

Local Complement Activation in Nasal Polyposis

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Objectives/Hypothesis: The role of the complement system in nasal polyps (CRSwNP) has so far scarcely been studied. Because nasal polyps are characterized by bacterial colonization, and the complement system is an effective defense mechanism, it might be involved in the pathogenesis of CRSwNP. This study was designed to investigate the local and systemic activation of the complement system in CRSwNP versus control mucosa in relation to the local and systemic eosinophilic and neutrophilic inflammation and local plasma exudation.

Methods: Concentrations of complement factors C3a desArg and C5a desArg, and of albumin, α 2-macroglobulin, eosinophilic cationic protein, and myeloperoxidase were determined on nasal secretions and serum from 12 CRSwNP patients and 10 control patients. Tissue cryosections were stained for the membrane attack complex (C5b9)

Results: We found a significantly higher concentration of C3a desArg and C5a desArg in nasal secretions from CRSwNP patients compared to controls, whereas the serum levels between the two groups did not differ significantly. Significant correlations were found between C5a desArg and eosinophil cationic protein in nasal secretions. Staining for the membrane attack complex revealed a deposition around blood vessels and the basal membrane exclusively in nasal polyp tissue.

Conclusions: These data support the hypothesis that, in addition to the adaptive immune responses, the complement system is involved in the pathogene-

sis of CRSwNP and may contribute to typical features such as edema and granulocytic inflammation.

Key Words: Nasal polyposis, chronic rhinosinusitis with polyps, complement, C3a, C5a, membrane attack complex.

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INTRODUCTION

The sinonasal cavity is, both in healthy patients and in patients with chronic sinonasal disease, under a permanent threat of colonization with pathogenic bacteria. Therefore the nasal mucosal lining is defended by an innate immune system that supports the adaptive immunity to clear potential pathogens. The complement system is one of the central components of the human innate and adaptive immunity. The complement system consists of more than 30 complement factors and cell membrane proteins and has three activation pathways.¹ Central in these three pathways is the cleavage and activation after contact with an antigen-antibody complex or mannose on bacterial surfaces of C3 and C5 into C3a, C3b, C5a, and C5b. The final downstream of complement activation is the formation of the membrane attack complex, which causes lysis of microorganisms by formation of pores.² Furthermore, C3a and C5a have proinflammatory functions, such as contraction of smooth muscles, increased vascular permeability, vasodilatation, stimulation of neutrophil and eosinophil endothelial adhesion, and induction of proinflammatory cytokine secretion.³ Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease that has clearly been linked to bacterial colonization.⁴ CRSwNP is clinically characterized by the presence of polypoid structures in the paranasal sinuses and nasal cavities. The prevalence varies between 1% and 4%, and a clear association has been observed with asthma and aspirin intolerance in adults. Nasal polyps show typical histological features, such as plasma exudation, paucity of vascular structures, and damage of the respiratory epithelium. Almost 75% of the eosinophils present in nasal polyps are activated, causing high levels of eosinophil cationic protein (ECP) in the polyp tissue.^{5,6} Besides eosinophils, other inflammatory cells, such as lymphocytes, neutrophils, and mast

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cells are highly present in nasal polyp tissue.^{7,8} Since nasal polyps have a predominant eosinophilic inflammation, and bacteria play a role in the pathogenesis,^{4,9} the complement system might be involved in the chemotaxis and activation of eosinophils, and thus in sustaining the inflammatory processes in nasal polyps. The current study investigates the local and systemic activation of the complement system in CRSwNP in relation to the local and systemic eosinophilic and neutrophilic inflammation and local plasma exudation. Therefore, the anaphylatoxins C3a and C5a were measured in serum and nasal secretions together with albumin, α 2-macroglobulin, ECP, and myeloperoxidase (MPO). As C3a and C5a in serum are rapidly cleaved into the C3a desArg/C5a desArg forms by endogenous carboxypeptidase N-enzyme, the quantification of C3a desArg and C5a desArg is a reliable measurement for the level of complement activation.

