

## Eosinophilia in nasal polyposis: its objective quantification and clinical relevance

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### Summary

**Background** Eosinophilia within nasal polyps is often taken as a criterion for adjuvant medical treatment postoperatively such as topical steroids.

**Objective** This study was performed in order to validate a new technique for objective quantification of eosinophilia by using laser scanning cytometry (LSC), to compare these results with manual scoring and routine histopathology, and to correlate them with the history of allergy or recurrence.

**Methods** LSC was used for semi-automated analysis of single-cell preparations from representative ethmoidal polyps obtained during routine paranasal sinus surgery ( $n = 41$ ). This microscope-based instrument scans the cells after immobilization of cells on a glass slide and after triple staining of cytokeratin, eosinophilic granula, and DNA. The location of each cell is stored with the fluorescence data. Therefore, the morphology of every cell can be documented by re-staining with haematoxylin and eosin and re-localization on the slide. Subsequently, slides were subjected to manual scoring. The remaining polyps were analysed by routine histopathology.

**Results** Data from LSC and manual scoring showed good correlation ( $r = 0.81$ ,  $P < 0.001$ ), whereas there were discrepancies with histopathology. Eosinophilia scored by LSC and histopathology was neither correlated with the history of allergy nor with recurrence as determined by Fisher's exact test independent of the definition of eosinophilia ( $\geq 2\%$ ,  $\geq 3\%$ , or  $\geq 5\%$  of all cells).

**Conclusion** Scoring eosinophilia by LSC in comparison with histopathology does not contribute to a more reliable basis for adjuvant medical therapy in nasal polyposis. Instead, functional parameters (cytokine production, apoptosis) may serve better.

**Keywords** allergy, comparative study, laser scanning cytometry (LSC), nasal polyposis, semi-automated quantification

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### Introduction

Chronic rhinosinusitis (CRS) is the most common chronic disease in the United States [1], and the situation for Europe is supposed to be similar [2]. Diffuse polyposis of the nose and sinuses is seen as the final stage of CRS. Eosinophilic infiltration of the oedematous polypoid tissue to a varying degree is considered to be one of the histologic hallmarks of CRS, irrespective of the presence or absence of atopy [3]. Eosinophilic inflammation of the subepithelial tissue was found in more than 80% of all cases [4]. The eosinophils were found to be activated and to account for 20% of all cells [5, 6]. The role of allergy has been under discussion, but there has been little evidence for the implication of IgE-mediated allergy in the development of nasal polyps [7, 8].

Eosinophilia is considered an important feature since therapeutic concepts nowadays include minimal invasive surgery with or without the pre- and postoperative application of topical steroids [9, 10]. In this context, it is very important to analyse eosinophilia in an objective, standardized, and quantitative way in order to have clearly defined cut-off levels at which to decide about expensive adjuvant medical treatment.

So far, the extent of eosinophilia in polypoid tissue is determined qualitatively by manual scoring of cells in tissue sections [6, 11, 12]. This approach is user-dependent and time-consuming. Furthermore, judgement on the density of eosinophilic infiltration is biased by the presence or absence of oedema within the tissue; this could only be avoided if all other cell types were quantified as well including epithelial cells and stromal cells, which in turn makes analysis further time-consuming and less favourable for routine investigation [13]. In conclusion, up to now there is no objective and quantitative assay available for analysis of eosinophilia that could be applied in a routine clinical setting as part of the

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standardized diagnostic and therapeutic regimen for patients with CRS.

