Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers

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Abstract

Background: Current phenotyping of chronic rhinosinusitis (CRS) into CRS with nasal polyps and without nasal polyps may not adequately reflect the pathophysiologic diversity within CRS.

Objective: We sought to identify inflammatory endotypes of chronic rhinosinusitis. Therefore, we aimed to cluster CRS subjects solely based on immune markers in a phenotype-free approach. Secondarily we aimed to match clusters to phenotypes.

Methods: In this multicenter case-control study, CRS cases and controls underwent surgery, and tissue was analyzed for IL-5, IFN-γ, IL-17A, TNF-α, IL-22, IL-1β, IL-6, IL-8, ECP, MPO, TGF-β1, IgE, Staphylococcus aureus enterotoxin specific IgE (SE-IgE), and albumin. We used using partition-based clustering.

Results: Clustering of 173 cases resulted in 4 clusters with low or undetectable IL-5, ECP, IgE and albumin concentrations, and 6 clusters having high concentrations of those markers. Three of four IL-5-negative clusters had no inflammation, a Th22, or a Th1 profile, and clinically resembled a predominant CRSsNP phenotype without increased asthma prevalence. One had a Th17 profile and had mixed CRSsNP/CRSwNP. The IL-5-positive clusters were divided in a group of three clusters with moderate IL-5 concentrations, mixed CRSsNP/CRSwNP and increased asthma phenotype, and a group with high IL-5 levels, almost exclusive nasal polyp phenotype and strongly increased asthma prevalence. In the latter, two clusters demonstrated highest concentrations of IgE and asthma prevalence with all samples expressing SE-IgE.

Conclusion: Distinct CRS clusters with diverse inflammatory mechanisms largely correlated with phenotypes and further differentiated them, and provide a more accurate description of the inflammatory mechanisms involved than phenotype information only.

Key messages: Chronic rhinosinusitis consists of several inflammatory endotypes defined by the composition of inflammatory markers; those endotypes correlate with the clinical expression of disease and asthma.

Key words: Chronic rhinosinusitis with and without nasal polyps (CRSwNP, CRSsNP), endotypes, cluster analysis, inflammation, comorbid asthma

Capsule summary: Phenotypes in chronic rhinosinusitis may further be differentiated in inflammatory endotypes, reflecting different pathophysiological mechanisms, and may help to determine therapeutic targets

Abbreviations:

CRS: chronic rhinosinusitis

CRSsNP: chronic rhinosinusitis without nasal polyps

CRSwNP: chronic rhinosinusitis with nasal polyps

ECP: eosinophilic cationic protein

IFN: interferon

IgE: immunoglobulin E

IL: interleukin

MPO: myeloperoxidase

SE-IgE: Staphylococcus aureus enterotoxin specific IgE

TGF: transforming growth factor

Th: T-helper

TNF: tumor necrosis factor

Introduction

Chronic rhinosinusitis (CRS) is a disabling disease affecting 10.9% of the European [1] and 13.4% of the American [2] general population. CRS is defined by symptoms and clinical signs, and diagnosis may be supported by nasal endoscopy and CT scanning [3]. In a European multicenter epidemiological study involving 56,000 individuals, it recently has been demonstrated that cigarette smoking increases the risk of CRS [1]; of interest, CRS is also associated with late-onset asthma [4]. However, questionnaire-based population studies are limited in further defining possible subgroups relevant for these associations [5]. Indeed, CRS shows remarkable heterogeneity, both at the clinical phenotype level and at the molecular pathophysiological level. Current consensus in Europe and the US discerns two major phenotypes - defined as subgroups of patients with homogeneous clinically observable characteristics [6] - based on nasal endoscopic findings: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP). Furthermore, there are additional subtypes such as allergic fungal rhinosinusitis, CRS associated with aspirin-exacerbated respiratory disease, and with cystic fibrosis which may present as CRSwNP or CRSsNP [3].

The clinical dichotomization of CRSwNP *vs.* CRSsNP was initially reflected at the molecular level, showing a predominance of Th1 cells in CRSsNP and of Th2 cells and eosinophils in CRSwNP in Caucasians [7, 8]. However, subsequent studies reported a wider spectrum of immunologic profiles, especially in non-Caucasian phenotypes [9], expressing a neutrophilic type of inflammation with involvement of other T-cell subsets such as Th1 and Th17 cells. Furthermore, the simultaneous expression of different Th cell types within a single tissue was demonstrated [10].

Thus, the simple differentiation in Th1 and Th2 disease does not encompass the molecular diversity in CRS, and the clinical phenotype does not adequately identify the immunological profile. Therefore we sought to identify inflammatory endotypes of CRS – defined as "subtypes of disease with a unique pathomechanism, functionally and pathologically different from others by the involvement of a specific molecule or cell". [11] For this, we aimed to cluster CRS subjects solely based on immune markers in a clinical phenotype-free approach. Secondarily, we aimed to match these endotypes with clinical phenotypes and with selected clinical parameters, of which we already have identified association with immune markers [12, 13]. This approach would also facilitate the identification of therapeutic targets and predict response to those approaches, e.g. biologicals.

Methods

Study design

The study was designed as a multicenter case-control study carried out by the Ga²len Sinusitis Cohort group (principal investigator C. Bachert) in the framework of the European FP6 research initiative. The ENT departments in tertiary referral academic hospitals of Ghent, Leuven, Amsterdam, Barcelona, London, Berlin, Helsinki, Lodz, Malmö, and Stockholm participated in this study. This study was approved by the ethical committees of all individual institutions involved in data and tissue collection. Informed consent was obtained from all subjects before sample collection.

Patient population

Patients with CRS (defined by EPOS-criteria based on symptoms, nasal endoscopy and computed tomography of the sinuses [3]) were included as cases, and subjects undergoing inferior turbinate surgery with no signs of CRS were included as controls. Excluded were subjects with an acute exacerbation of rhinosinusitis 2 weeks preceding inclusion, subjects with immunodeficiencies, cystic fibrosis patients, and subjects who used oral or nasal steroids in the 4 weeks preceding surgery, or anti-leukotrienes in 2 weeks preceding inclusion. In total, 917 subjects were recruited, of whom 682 cases (of which 65% CRSwNP) and 187 controls. Subjects underwent a standardized skin prick test [14] and subjects were registered as asthmatic based on a clinical diagnosis. Patients were considered allergic on clinical grounds (positive skin prick test or provocation tests or specific IgE to inhalant allergens plus allergy symptoms present).