MATERIALS AND METHODS

Patients

Twelve subjects with nasal polyposis were included in this study (12 men, range 21–63 years, mean 44.27 years) and 10 control patients (5 men and 5 women, range 18–63 years, mean 39.7 years). The control group consisted of patients without mucosal disease who underwent a septoplasty with inferior conchotomy for anatomical obstruction. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EP³OS) criteria¹ and based on history, clinical examination, nasal endoscopy, and sinus computed tomography scanning. Nasal secretion and serum samples were collected before routine endonasal sinus surgery unrelated to this study. Skin prick tests (SPT) to 14 common inhalant allergens were used to evaluate the atopic status. In both groups one patient was SPT-positive. A history of asthma was reported in five nasal polyp patients. None of the controls had a history of asthma. Three nasal polyps were aspirin intolerant. For all patients a washout period of at least 1 month was kept for antibiotics, oral, or nasal corticosteroids. The ethics committee of the Ghent University Hospital approved this study and all patients gave their written informed consent.

Processing and Measurement of Serum and Nasal Secretions

Nasal secretions were obtained using sinus packs (IVALON 4000 plus 3.5 × 0.9 × 1.2 cm surgical products (M-Pact, Eudora, KS) as previously described.¹⁰ Sinus packs were placed in both nasal cavities for exactly 5 minutes. All freshly obtained nasal secretion samples were immediately processed, separated, and stored in aliquots at –20°C until analysis as previously described. Serum and nasal secretions were assayed for C3adesArg, C5adesArg, and MPO by commercially available enzyme linked immunosorbent assay kits (C3adesArg, C5adesArg: BDoptEIA, BD Biosciences-Pharmingen, Erembodegem, Belgium; MPO: Bioxytech MPO-EIA, OXIS Health Products, Portland, OR).

ECP concentrations were measured using the Uni-CAP system (Pharmacia & Upjohn, Uppsala, Sweden). Albumin and α 2-macroglobulin were determined both in serum and nasal secretions using a Behring nephelometer II (Dade-Behring, Marburg, Germany).¹¹

Immunohistochemistry

Specimens were snap frozen in liquid nitrogen-cooled methyl butane and stored at –80°C. Cryostat sections were pre-

pared (6 μ m) and mounted on SuperFrost Plus glass slides, packed in aluminum paper and stored at –30°C until staining. Sections were stained for C5b-9 (membrane attack complex) (Dako, Copenhagen, Denmark). Specimens were fixed in acetone and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in TBS containing 0.1% sodium azide for 20 minutes. Primary antibodies or negative controls, consisting of the corresponding isotype controls, were incubated for 1 hour and detected using the LSAB technique conjugated with peroxidase according to the manufacturer's instructions (labeled streptavidin-biotin, Dako). The peroxidase activity was detected using AEC substrate chromogen (Dako), which results in a red-stained precipitate. Finally sections were counterstained with hematoxylin and mounted.

Statistical Analysis

Statistical analysis was performed with the SPSS 12.0 software (SPSS Inc., Chicago, IL). Statistical analysis was performed by the Kruskal-Wallis test and Mann-Whitney *U* two-tailed test for unpaired comparisons. When comparisons were made between groups, the Kruskal-Wallis test was used to establish the significant intergroup variability. The Mann-Whitney *U* test was then used for between-group comparison. Spearman rank correlation coefficient was used to assess the relationships between parameters. The significance level was set at $\alpha = .05$.

RESULTS

In an approach to quantify the complement activation we measured the concentrations of C3a desArg and C5a desArg in serum and nasal secretions (Table I and Fig. 1). Levels of C3a desArg and C5a desArg were significantly higher in nasal secretions from CRSwNP patients compared to controls ($P = .048$ and $P = .035$, respectively). On serum level, no difference was observed in C3a desArg and C5a desArg between CRSwNP patients and controls.

ECP and MPO were measured in this study to reflect to local and systemic eosinophilic and neutrophilic inflammation. Both ECP and MPO concentrations were higher in the nasal secretions of nasal polyp patients compared to controls; however for MPO the difference did not reach statistical significance ($P = .668$) and for ECP it was nearly significant ($P = .0507$). Furthermore, no difference was observed between the two patient groups in serum.