In order to develop such an assay, we applied the slide-based technology of laser scanning cytometry (LSC). This instrument is built around a routine epi-fluorescence microscope; its instrumentation, software, and clinical applications are explained elsewhere [14–17] and will only be described briefly. It is equipped with a motorized stage so that a specimen immobilized on a conventional glass slide is scanned stepwise by two lasers (488 nm, argon laser; and 630 nm, helium–neon laser). The fluorescence of the cells is detected by four photomultipliers for different fluorochromes. Per cell, a number of data are calculated automatically (e.g. the integral fluorescence, the intensity of its brightest pixel, its size), and its exact position on the slide is recorded together with these fluorescence data; therefore, each individual object can be directly visualized at any time after completing the analysis. This allows one to verify if objects are single cells, doublets, debris, or artefacts, and to document the cells' morphology; to this end the slide can be removed from the stage, stained by conventional cytological methods (Giemsa, haematoxylin and eosin (H&E)), and placed on the stage again [18, 19]. Up to 5000 cells/min can be analysed by LSC. LSC therefore fills the gap between high-throughput multiparametric cytometry on the one hand and morphological analysis and documentation at the other. Since exact analysis of the cells' morphology is of special importance in pathologically altered tissue (such as polyps), we decided to perform our analyses by LSC. In addition, this technology needs only minimal sample volumes so that tests could be performed on nasal brushes or washes in future.

## Materials and methods

### *Specimens and preparation*

Polyps of the nose and sinuses were resected in general anaesthesia as part of microscopic and endoscopic sinus surgery ( $n = 41$  patients). Special interest was given to the history of allergy or recurrence, and standardized skin prick tests were performed where appropriate. Patients with known asthma, ASA intolerance, or cystic fibrosis were excluded. The set-up of this study was approved by the local ethics committee of the University of Leipzig (#593). Representative polyps of the ethmoid were prepared as single-cell suspension by mechanical disaggregation using the MediMachine (Dako, Carpinteria, CA, USA); the remaining polyps were sent for routine histopathological examination. Erythrocytes were lysed by FACS Lysis (BD Biosciences, San Jose, CA, USA). Cells were then transferred to microscope slides, air-dried, and stored in 70% ethanol. For staining of epithelial cells and eosinophils, slides were washed in phosphate-buffered saline (PBS; Gibco BRL, Paisley, Scotland, UK) and incubated for 30 min at room temperature in a humidified chamber with propidium iodide (PI; 50 µg PI/mL) and RNase (0.1 mg/mL; both: Sigma, St Louis, MO, USA), fluorescein isothiocyanate (FITC; 50 µg FITC/mL; Sigma, St Louis, MO, USA) (for optimization study on peripheral blood, see below), and either with anti-cytokeratin-antibody or control-antibody (both: mouse-IgG1, Dako, Carpinteria, CA, USA), both

indirectly immunolabelled via biotin–streptavidin with allophycocyanine (APC; Caltag, Hamburg, Germany) as described previously [20]. Finally, slides were covered with glycerol in PBS 75%/25% with 25 µg PI/mL.

### *Scoring by routine histopathology*

Scoring of eosinophilia by routine histopathology was performed by an experienced pathologist (F. S.) on the larger remaining portion of the resected material. Scoring was as follows: 0° = no, 1° = slightly, 2° = moderately, 3° = grossly increased eosinophilic infiltration as observed per high-power field. In order to describe the correlation of routine histopathology with the history of allergy or recurrence, 0° and 1° were grouped as negative and 2° and 3° were grouped as positive (for statistical tests, see below).

### *Preparation of peripheral blood*

In order to establish the optimal concentration of FITC for staining of eosinophils, titration experiments were performed on normal peripheral blood. An assay described previously [18] was slightly modified: cells were stained with anti-CD45 conjugated with APC (Caltag Int., Hamburg, Germany), with DNA dye (7-aminoactinomycin-D), and with varying concentrations of FITC (10 µg/mL through 10 mg/mL; both: Sigma-Aldrich, Munich, Germany). Eosinophil counts by LSC were compared with the data obtained by conventional full blood count.

### *Data acquisition in LSC for polyps*

Analysis was performed using the 20 × objective and triggering on the nuclear fluorescence of PI. Data were acquired for FITC and PI by excitation with the argon laser, and for APC by excitation with the helium–neon laser, and were stored together with the  $x$ - $y$  co-ordinates of every cell. For documentation and quality control, cells from specific sub-populations were re-localized, re-scanned with both lasers, and pseudo-coloured according to their fluorescence; the resulting pixel maps were stored as figures (Fig. 1).