At a maximum of 12 months after inclusion, a portion of the subjects underwent functional endoscopic sinus surgery, and controls underwent partial inferior turbinotomy during septal surgery (226 CRS patients and 106 controls). Controls having allergic rhinitis were not excluded. The indications for surgery and its procedures were based on clinical decisions independent from the participation in the study. Tissue was collected from nasal polyps or sinus mucosa in patients with CRS, and from the inferior turbinates in control subjects. Tissue was separated from bone fragments, snap-frozen in liquid nitrogen and stored at -80°C. Control tissues were not used in the cluster analyses of the CRS patients, but solely served to determine increased concentrations of markers above normal.

Measurement of inflammatory markers

For tissue analysis, we selected 14 markers (Table 1) based on earlier publications; those markers reflect the inflammatory patterns observed in CRSsNP and CRSwNP. Only subjects of which there was an adequate amount of tissue that was needed for these analyses (typically 0.15g), were included. Briefly, as described before [15], each 0.1g of tissue was diluted in 1mL of 0.9% NaCl solution containing a protease inhibitor cocktail (Complete®, Roche Diagnostics, Mannheim, Germany), homogenized at 1000 rpm for 5 min and centrifuged at 1500 g for 10 min at 4°C. Concentrations of eosinophilic cationic protein (ECP), total immunoglobulin E (IgE) and IgE specific to a mixture of *S. aureus* enterotoxins (SEA, SEC, and TSST-1) were assayed using the UNICAP system (Phadia, Uppsala, Sweden). Concentrations of IL-22, IFN-γ, and TGF-β1 were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kits from R&D systems (Minneapolis, MN, USA). Myeloperoxidase (MPO) concentration was measured using a commercially available ELISA from BioCheck, Inc (Foster City, CA, USA). For albumin we used kits from AssayPro (St. Charles, MO, USA). Concentrations of IL-1β, IL-5, IL-6, IL-8, IL17A and TNF-α were assayed using the Luminex 100 system (Luminex, Austin, TX, USA).

Concentrations in tissue homogenates were expressed as mass versus volume after multiplication with the homogenization dilution factor of 11. Values below the limit of detection were considered negative for categorical analysis and were given a value equal to half of the detection limit for continuous analysis.

Statistical methods

Statistical software R version 2.15.2 was used [16]. The following variables which had a high proportion (>33%) of values below detection limit were used as binomial variables, with their detection limits used as cut-off: SE-IgE (cut-off 3.85 kUA/L), IFN-γ (85.8 pg/mL), TNF-α (38.94 pg/mL), IL-17A (25.06 pg/mL), IL-5 (12.98 pg/mL). Baseline parameters were tested for difference between cases and controls using t-test on continuous and chi-squared test on discrete variables.

To analyze relationships between variables, we performed principal component analysis, after which orthogonal rotation with Kaiser normalization was performed, and only variables with loadings higher than 0.4 were retained. Next, we performed cluster analysis on the variables using ascendant hierarchical clustering based on the correlation ratio and the mixed principal component analysis ([17], and the optimal number of cluster was determined using the Rand statistic.

For cluster analysis of individual cases, a dissimilarity matrix according to Gower was calculated, which allows both ordinal and binomial data. Next, clusters were calculated using the partitioning around medoids method, of which outcomes with the number of clusters ranging from 2 to 15 clusters were generated. The optimal number of clusters was based on the elbow (maximum change) of the scree plot of the mean silhouette width, Baker-Hubert Gamma statistic [18], and Hubert-Levin C index [19]. Additionally, the optimal number of clusters was assessed using Jaccard stability after bootstrap resampling [20] and visual inspection of the clusplot after multidimensional scaling[20].

For descriptive characterization of individual clusters, cytokine and phenotype data from each cluster were tested for difference from the control group (Mann Whitney U and logistic regression). Next, between-cluster differences of all parameters, as well as phenotype parameters, were tested using Kruskal-Wallis with multiple group comparison with Benjamini-Hochberg adjustment for multiple testing, or Tukey contrasts in logistic models for binomial parameters. For visualization of clusters, a "clusplot" was drawn, plotting individuals in two dimensions after multidimensional scaling.

As an aid in interpretation of inflammatory patterns, each cytokine in each cluster was categorized as increased compared to controls only, and increased compared to controls and to 2, 3 or 6 other clusters, or no difference from control or other clusters. Furthermore, for each cluster we interpreted the inflammatory patterns based on a high proportion of positive categorical variables or on increased concentrations of continuous variables, compared to controls and other clusters. The pattern for interpretation is listed in Table 1. A modified heatmap was created (Figure 3), after ordering and grouping clusters with similar characteristics, and ordering variables according to the previous principal component analysis. The variables that were used categorically in the cluster analysis, were additionally tabulated with their continuous values.

Results

Of the 226 cases and 106 controls who underwent surgery, 173 cases and 89 controls had adequate amount of tissue prelevated to carry out all intended analyses. Demographic, phenotypic and cytokine data of cases and controls are tabulated in Table 2; demographic data of the sample included were not different from the total cohort. Cases had a significantly higher age, and had a higher prevalence of smoking history and asthma. Cases had significantly higher concentrations than controls of IL-6, IL-8, albumin, MPO, ECP, IgE, and

had significantly higher proportion of concentration above detection limit for IL-5, IFN-γ and SE-IgE (see Table 2).

Principal component analysis (illustrated in Table E1 and Figure 1A-B) retained 5 components, explaining 74% of all variance in the data. The first component was composed of MPO, IL-1β, IL-6 and IL-8. The second component consisted of IgE, ECP, IL-5 and albumin. The third component had TNF-α, IL-17 and IL-22. The fourth component was composed of TGF-β and SE-IgE, and the last component of IFN-γ and SE-IgE. In a hierarchical cluster analysis of variables (illustrated in figure 1C), these were optimally clustered in the 6 following clusters: (1) IgE, ECP, IL-5, albumin, SE-IgE (2) IL1, IL6, IL8, MPO, (3) IL17, TNF-α, (4) TGF-β1 (5) IL-22 and (6) IFN-γ.