To evaluate the plasma exudation in polyps, albumin and α 2-acroglobulin concentrations were measured in serum and nasal secretions in both groups. In nasal polyps a significantly higher concentration of albumin was found compared to the control group ($P = .035$), whereas no significant difference between the two groups was found for α 2-macroglobulin. Again, no differences were observed for both plasma proteins in serum.

Comparison of C3a desArg, C5a desArg, MPO, ECP, albumin and α 2-acroglobulin levels between nasal secretion and serum in controls demonstrated that the concentrations of C3a desArg, albumin, and α 2-macroglobulin were significantly higher in serum ($P = .028$, $P = .005$, $P = .005$, respectively). In contrast ECP and MPO concentrations were significantly higher in nasal secretions compared to serum of controls ($P = .005$). No

TABLE I.
Median Concentrations and Interquartile Range of C3a desArg, C5a desArg, Eosinophil Cationic Protein, Myeloperoxidase, Albumin, and α 2-Macroglobulin in Controls and Nasal Polyps.

	Controls Median (IQR)		CRSwNP Median (IQR)		P value			
					CO vs. CRSwNP		Serum vs. Nasal Secretions	
	Serum	Nasal Secretions	Serum	Nasal Secretions	Serum	Nasal Secretions	Controls	Nasal Polyps
C3a desArg (ng/ml)	12985 (8175)	2434.75 (6765.86)	14820 (11065)	6456.62 (7829.35)	NS	.048	.028	.015
C5a desArg (ng/ml)	6,51 (3.7)	7,27 (32.43)	7,25 (3.01)	35,78 (48.47)	NS	.035	NS	.002
ECP (μ g/l)	10,14 (24.42)	214,26 (202.94)	13,9 (12.4)	867,1 (1031.01)	NS	NS	.005	.002
MPO (ng/ml)	9,85 (5.84)	2891,33 (6002.94)	9,76 (6.98)	4823,82 (8158.22)	NS	NS	.005	.002
Albumin (mg/l)	33400 (12875)	4825,01 (10730.77)	38450 (12200)	11803,8 (15161.17)	NS	.035	.005	.002
α 2-macro-globulin (mg/l)	1005 (412.5)	111,91 (366.56)	1015 (400.0)	277,20 (351.27)	NS	NS	.005	.002

Significant differences between serum and nasal secretions for controls and nasal polyps and significant differences between controls and nasal polyps are indicated with the appropriate P value; nonsignificant differences are indicated with NS. CO = control; CRSwNP = chronic rhinosinusitis with nasal polyps; ECP = eosinophil cationic protein; IQR = interquartile range; MPO = myeloperoxidase.

significant differences were found for C5a desArg in controls.

Similarly the concentrations of C3a desArg albumin and α 2-macroglobulin in the CRSwNP group were significantly higher in serum compared to nasal secretions ($P = .015$, $P = .002$, $P = .002$, respectively), whereas ECP and MPO concentrations were significantly higher in nasal secretions of CRSwNP patients ($P = .002$). Contrary to the findings in controls, nasal secretions of nasal polyp patients contained significantly higher levels of C5a desArg compared to serum levels ($P = .002$).

C5a desArg in nasal polyp patients both in serum and in nasal secretions were positively correlated with ECP concentrations ($r = 0.615$, $P = .033$; $r = 0.608$, $P = .036$, respectively). Both in serum and nasal secretions, a positive correlation ($r = 0.671$, $P = .017$; $r = 0.846$, $P = .001$, respectively) was observed between C3a desArg and albumin in the nasal polyp group, whereas there was no correlation between C5a desArg and albumin. Inversely a significant correlation was found between C5a desArg and α 2-macroglobulin and C3a desArg and α 2-macroglobulin in nasal secretions from nasal polyp patients ($r = 0.692$, $P = .013$; $r = 0.902$, $P = .001$, respectively).

Immunohistochemistry showed a positive staining for the membrane attack complex I in all polyp samples, whereas such staining completely lacked in controls. In polyps, the basal membrane and endothelial cells were specifically stained for membrane attack complex (MAC) (Fig. 2).