### *Cytomorphological staining*

After analysis by LSC, the slide was removed from the stage and the coverslip was detached gently to perform a conventional H&E staining [19]. Slides were covered again using Eukitt (Kindler GmbH & Co, Freiburg, Germany) and were stored as normal cytological samples.

### *Data analysis and interpretation*

For analysis, dot plots showing the integral fluorescence values of PI vs. FITC and PI vs. APC were created. The ratio of the integral APC fluorescence to the cell area was calculated, and a cut-off at 5% was set in the negative control in order to separate cytokeratin-positive vs. negative cells in the specific staining (Fig. 1). In the specifically stained sample, gates were set on the FITC-positive population (= eosinophils), the cytokeratin-positive population (= epithelial cells), and the remaining DNA-diploid population (= stromal cells), and the cell count within these subsets

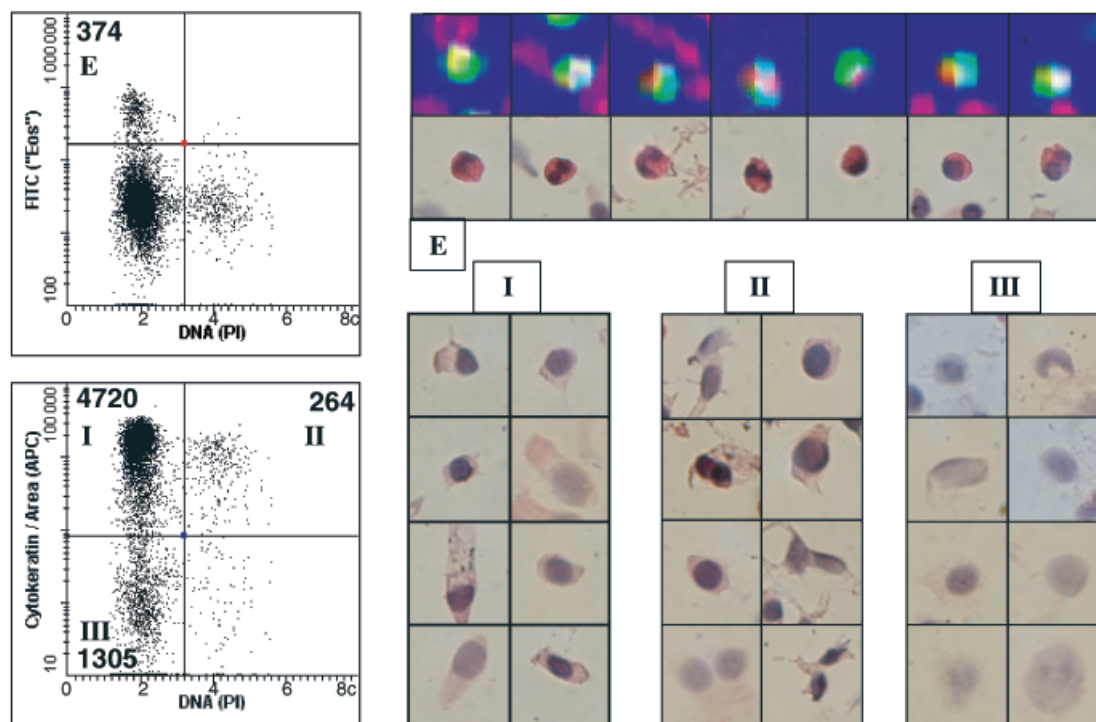


**Fig. 1.** Quantitative analysis of eosinophilia in paranasal polyps by laser scanning cytometry. Analysis of a mechanically disaggregated human ethmoidal polyp prepared and incubated as described. DNA content (x-axis, linear) vs. FITC integral fluorescence (y-axis, logarithmic) of all measured cells (upper dot plot) and DNA content (x-axis, linear) vs. cytokeratin expression per area (y-axis, logarithmic) (lower dot plot; eosinophils were excluded) are shown. The absolute cell counts within the different sub-populations are given. In this example, the following ratios are determined: %-Eos = 5.8% and Q-Eos = 7.3%. For eosinophils, pseudo-coloured fluorescence images were generated by re-scanning (upper row: FITC = green, PI = red, APC = pink). After haematoxylin and eosin staining, representative cells from the regions E and I–III were re-localized and micrographs were taken.