Clustering of all CRS individuals using cytokine measurements, irrespective of phenotype information, resulted in an optimal outcome of 10 clusters (details are given in Figure E1 and Table E2). The clusters were well separated from each other, as illustrated in the cluster plot (Figure 2). Tests for between-cluster differences and differences from controls showed that for all cytokines, except TGF-β1 and IL-1β, there was at least one cluster having significantly higher concentrations than any other cluster. Compared to the control group, all cytokines except TGF-β1 were significantly increased ($p<0,05$) in at least one cluster. Not all clusters that had a significant difference from the control group, had differences with other clusters. IL-22 concentrations did not differ significantly from controls, however there were multiple significant between-group differences.

Analogously, clinical parameters (which were not used in the cluster analysis) were analyzed within clusters and compared with controls (Figure 3). The proportion of CRSwNP was significantly different between clusters, with clusters 1-3 significantly lower than most clusters and clusters 8-10 significantly higher than most clusters. Asthma prevalence was significantly different from controls , and differed significantly between clusters. Age, gender, prevalence of allergy and of reported aspirin sensitivity were not significantly different between clusters (Table E3).

To characterize the clusters, means and ratios of cytokine and phenotype data were calculated and tabulated as a heatmap (Figure 3). The most striking differentiation was formed by the IL-5/ECP/IgE/albumin axis, resulting in four clusters having undetectable or low concentrations of these biomarkers, and six clusters having high concentrations. The endotype differentiation was also reflected in the clinical phenotypes, with three clusters (1-3) showing a predominant

CRSsNP appearance, four clusters (4-7) a mixed phenotype, and the three clusters (8-10) showing a predominant CRSwNP appearance. Clusters 1-3 showed no increase in asthma prevalence, whereas clusters 4-10 the asthma prevalence was increased compared to controls and to clusters 1-3, with further increases in clusters 9 and 10.

The 4 clusters with predominant non-eosinophilic appearance could further be differentiated as follows: cluster 1 did not show any signs of inflammation (no single cytokine was significantly increased compared to controls in this cluster); cluster 2, a pure CRSsNP cluster, overexpressed TNF-α and IL-22, and cluster 3 overexpressed IFN-γ and MPO/IL-8. Cluster 4, in contrast, showed a neutrophilic inflammation with upregulation of pro-inflammatory cytokines IL-1β/6, IL-8/MPO, as well as IL-17A, -/22, TNF-α and partially IFN-γ.

The latter 6 clusters were characterized by the expression of IL-5 in every single subject. These clusters also expressed increased ECP and total IgE concentrations. This IL-5 positive group could be further differentiated in a group of three clusters with mean concentrations of IL-5 between 100 to 151 pg/ml (moderate expression group), and a high expression group (257 to 483 pg/ml) including clusters 8 to 10. In parallel, with the eosinophilic inflammation, there was a neutrophilic inflammation with increased concentrations of IL-8, MPO in clusters 6 -10.

The IL-5 moderate expression group (clusters 5-7) was predominantly, but not exclusively consisting of the CRSwNP phenotype. Further differentiation within these groups was made by co-expression of IFN-γ (cluster 6), or TNF-α and increased IL-22 (cluster 7). The high expression group (clusters 8-10) was almost exclusively composed of nasal polyps, and had highest levels of albumin, IL-6 and IL-8. Additionally cluster 8 and 10 showed a Th17-family based inflammation. Clusters 9 and 10 demonstrated the highest concentrations of tIgE (around 1000 kU/l) and the highest rate of co-morbid asthma with 64-71%. All samples in these two clusters expressed SE-IgE antibodies in the tissue.

Discussion

We here present inflammatory endotypes of chronic rhinosinusitis, based on a cluster analysis solely based on biomarkers, and secondarily we correlate these to clinical characteristics (phenotypes). In this cohort of European subjects who underwent surgery for CRS, we observed considerable variability in the data, so that multiple homogeneous subgroups (clusters) could be differentiated. This observation indicates that CRS is not a homogeneous inflammatory disease, and endotypes are present with a wide diversity of inflammatory profiles. To our knowledge, this is the first study to report the existence of inflammatory endotypes within CRS, unbiased by phenotype data. Furthermore, we demonstrated that the newly defined endotypes mirror clinical phenotypes, supporting their clinical relevance.

To determine which parameters were relevant in the generation of this diversity, principal component analysis and clustering of variables showed that diversity in our samples was driven by five groups of related cytokines: (1) markers of eosinophilic, Th2-driven inflammation and antibody production (ECP, IL-5, IgE and SE-IgE) together with albumin (2) neutrophilic and pro-inflammatory cytokines (IL1β, IL6, IL8, MPO) (3) Th17 or Th22 related markers (IL-17A, IL-22, TNF-α, and (4) the Th1 marker IFN-γ. Moreover, TGF-β1 had a separate but small contribution to variability in our subjects.

These observations have previously been observed, albeit in specific CRS subgroups. The correlations of ECP, IL5 and IgE confirm previous data in Caucasian nasal polyps, where inflammation is dominated by activated eosinophils [15] and IgE concentrations were related to eosinophilic inflammation[21]. The association of albumin with eosinophil products can be related to observations in nasal polyps where increased albumin deposition has been thought to be caused by eosinophil-driven inflammation [15]. The expression of neutrophilic and pro-inflammatory cytokines has been observed previously in Chinese nasal polyp patients [9] and more specifically in Chinese nasal polyp patients that were IL-5/IL-17/IFN-γ negative [22].