DISCUSSION

We found a significantly higher concentration of C3a desArg and C5a desArg in nasal secretions from nasal polyp patients compared to controls, whereas the serum levels between controls and nasal polyp patients did not differ significantly. The MAC was exclusively found in nasal polyp tissue, situated at the basal membrane and around blood vessels. These data support the hypothesis that, in addition to the adaptive immune

responses,⁹ the complement system is locally activated and involved in the pathogenesis of nasal polyposis.

The significantly higher concentration of albumin in nasal secretion from nasal polyp patients underlines our previous findings of albumin deposition with pseudocyst formation and plasma-exudation in nasal polyps.^{5,12} Albumin deposition is a typical feature of nasal polyps^{5,13} and may be regulated by increased C3a and C5a levels.^{2,3} In this study, the strong correlations between C3a desArg and both albumin or α 2-macroglobulin and between C5a desArg and α 2 macroglobulin in nasal secretions nicely show the relationship between plasma exudation and complement activation in nasal polyps.

To assess the local activation or generation of the C3a and C5a, nasal levels of C3adesArg and C5adesArg were compared to serum concentrations. C3a desArg concentrations were significantly higher in serum than in nasal secretions, both in controls and CRSwNP patients. For C5a desArg the concentrations in nasal secretions of CRSwNP significantly exceeded the serum levels, suggesting a local generation of C5adesArg in nasal polyp tissue. Since concentrations of C3a desArg and C5a desArg correlate well with serum markers of increased vascular permeability, we cannot exclude that high levels of C5a desArg in nasal secretions of CRSwNP are caused by increased vascular permeability. However, one study has shown an extrahepatic expression of complement mRNA in sinonasal tissue.¹⁴

C3a and C5a play a major role in the chemotaxis and activation of eosinophils and neutrophils, and in this study C5a desArg levels in CRSwNP were strongly correlated to ECP in nasal secretions. There was no significant correlation to eosinophilic inflammation with C3a desArg. This may be explained by the rapid inactivation of C3a into C3a desArg losing its ability to attract and stimulate eosinophils, whereas C5a desArg remains an active eosinophil stimulus. Furthermore, C5a can stimulate eosinophils more powerfully than C3a.^{15,16}

Several pathomechanisms in CRSwNP could lead to the activation of complement. First, it has been shown in asthmatics and patients with allergic rhinitis that allergen exposure in sensitized individuals may induce the local