( $n_{\text{Eos}}$ ,  $n_{\text{Epi}}$ , and  $n_{\text{Stromal}}$ ) was calculated. Additionally, the remaining DNA-tetraploid population was gated. For interpretation, the slide was placed back onto the LSC stage after H&E staining, cells were re-localized, and micrographs were taken with a digital camera (Olympus C2020, Olympus Optical Co. Ltd, Tokyo, Japan) and a  $40\times$  objective. For comparison with histopathology and for correlation with the history of recurrence or allergy, the percentage of eosinophils (%-Eos) per all DNA-diploid cells was calculated:

$$\%-\text{Eos} = n_{\text{Eos}} / (n_{\text{Eos}} + n_{\text{Epi}} + n_{\text{Stromal}}) \times 100\%$$

#### Manual scoring

After H&E staining, for validation of the LSC data, the slides of 18 patients were scored by manual counting with a standardized grid. To this aim, only clear epithelial cells and clear eosinophils were taken into account; slides were scored grid by grid until 3000 single epithelial cells were counted as a minimum. The ratio of the respective eosinophilic cells to the sum of epithelial plus eosinophilic cells (Q-Eos) was calculated:

$$\text{Q-Eos}_{\text{Man}} = n_{\text{Eos}} / (n_{\text{Eos}} + n_{\text{Epi}}) \times 100\%$$

For comparison with the scoring by LSC, this ratio of  $\text{Q-Eos}_{\text{LSC}}$  was also calculated based on the LSC data. Pearson's coefficient of correlation was calculated.

#### Statistical tests

Where appropriate, the correlation of two parameters – i.e. recurrence (yes/no) or allergy (yes/no) with eosinophilia (yes/no) – was tested by Fisher's exact test in order to determine the value of  $P$  for an observed combination to occur (Figs 4a and b). The correlation of scoring eosinophilic infiltration by LSC and by routine histopathology was tested by the Mann–Whitney  $U$ -test.

## Results

#### Staining of eosinophils with FITC and re-localization in LSC

The accuracy and reliability of the re-localization feature of LSC was evaluated by re-localizing a series of up to 50 cells from the different cell subsets: the cell loss produced by the H&E staining never exceeded 5% and was not cell-type specific as shown in previous studies [18, 20, 21]. In peripheral blood, comparing eosinophil counts by conventional full blood count and by LSC after staining with FITC showed that  $50\mu\text{g/mL}$  FITC was optimal for selective staining and detection of eosinophils. At higher concentrations neutrophils also exhibited an affinity for FITC, whereas at lower concentrations some eosinophils did not stain with FITC, but could be clearly classified by direct visual observation and by re-analysis after H&E staining. For polyps, staining with FITC, anti-cytokeratin indirectly labelled with APC, and