Importantly, our observations were present in the whole CRS group, whereas previous results were limited to specific subgroups, implicating that that these previously described mechanisms can be extrapolated to the overall CRS group, and might not be restricted to the CRSwNP phenotype. Indeed, separate post hoc analyses in the CRSsNP and CRSwNP subgroup yielded similar principal components (data not shown), indicating that even in the lower concentration ranges, concentration gradients of cytokines are present and meaningful. These findings indicate that CRS is not a dichotomal or a one-dimensional linear spectrum of

diseases but rather multidimensional with different polarizations on the main inflammatory axes.

When individuals were clustered into inflammatory endotypes, our analysis resulted in 10 clusters of which each cytokine pattern was interpreted, and accordingly, clusters could be grouped together (summarized in Figure 4). To characterize each cluster's phenotype, we correlated clusters with selected phenotype parameters such as nasal polyp prevalence and asthma comorbidity. Importantly, this was a post-hoc analysis, and the phenotype information was not used during cluster analysis.

We show a strong correlation with the phenotype, with three clusters almost exclusively composed of CRSsNP without asthma comorbidity, and three clusters almost exclusively of CRSwNP with strongly increased asthma prevalence. Presence of IL-5 remains the paramount factor dictating the phenotype with nasal polyps and asthma, in line with previous evidence [13]. SE-IgE presence remains associated with nasal polyps with intense eosinophilic inflammation with very high IgE concentrations, and asthma comorbidity, as demonstrated before [13]. As summarized in Figure 4, clusters can accordingly be grouped in four IL-5 negative clusters, three moderately increased IL-5 positive clusters, and three IL-5 high clusters of which two SE-IgE positive clusters.

However, in clusters 4 to 7 there is a mixture of phenotypes, with polyp prevalence around 50 to 60% and albumin moderately increased, although each of these clusters had a distinct inflammatory profile. Three of these clusters had moderate increase in IL-5 concentrations and further had an eosinophilic pattern, but differed in terms of IFN-γ and TNF-α expression. This might indicate that for some groups, IL-5 is not the only key to edema and polyp formation, and other, possibly unmeasured factors might play a role in differentiation towards a polyp. Our study was limited to the major cytokines, and the amount of tissue available limited further analyses. Also, subjects were allocated to a phenotype during office endoscopy before surgery, and the clinical differentiation might be unclear in some cases were polyps were only observed during surgery. In cases of middle turbinate-confined polypoid oedema, the phenotype might not be clear-cut during office endoscopy, and it has been shown that early stage nasal polyps already show eosinophilic inflammation [15, 23]. Furthermore this overlap may be inherent to the concept of phenotype, which, as has been shown in asthma phenotyping, fail to provide adequate insight in the underlying pathogenic mechanisms [24].

Within the non Th2 group, inflammation is variable, from being completely absent to broad, involving Th1, Th17 or Th22. The role for Th1 in only a part of the IL-5 negative subjects contrasts with earlier findings, showing increased levels of IFN-γ protein and mRNA in CRSsNP subjects[8]. This could be explained, however, by the averaging effect of former analysis approaches. One of the overlapping clusters (4) had no eosinophils or IL-5, and in contrast a Th17 – neutrophilic type inflammation. Possibly this endotype is the same as what has been observed before in Chinese nasal polyps[9]. Interestingly, it was recently shown that second-generation Asian CRSwNP patients in USA showed a higher rate of non-eosinophilic polyps than Caucasians [25]. Unfortunately, we had no ancestry information in our study to further analyze this relationship.

The production of Th17-family derived cytokines IL-17A and IL-22 correlated modestly as was shown by the principal components analysis. Reasons for this modest correlation are apparent when analyzing the cluster profiles; in cluster 4 and 8 the IL-17A production was paired with increased IL-22 production whereas in clusters 2 and 7, only increased IL-22 production was present, possibly indicating Th22 involvement in these clusters. Interestingly, in cluster 10, which was a SE-IgE positive cluster, the increased IL-22 production was paired with only marginal IL-17A production, indicating a Th22 involvement which also was observed to be induced by staphylococcal enterotoxins in atopic dermatitis [26]. Additionally, we observed increased TNF-α levels in all of these clusters, consistent with the observation that both Th17 and Th22 cells may induce TNF-α production[27]. However, IL-22 concentrations did not differ from controls, and this effect remained after excluding allergic controls (data not shown). The reasons for this discrepancy with coherent between-cluster differences are not clear and the relevance of Th22 in the pathology of CRS should be further elucidated.

Surprisingly, we found little contribution of TGF-β1 to the variability of our data; the TGF-β1 concentration had no significant between-cluster differences and did not differ between clusters and the control group. Previously, expression of TGF-β signaling was in Caucasians found to be increased in CRSsNP, sharply contrasting to a decreased signaling in CRSwNP [28]. These findings were later confirmed in Chinese CRS subjects[29], although these are characterized by different T effector cell profiles than their Caucasian counterparts [9]. In our study, CRSsNP subjects were confirmed to have increased TGF-β1 concentrations compared to CRSwNP subjects and controls ($p<0.001$, data not shown). However, we did not have adequate amounts of tissue to further analyze remodeling patterns and T regulatory cell

involvement. Taken together, these findings might indicate that remodeling is strongly correlated with the phenotype (fibrosis vs. edema formation), irrespective of the inflammatory endotype.

Some of the broad characteristics of this clustering are found in a previous cluster analysis by Nakayama[30] who proposed a clustering where nasal polyp score and tissue eosinophilia mainly determined the phenotype. Interestingly, in a recent cluster analysis of CRS subjects focusing on symptoms and clinical information, nasal polyposis did not differ between phenotypes[31]. However, these approaches are inherently different from our study, as we based our cluster analysis solely on inflammatory mediators to obtain endotypes, and only in a post-hoc analysis we compared these with selected phenotype parameters such as nasal polyp prevalence and asthma comorbidity.

Our study has some limitations. Our definition of endotypes was entirely based on inflammatory biomarkers. However, for asthma, it is proposed that more parameters, such as clinical characteristics, genetics and treatment responses should be included for the definition of an endotype[33]. We aimed to propose 'inflammatory endotypes', and further studies are needed incorporating clinical characteristics, genetics, remodeling parameters, and treatment responses to further define these endotypes. Some of these endotypes might eventually be merged or split with other endotypes given these additional studies.

