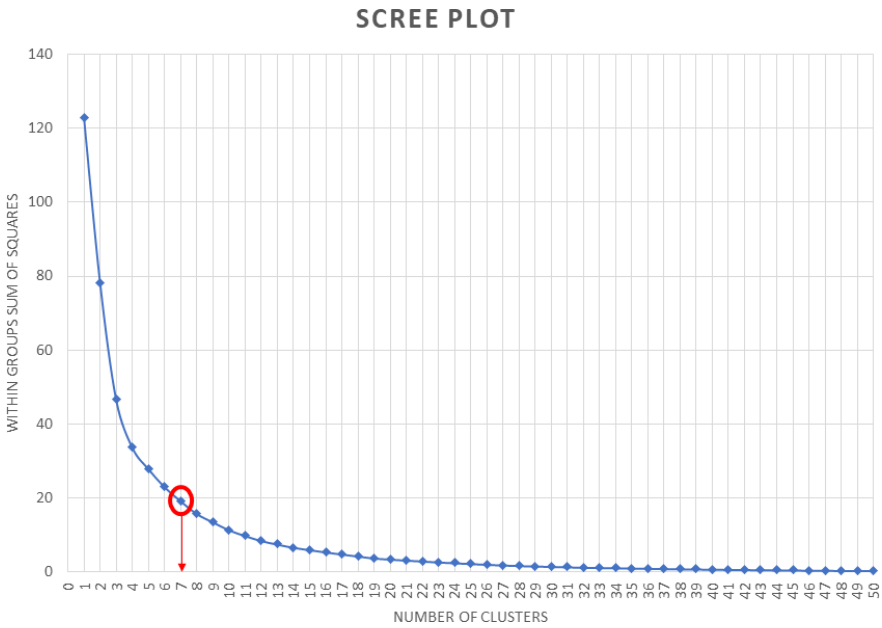
Simplified schema of the clusters and their characteristic chemokines, as well as the proportion of CRSwNP and CRSsNP, asthma and N-ERD. One cluster with negative IL-5, 3 clusters with low IL-5 and 3 clusters with high IL-5 values are given.

Cluster	Number of cases	IL-5	IL-5 positive Ratio %	IL-5	ECP	Total IgE	PARC (CCL18)	TARC (CCL17)	Eotaxin (CCL11)	MCP3 (CCL7)	MIII1a (CCL3)	ENA-78 (CCL5)	MPO	IP-10 (CXCL10	IL 22
1	9	**IL-5 Negative**	0.0										>2		>3
2	11	**IL-5 low**	18.2							>3					
3	8		25.0		>2			>2	>2	>5	>5			>5	>2
4	13		53.8	>2	>2	>2		>2	>2				>3		>5
5	7	**IL-5 high**	85.7	>3	>5	>3	>5	>5	>5	>5	>5		>5		
6	16		100	>5	>3	>5 +SE IgE	>3	>3	>5				>2		
7	2		100	>5	>5	>5 +SE IgE	>5	>5	>3				>5		

Figure Legends:

Figure 1 Scree plot:

The scree plot was used to determine the optimal number of clusters to be retained in further analysis, as described elsewhere (12).



Conflict of interest:

The authors declare no conflict of interest.

Contributions:

UFR data collection and laboratory measurements, analyzed and interpreted data, manuscript draft

GP data collection, critical input to the manuscript

AJS data interpretation, manuscript draft and final version

JWF critical input to the manuscript

MA laboratory measurements, critical input to the manuscript

TZ critical input to the manuscript

CB study concept and design, critical input to the manuscript

HO study concept and design, study lead, data analysis and interpretation, manuscript draft and final version