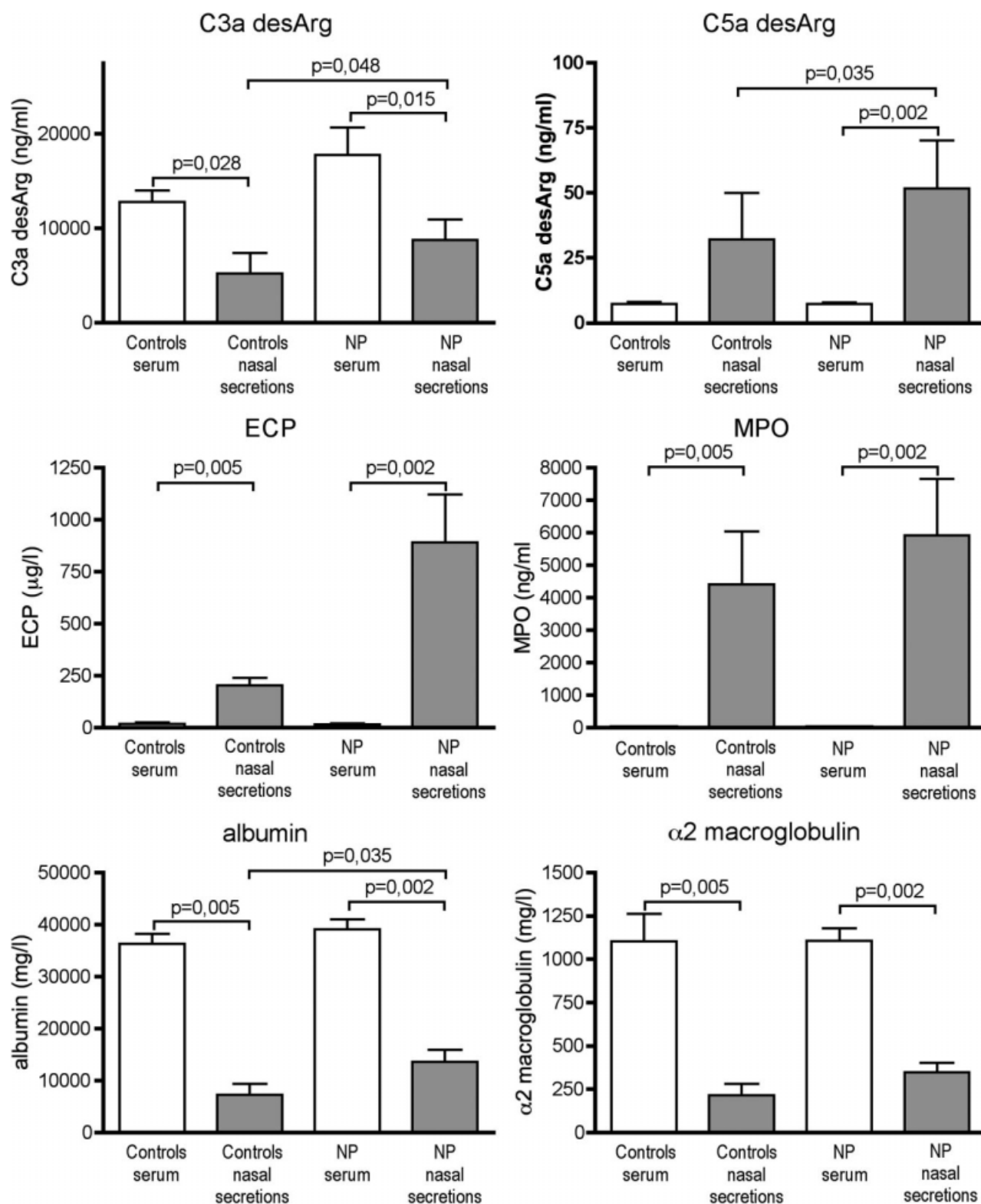


Fig. 1. Comparison of the levels of C3a desArg, C5a desArg, eosinophil cationic protein (ECP), myeloperoxidase (MPO), albumin, and α 2-acroglobulin in serum and nasal secretions from 10 controls (CO) and 12 CRSwNP patients. Data are expressed in box-and-whisker plots. Arrows represent a statistical difference between groups.

activation of C3a and C5a.^{17,18} However, in the current study no significant difference was found between SPT-positive and SPT-negative nasal polyp patients in terms of C3a

and C5a levels in nasal secretions, supporting the idea that factors other than allergy play a role in the complement activation in CRSwNP.