The use of cluster analysis is potentially a limiting factor in this study. As cluster analysis is no inferential statistical technique, there is no probability outcome. The technique generates multiple possible solutions in terms of number of clusters, of which the most relevant solution is selected by the examiner. To scrutinize this process, the selection of the best number of clusters was based on objective criteria such as the Hubert-Levine index as a measure of internal cluster validity, as well as bootstrap resampling of the cluster analysis. Even though all these measure resulted in a certain optimal number of clusters, it is not our aim to claim a final definite classification in which all CRS subjects should be categorized, but instead, illustrate the diversity in inflammatory profiles.

The inflammatory profile of asthmatics has been found to evolve over time within subjects. As we only analyzed our subjects on one time point, possible variability of the inflammatory endotype over time limits our findings. Indeed, when comparing the profile of nasal polyp patients undergoing two surgeries due to disease recurrence, it has been found that all nasal polyps remained IL-5 positive over a period of in average 5 years, however in some patients IL-17 became co-expressed[12]. To determine the clinical and therapeutic utility of the

inflammatory endotypes, further longitudinal studies are needed, evaluating their stability over time, in relation to possible treatment and environmental changes.

The applicability of our study, which was done in a European population, to other regions in the world, remains at question as well. For example, the high smoking prevalence in our study was comparable to the European average, but lower smoking prevalences in North America might affect the inflammatory endotype. Other lifestyle and environmental factors might affect the endotype, and global studies are needed to address these.

Furthermore, Our choice of biomarkers did not cover remodeling parameters, and we have not included cellular contents. However, the current analysis enables the appreciation of the possible target groups for various therapeutic monoclonal antibodies currently in study.

In summary, we have identified distinct inflammatory endotypes within chronic rhinosinusitis, which largely correlated with phenotypes and further differentiated them. Endotypes clearly provide a more accurate description of the inflammatory mechanisms involved than phenotype information only, and inflammation in CRS may be more diverse than previously assumed. CRS inflammation should be considered as multidimensionally heterogeneous on the Th1, Th2, Th17, eosinophilic/neutrophilic, pro-inflammatory, superantigen and possibly Th22 axes. Inflammatory endotypes might be of importance for the prognosis of comorbid asthma development or disease recurrence after surgery[12]. As recent monoclonal antibody approaches such as omalizumab [34], mepolizumab [35] or dupilumab target specific cytokines or pathways, it is expected that an approach based on biomarker-defined clusters would identify responders to these drugs. Nasal levels of IL-5 for example were found to predict responders to reslizumab, an anti-IL5 monoclonal antibody[36]. The clinical applicability of inflammatory endotypes, however, would be aided by the availability of noninvasive serum or nasal secretion markers; a range of markers is currently under investigation in the same cohort of CRS patients.

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Figure legends

Figure 1. Principal component analysis and clustering of variables. A: Vectors of each parameter are plotted against the first four rotated principal components. A1: First two principal components, B: Third and fourth principal component. The fifth rotated component is not depicted, it consisted of IFN-γ and SE-IgE. C. Dendrogram of clustered variables. Horizontal dashed line indicates the tree cut used in this analysis, based on the Rand index and resulting in 6 variable clusters.

Figure 2 Cluster plot of cases. Clustering of cases illustrated in a clusplot where individuals are plotted in a two-dimensional space after multidimensional scaling. Here, dissimilarities between individuals, taking in account all variables, are represented by distances between the individuals in two dimensions.

Figure 3. Modified heat map of clustering of individual cases.

Rows define clusters of CRS cases; these were arbitrarily (non-hierarchically) ordered according to the cytokine pattern. Columns indicate the variables used for cluster analysis, which were ordered according to their interrelationship in component analysis. Geometric mean concentrations are given for each cluster. Some variables were used in the cluster analysis in a categorical manner because of a high rate of below detection limit measurements (IFN-γ, IL-5, SE-IgE, TNF-α and IL-17). For these, proportion of positive values are given, and additionally geometric means are given for illustrative purposes. Selected phenotypic information (proportion with nasal polyps, with asthma, allergy) is tabulated, although not used in cluster analysis. For characterization of the clusters, multiple group comparison for between-cluster differences, and for differences with the control group, is visualized with a color code as in legend. For CRSwNP proportion, only between-cluster differences were calculated. *In clusters 2,4,7,8, IL-22 concentrations were significantly higher than in clusters 1,5,6 and 9, but were not different from controls.

Figure 4. Summary graph. Simplified graphical depiction of the clusters and their characteristic cytokines as well as the distribution of CRSsNP vs CRSwNP and asthma. For cytokines, white color indicates no increased concentration, light colors indicate moderately increased concentration and dark colors indicate strongly increased concentration. Horizontal lines indicate groups of clusters as determined by IL-5, SE-IgE and CRSwNP and asthma characteristics.

Figure E1. Validation of clustering.
We generated different clusterings with a number of clusters (k) ranging from 2 to 15. To validate clustering outcomes, we used internal cluster quality measures, as indicated in graphs A to F. For each of the possible number of clusters, an index is calculated reflecting the between-subject similarity within clusters, and the dissimilarity between clusters. This index usually increases monotonically with increasing number of clusters, and the optimal is determined to be at the elbow of its plot, where the change in index (difference with k-1 and k+1) is at a maximum. There are several possible indices available. We used mean silhouette width, Baker-Hubert Gamma statistic, and Hubert-Levin C index as they are relying on dissimilarity data (allowing mixed continuous and categorical data, as is the case in our

sample). We mostly relied on the C index as this is especially fit for mixed type data. The following plots show the index for k = 2-15 possible clusters, and the change (delta) in index compared to k-1. Note that for the C index lower values are better. The results from the internal cluster quality indexes produce a good signal at either 4-5 clusters and 8-10 clusters.
Secondly we assessed clustering stability after resampling of the cluster analysis. Here, data is resampled (for 1,000 iterations) using several schemes (bootstrap, subsetting of the data) and clusters are recalculated. The Jaccard similarities of the original clusters to the most similar clusters in the resampled data are computed, giving an estimate of the stability of a cluster. In the range of k = 2-15, average stability peaked at 5 and 10 clusters.
Lastly we assessed cluster validity using visual inspection of the clusterplot. The subjects are plotted in a two-dimensional space after multidimensional scaling, which is a technique maximizing the dissimilarities between subjects projected in two dimensions. Plots were created for the 5 and 10-cluster solution, and are illustrated in pane G and H. The five cluster solution clearly showed unresolved clusters, which were correctly discovered in the 10-cluster solution.