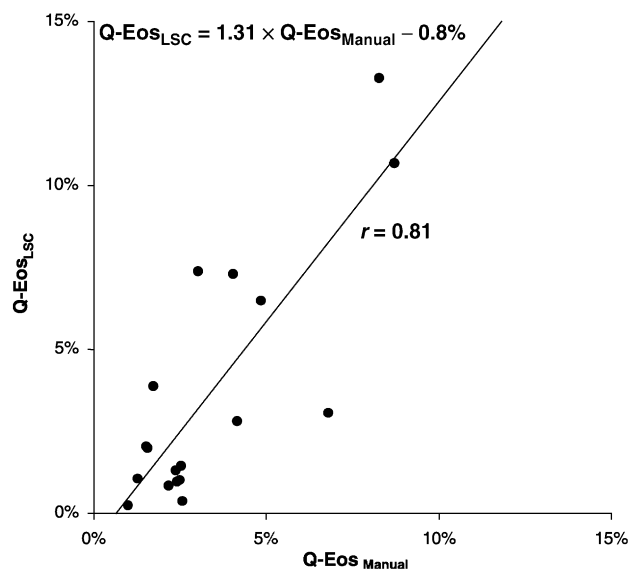


**Fig. 2.** Correlation of manual vs. automated quantification of eosinophilia. Mechanically disaggregated human ethmoidal polyps ( $n = 18$ ) were manually scored as well as analysed by laser scanning cytometry (LSC) and the ratio Q-Eos was calculated for both methods (x-axis: manual scoring; y-axis: LSC analysis). The correlation of both methods was determined by calculating Pearson's coefficient  $r = 0.81$  ( $P < 0.001$ ).

PI gave clear results after analysis by LSC as exemplified in Fig. 1.

#### *Correlation of scoring eosinophilia by LSC, by manual counting, and by histopathology*

The ratio of eosinophils Q-Eos as determined by LSC and by manual scoring showed good correlation ( $r = 0.81$ ) with high significance ( $P < 0.001$ ) (Fig. 2); however, scoring of eosinophilic infiltration as %Eos determined by LSC showed no correlation with scoring by routine histopathology (Fig. 3); there was no significant difference in %Eos values of the polyps with different histopathological grades.

#### *Correlation of eosinophilia with a history of allergy or recurrence*

The ratio of eosinophils %Eos as determined by LSC was plotted against the history of allergy or recurrence. For statistical tests, different cut-off levels were applied: independently of the definition of eosinophilia (cut-off at 2%, 3%, or 5%),  $P$  never reached the level of significance (Fig. 4a). In addition, Fisher's exact test was performed with the data of histopathology and the history of allergy and recurrence, respectively (Fig. 4b); the hypothetical probability of the observed distribution alone was 0.22 and 0.35, respectively, leaving no doubt about the independence of the tested criteria.

## **Discussion**

The LSC-based assay applied in this study provides a means for the quantitative determination of eosinophilic infiltration of nasal and paranasal polyps. To our knowledge, this is the

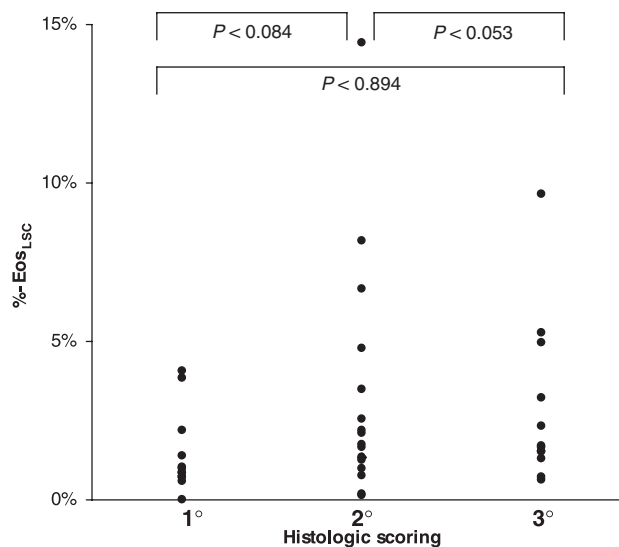


**Fig. 3.** Comparison of automated quantification of eosinophilia with histologic scoring. Freshly resected human nasal polyps were aliquoted for analysis by laser scanning cytometry (LSC) and sent for routine histopathology. For LSC, the ratio %Eos was calculated (y-axis), whereas histologic scoring was graded as 0–3° (x-axis). The Mann–Whitney  $U$ -test was performed to test for a significant difference of %Eos values between the different histopathological groups.

first time that this cell subset is objectively quantified among the studies published so far. In our retrospective setting, the sheer number of eosinophils as a single parameter is not correlated with the clinical outcome (i.e. recurrence) or with the history of allergy. This assay can be used in future prospective studies for the initial objective quantification of eosinophilic infiltration since the specimen can be obtained by a minimal-invasive approach in the out-patient clinic. It furthermore might be applied to nasal washes combining quantitative and qualitative aspects (number of eosinophils, presence of fungi, formation of eosinophilic clusters [22, 23]), and therefore could help to further clarify the role of eosinophils in polyp formation, among others.