Controls

Nasal polyps

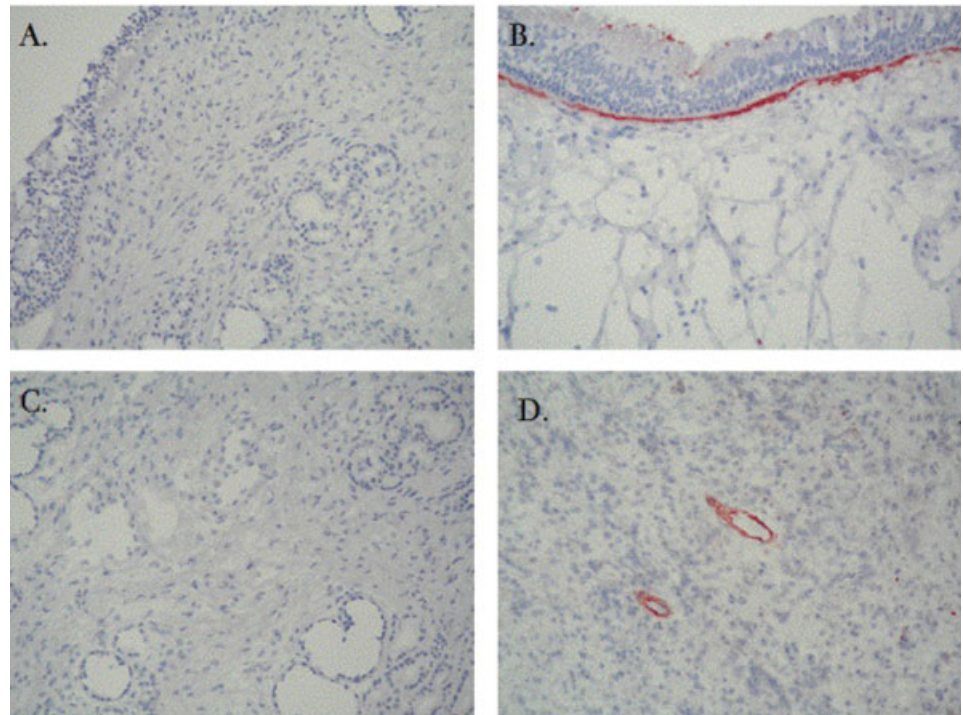


Fig. 2. Immunohistochemical staining for membrane attack complex in nasal polyposis (CRSwNP) and controls: membrane attack complex was found adherent to basal membrane and vascular structures in all polyps, but not in any controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The complement system can be activated through three activation pathways, all leading to the generation of C3a.² There is the classical pathway, which is activated by immune complexes with IgE or IgG.^{19,20} In nasal polyps, massive concentrations of polyclonal IgE and increased IgG levels are present in the polyp tissue¹² indicating that complement activation in nasal polyps could partially be due to activation of the classical pathway by immunoglobulin complexes. Furthermore, colonization with *Staphylococcus aureus*, frequently found in nasal polyp patients,^{4,9} can cause a supplementary activation of the complement system through the alternative pathway. The third pathway causing complement activation is the mannose binding lectin pathway, by recognition of foreign carbohydrates. Although there is no direct evidence to assume an activation of this pathway, previous results from our group showed a significant upregulation of the macrophage mannose receptor in nasal polyps²¹ that could lead to the activation of the mannose binding lectin pathway.

After cleavage of the C5 into the anaphylatoxin C5a and C5b-9, the membrane attack complex is formed through the self-association of C5b with proteins C6, C7, C8, and C9 and functions mainly as a pore former in cell membranes of pathogens.²² However, C5b-9 can also influence, independently of the engagement of C5a, the downstream inflammatory cascade by triggering releases of multiple mediators, such as proMMP9.²³ In the current study, MAC was exclusively found in nasal polyp tissue, mainly situated subepithelially at the basal membrane. Earlier histomorphological studies revealed frequent epithelial damage and a thickened basal mem-

brane.²⁴ The deposition of the MAC at the basal membrane of nasal polyps might be implicated in epithelial damage and high turnover in nasal polyps. Secondly C5b-9 can lead to the overproduction of extracellular matrix components, such as collagen IV and fibronectin, even in the absence of TGF- β over-expression, and cause basal membrane thickening.²⁵ Furthermore, we describe for the first time that MAC was also situated around blood vessels in nasal polyps but not in controls. Although nasal polyps have a reduced number of vessels, they are characterized by a massive edema with pseudocyst formation, filled with large amounts of albumin and other plasma proteins.⁵ An increased leakage by anaphylatoxins or other inflammatory products might be instrumental in causing this edema. The deposition of MAC around these vessels may induce damage of the vascular epithelium, leading to increased vascular permeability, as has been described in glomerular kidney disease and demyelination and axonal damage in ischemic brain injury.^{26,27}

CONCLUSION

These data support the hypothesis that, in addition to the adaptive immune responses, the complement system contributes in the pathogenesis of nasal polyposis. The involvement of complement in nasal polyps is new to the current understanding of the pathophysiology of nasal polyposis, and typical features like edema, vascular leakage, high epithelial turnover, and the attraction of eosinophils may be influenced by the complement activation in CRSwNP.

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