Table 1: Markers used for the component and cluster analysis*

Marker	Cut-off value	Interpretation of increased concentrations
IFN-γ	85.8 pg/mL	T-helper 1 (Th1) activity
IL-5	12.98 pg/mL	T-helper 2 (Th2) activity
IL-17A	25.06 pg/mL	T-helper 17 (Th17) activity
IL-22	n.a.	T-helper 22 (Th22) activity
TNF-α	38.94 pg/mL	Pro-inflammatory action
IL-1β	n.a.	Pro-inflammatory action
IL-6	n.a.	Pro-inflammatory action
IL-8	n.a.	Neutrophilic chemotaxis
MPO	n.a.	Neutrophilic activity marker
ECP	n.a.	Eosinophilic activity marker
IgE	n.a.	Adaptive immunity marker
SE-IgE	3.85 kUA/L	Marker for superantigen impact on local mucosa
Albumin	n.a.	Edema
TGF-β1	n.a.	Fibrosis/regulatory T cell activation

* Cut-off values are given for markers that were analyzed as categorical, and a short description of the interpretation given regarding the underlying inflammatory pattern when an increased concentration or an increased proportion above cut-off was observed. Cut-off values are expressed as mass per volume undiluted homogenized tissue, i.e. calculated as the detection limit x dilution factor.

Table 2. Characteristics of cases and controls.

	cases	controls	cases vs. controls (p)	
Mean age (years)	42.8	32.8	**<0.001**	*
Gender (% male)	58.2	58.1	0.995	*
Proportion ever smoker (%)	64.7	47.1	**0.005**	°
Proportion current smoker (%)	29.0	25.9	0.592	°
Proportion with allergy (%)	45.0	34.1	0.084	°
Proportion with asthma (%)	28.2	15.3	**0.019**	°
Proportion with aspirin sensitivity (%)	4.5	1.1	0.148	°
Proportion with CRSwNP (%)	56.6	-	-	
Total IgE (kU/L, geometric mean)	87.6	8.9	**<0.001**	*
ECP (µg/L, geometric mean)	2348.5	116.6	**<0.001**	*
IL-8 (pg/mL, geometric mean)	1798	644	**<0.001**	*
IL-6 (pg/mL, geometric mean)	72.8	41.0	**0.021**	*
IL-1β (pg/mL, geometric mean)	28.6	23.7	0.3923	*
TGF-β1 (pg/mL, geometric mean)	9358	8248	0.2282	*
MPO (ng/mL, geometric mean)	2122	966	**<0.001**	*
IL-22 (pg/mL, geometric mean)	363.2	347.5	0.4876	*
Albumin (µg/dL, geometric mean)	965.2	552.4	**<0.001**	*
IL-17A (% pos.)	32.1	25.3	0.239	°
TNF-α (% pos.)	58.4	48.3	0.108	°
SE-IgE (% pos.)	12.9	0.0	**<0.001**	°
IFN-γ (% pos.)	26.5	12.5	**0.015**	°
IL-5 (% pos.)	63.4	3.5	**<0.001**	°

Concentrations are expressed as mass per volume undiluted homogenate. *Student's T-test °Pearson's Chi-square. %pos: proportion samples above cut-off value.

Table E1. Coordinates of principal component analysis for the first five orthogonally rotated principal components*.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5
IgE		0.846			
ECP		0.84			
TNF-α			0.678		
IL-8	0.876				
IL-17A	0.611		0.442		
IL-6	0.76	0.432			
IL-5		0.904			
IL-1β	0.867				
IFN-γ					0.857
MPO	0.811				
IL-22			0.741		
TGF-β1				0.873	
Albumin		0.69			
SE-IgE				0.423	0.47
Variance explained (%)	25%	22%	10%	9%	8%
Cumulative variance explained (%)	25%	47%	57%	66%	74%

*Variables with coordinates lower than 0.4 were omitted from the component. The proportion of total variance in the dataset, explained by each component, is given. Also the cumulative proportion of total variance explained by the sum of each of the components and its preceding components is given. Comp.: primary component.

Table E2. Bootstrap stability of individual clusters in the 10-cluster solution.

Data is was resampled (for 1,000 iterations) using several schemes (bootstrap, subsetting of the data) and clusters are recalculated. The Jaccard similarities of the original clusters to the most similar clusters in the resampled data are computed, giving an estimate of the stability of a cluster. In general, values lower than 0,5 indicate unstable clusters, values higher than 0,6 (*) plausible structure, values higher than 0,75 (**) indicate stable and valid clusters and values higher than 0,85 (***) indicate highly stable clusters.

Cluster	Bootstrap stability	
1	0.98	***
2	0.95	***
3	0.82	**
4	0.87	**
5	0.96	***
6	0.70	*
7	0.94	***
8	0.77	**
9	0.61	*
10	0.68	*

Table E3. Clinical characteristics of clusters.