Staining of eosinophils with FITC is considered to be an unwanted effect in most applications [20, 24, 25]. However, this phenomenon can be extremely useful in studies on eosinophils like ours where it replaces the use of expensive antibodies. Our data show that eosinophils can selectively be detected by incubation with pure FITC. Scoring eosinophilia in tissue is difficult; in general, sections are qualitatively or semi-quantitatively analysed after H&E staining during routine histopathology [6, 11, 12]. This is convenient and cost-effective since this staining is performed routinely, but it takes extra time and the data show inter- and intra-observer variability. Furthermore, the structure of the polyps, its cellular components, and its interstitial space are influenced by manipulations during surgery such as shearing, friction, or infiltration with local anaesthesia. For these reasons, it seemed most sensible to analyse single-cell suspensions rather than sections.

For the optimal combination of high-throughput cytometric analysis with morphologic documentation, LSC is the technology of choice. Its resolution of fluorescence intensity is equivalent to flow cytometry [17] and its morphologic analysis is accurate. In the present study, for quantification

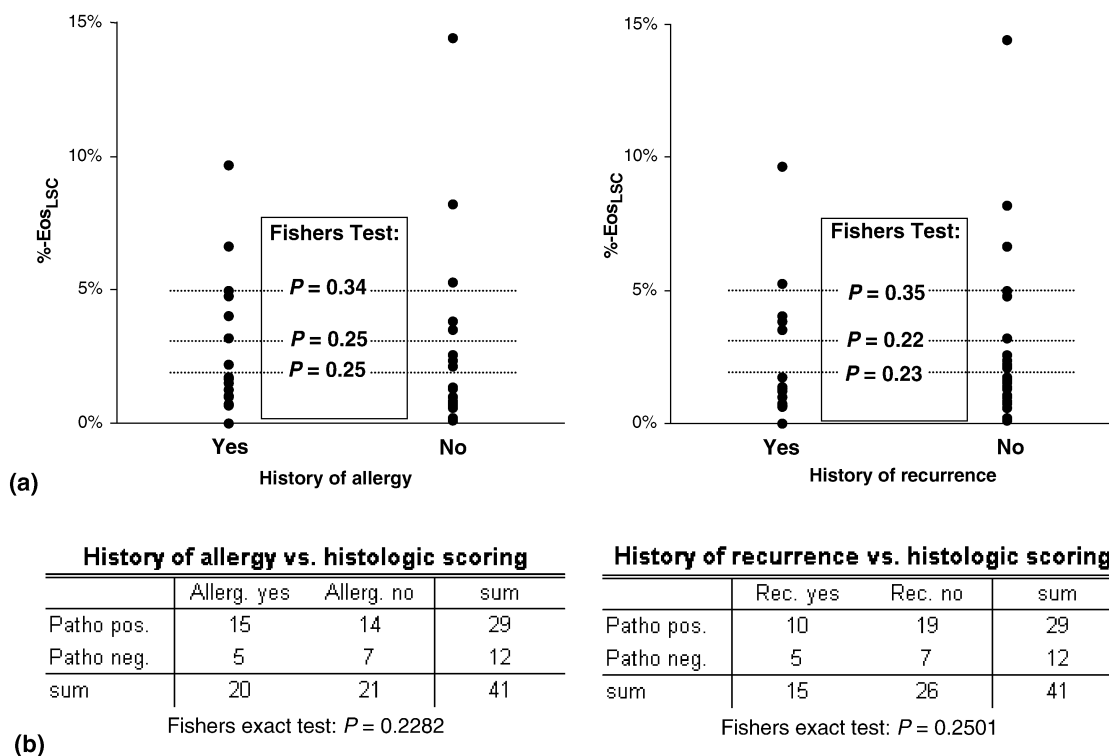


**Fig. 4.** Comparison of eosinophilia with a history of allergy or recurrence. Eosinophilia in human nasal polyps was calculated by laser scanning cytometry (LSC) and determined by routine histopathology. (a) The degree of eosinophilic infiltration as determined by LSC with the ratio %Eos (y-axis) was plotted against the history of allergy (left) and recurrence (right; x-axis). Fisher's exact test was performed with different levels of eosinophilic infiltration as cut-off ( $\geq 2\%$ ,  $\geq 3\%$ , or  $\geq 5\%$ ) and the respective  $P$ -values were determined as indicated. (b) The scoring by routine histopathology was classified as negative ( $0+1^+$ ) or positive ( $2+3^+$ ). Fisher's exact test was performed to determine the respective  $P$ -values when correlating this classification with the history of allergy (left) and recurrence (right).

of eosinophilia by LSC, two different ratios were calculated. First, the number of eosinophils per sum of eosinophils plus epithelia was calculated; this ratio (Q-Eos) was also obtained by manual scoring so that both methods, i.e. manual scoring and LSC, could be compared on the same basis (i.e. single-cell preparations) showing good correlation. However, in histopathology eosinophilia is rather judged on taking all cells of a polyp into account. In order to compare both methods, the second ratio %Eos was calculated on the LSC data as the number of eosinophils per sum of eosinophils plus epithelia plus remaining DNA-diploid cells (i.e. stromal cells). %Eos was also used to correlate eosinophilia with the history of allergy or recurrence, since these biological processes take place with the different cell types cross-acting together.