Cluster	**Ever smoker (%)**	**Current smoker (%)**	**Aspirin sensitivity (%)**	**Gender (% female)**	**Previous surgery rate (%)**
1	72	40	0	28	0
2	81	56	0	31	21
3	73	53	0	27	8
4	87	33	7	53	27
5	46	8	11	35	39
6	73	18	9	73	30
7	69	31	7	54	33
8	64	14	7	29	39
9	73	36	18	36	50
10	14	0	0	14	64

Figure No.1

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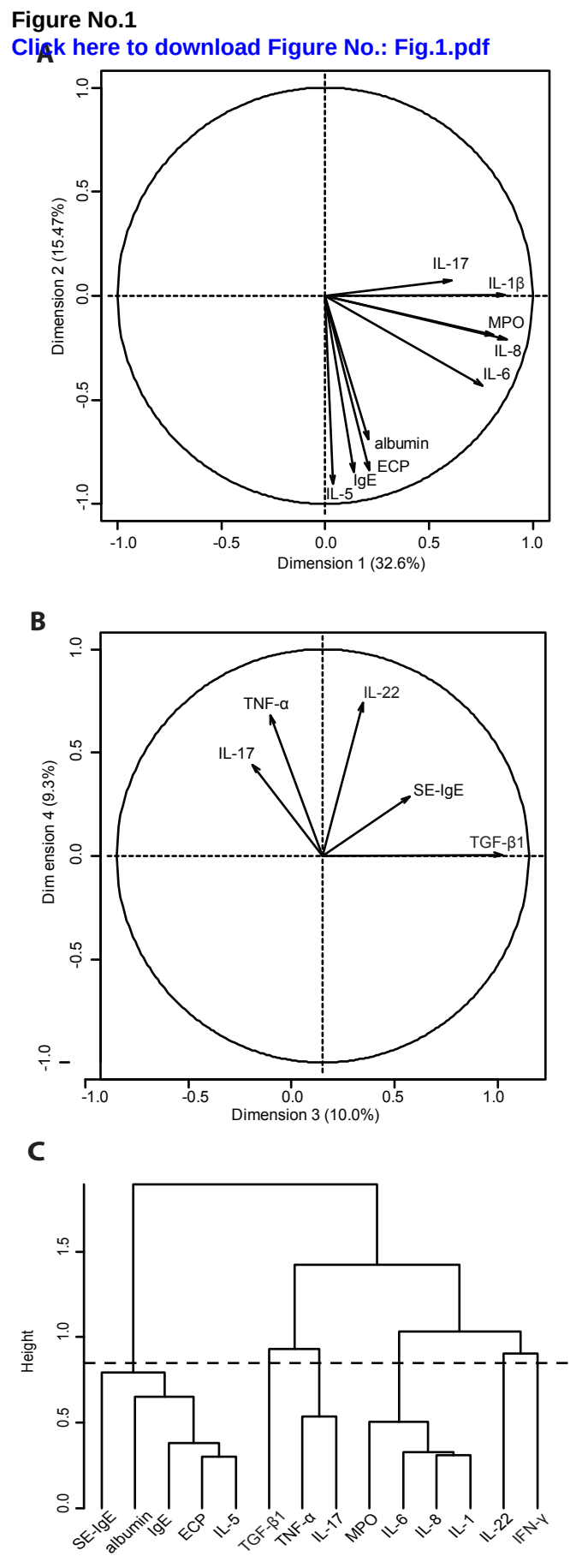

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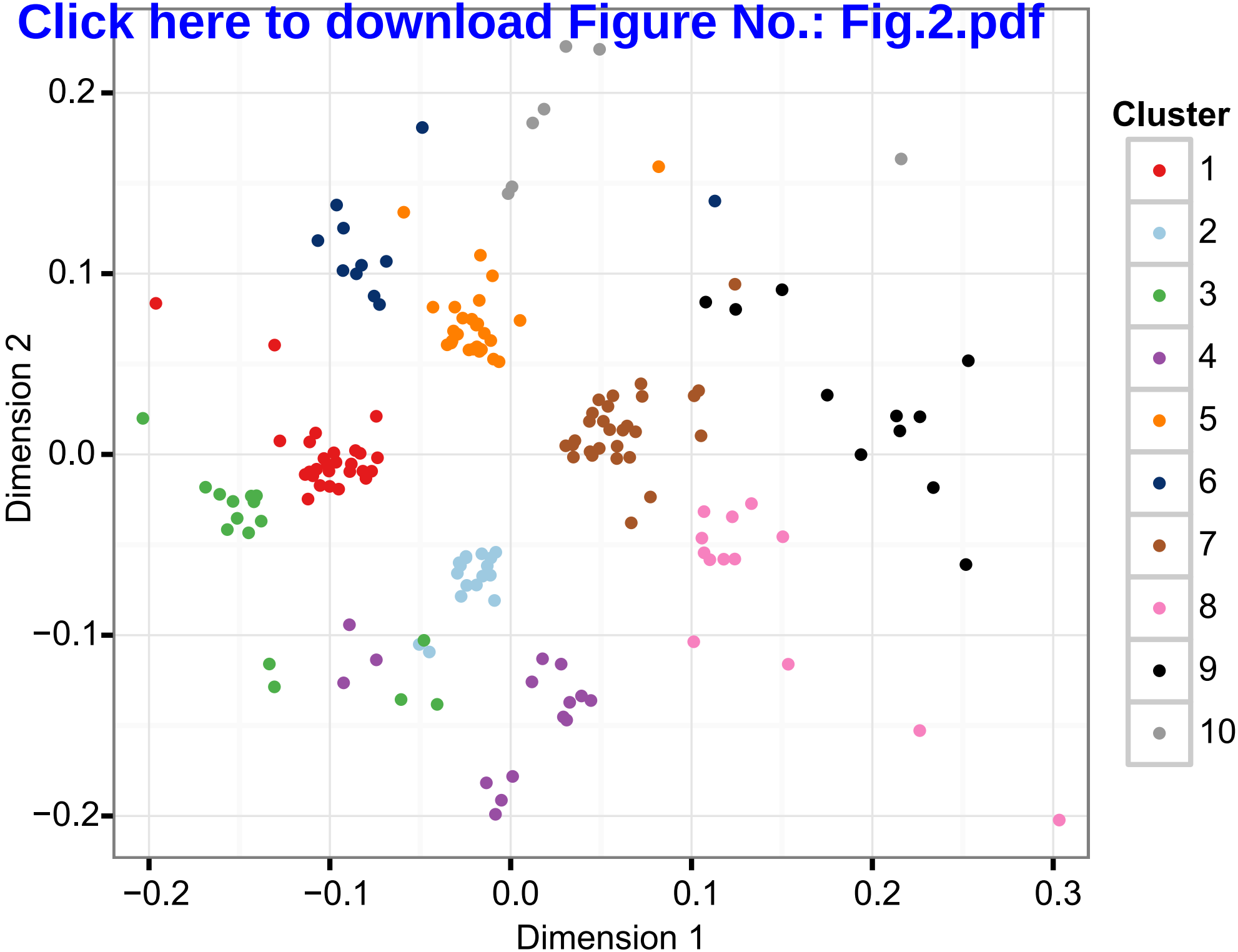