Although commonly eosinophilia is considered to be present in 86% of all polyps [26], this feature in general is not determined quantitatively. Our data show that the determination of eosinophilia by routine histopathology does not yield quantitative results; in fact there is no clear-cut definition of tissue eosinophilia at all. A cut-off has been set to 20% by others [27]. However, our LSC-based data showed a very weak eosinophilic infiltration in most polyps (mean %Eos = 2.6%), which never reached 20%. Interestingly, scoring eosinophilic infiltration by routine histopathology per high-power field always showed infiltration to some degree ( $1-3^+$ ), but never showed no infiltration ( $0^+$ ) (see Fig. 3). Moreover, our LSC-based data showed no correlation of the

degree of eosinophilic infiltration either with the history of allergy or the history of recurrence in agreement with others [28]. The data available so far therefore do not support the hypothesis that the number of eosinophils is correlated with the clinical course of nasal polyposis.

Indeed, recent studies concentrate on the functional aspects of polyp constituents rather than on their number. So far, several cytokines were identified that are overexpressed in polyps such as IL-5, ECP, or eotaxin, whereas others are down-regulated such as TGF- $\beta 1$  [4]. Other studies identified survival factors of eosinophils in polyps (IL-3, IL-5, GM-CSF) [29, 30], whereas glucocorticoids were shown to cause apoptosis of eosinophils *in vitro* [31] and to reduce the total lymphocyte number in polyps [32]. Based on these results, topical steroids are applied in nasal polyposis [33].

Since the degree of eosinophilic infiltration *per se* is non-informative, but eosinophilic function seems to be useful in predicting corticosteroid responsiveness in inflammatory conditions such as nasal polyposis [34], further clinical treatment should be additionally based on parameters of cell function and their regulators (cytokine production, expression of adhesion molecules, leukotriene antagonists) [12]. Similar to the use of LSC analysis as a tool of predictive medicine in other areas [21], these data should be obtained pre-operatively using minimal-invasive approaches such as nasal brushes or nasal washes; on this basis of quantitative and objective data, medical treatment of nasal polyps could be given prior to or instead of surgery.

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