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Cluster	N	N/total	MPO (ng/mL)	IL-8 (pg/mL)	IL-6 (pg/mL)	IL-1b (pg/mL)	Albumin (mg/dL)	IL-5 (pg/mL)	IL-5 (ratio pos.)	ECP (µg/L)	IgE (kU/L)	SE-IgE (kUA/L)	SE-IgE (ratio pos.)	TGF-b1 (pg/mL)	IL-17 (pg/mL)	IL-17 (ratio pos.)	TNF-a (pg/mL)	TNF-a (ratio pos.)	IL-22 (pg/mL)	IFN-g (pg/mL)	IFN-g (ratio pos.)	prop. CRSwNP	prop. asthma	prop. allergy
1	27	16%	973	956	30	24	65	1	0%	407	20	1.29	7%	17853	0.1	0%	0.8	0%	307	43	0%	11%	15%	42%
2	17	10%	1299	442	5	14	54	1	0%	284	13	1.01	0%	13676	0.1	0%	5.4	100%	463*	48	12%	0%	7%	63%
3	16	9%	2714	2908	91	69	70	2	0%	498	28	1.18	6%	17111	0.1	13%	1.2	19%	311	213	100%	13%	14%	50%
4	15	9%	4582	6614	191	230	77	0	0%	601	16	1.01	0%	10598	36.9	100%	10.3	80%	493*	57	27%	47%	20%	40%
5	27	16%	1997	1964	79	21	104	109	100%	4333	122	1.01	0%	16055	0.1	4%	0.8	0%	335	43	0%	59%	27%	50%
6	11	6%	2096	2956	204	55	82	100	100%	7631	170	1.70	18%	21428	0.1	0%	1.1	9%	303	325	100%	64%	36%	45%
7	28	16%	2355	1711	108	29	112	151	100%	3690	148	1.06	4%	17489	0.1	0%	8.2	100%	457*	55	14%	64%	37%	37%
8	14	8%	4621	4777	487	114	168	406	100%	5588	154	1.01	0%	6790	10.7	100%	17.0	100%	349*	58	29%	93%	38%	23%
9	11	6%	3464	4916	184	52	167	483	100%	5626	1038	6.33	100%	13131	2.0	73%	18.5	100%	633	57	18%	91%	64%	82%
10	7	4%	4827	4806	637	59	133	257	100%	14143	988	5.69	100%	12380	0.1	0%	1.4	14%	237	43	0%	100%	71%	57%

Legend:

- Concentration significantly higher than controls and higher than 6 or more other clusters
- Concentration significantly higher than controls and higher than 3 or more other clusters
- Concentration significantly higher than controls and higher than 2 or more other clusters
- Concentration significantly higher than controls but not higher than other clusters

Figure No.4

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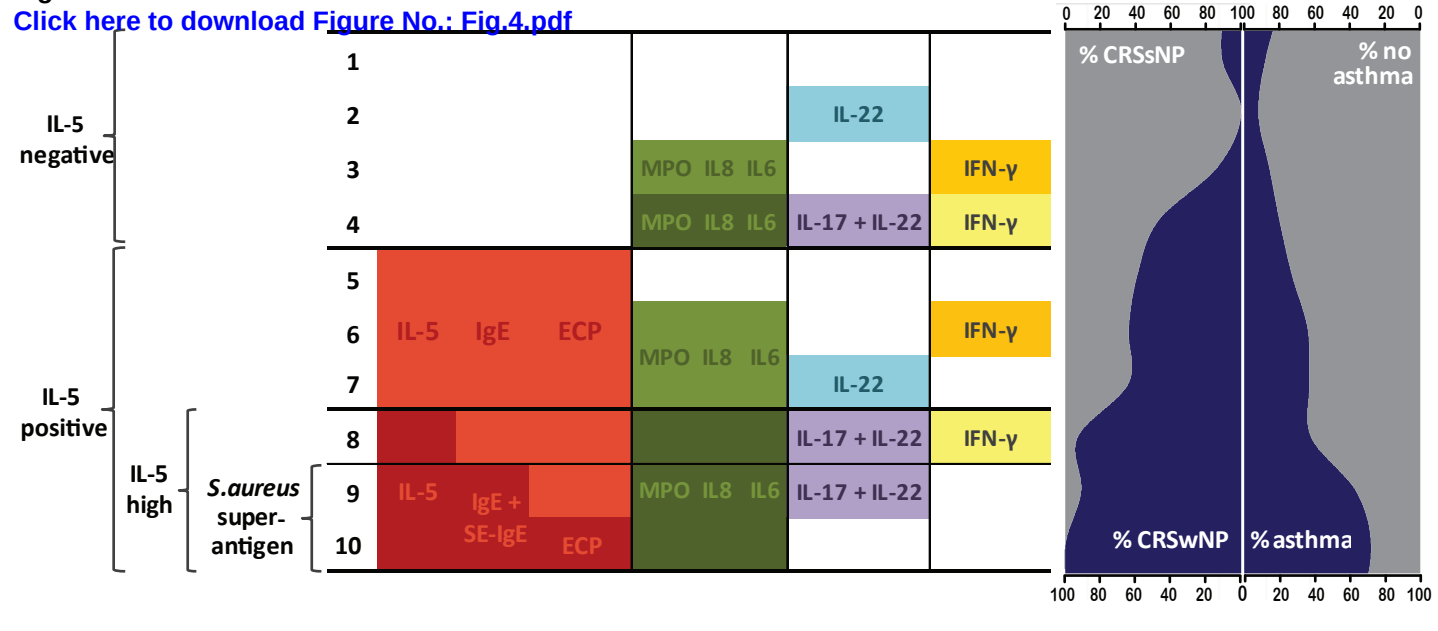

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