

Downregulation of polymeric immunoglobulin receptor and secretory IgA antibodies in eosinophilic upper airway diseases

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Abstract

Background: Immunoglobulin (Ig) A represents a first-line defence mechanism in the airways, but little is known regarding its implication in upper airway disorders. This study aimed to address the hypothesis that polymeric Ig receptor (pIgR)-mediated secretory IgA immunity could be impaired in chronic upper airway diseases. **Methods:** Nasal and ethmoidal biopsies, as well as nasal secretions, were collected from patients with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) or without nasal polyps (CRSsNP), allergic rhinitis (AR) and controls, and assayed for IgA1/IgA2 synthesis, pIgR expression, production of secretory component (SC), IgA and relevant IgA antibodies, and correlated with local eosinophils and inflammatory features (IL-12, IL-13 and ECP). **Results:** pIgR expression was decreased in the ethmoidal mucosa in patients with CRSwNP ($P = 0.003$) and in AR ($P = 0.006$). This pIgR defect was associated with reduced levels of SC ($P = 0.007$) and IgA antibodies to *Staphylococcus aureus* enterotoxin B (SAEB) ($P = 0.003$) in nasal secretions from patients with CRSwNP, and with increased IgA deposition in subepithelial areas. pIgR downregulation was selectively observed in patients with tissue eosinophilia, whilst no clear relation to smoking history was observed.

Conclusion: Epithelial pIgR expression is decreased in patients with CRSwNP and AR and results in decreased SC and IgA antibodies to certain bacterial antigens (SAEB) in nasal secretions of patients with CRSwNP in parallel to subepithelial accumulation of IgA. This defect in mucosal immunity is associated with eosinophilic, Th2-related inflammation.

Chronic rhinosinusitis (CRS) defines a group of disorders characterized by persistent inflammation of the sinonasal tract. The diagnosis is based upon the presence of persistent symptoms, nasal endoscopy and CT scan (1). Depending on the presence of polyps on endoscopic examination, CRS is

divided into two groups: CRS with nasal polyps (CRSwNP) or CRS without nasal polyps (CRSsNP). Whereas the pathogenesis of CRS remains controversial, Th2-driven eosinophilic inflammation is classically observed in CRSwNP, in contrast to CRSsNP where neutrophils are predominant. Allergic rhinitis (AR) represents another Th2-related nasal disorder. The role of pathogens, for both inception and exacerbations, is supported by several studies (2, 3), whereas only a few report on defective defence mechanisms in these disorders (4–6).

Immunoglobulin (Ig) A is the predominant immunoglobulin in mucosal tissues, endowed with several protective functions such as neutralization of pathogens and particles (7).

Abbreviations

AR, allergic rhinitis; COPD, chronic obstructive pulmonary disease; CRS, chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; ECP, eosinophil cationic protein; Ig, immunoglobulin; pIgR, polymeric Ig receptor; SA, *Staphylococcus aureus*; SAEB, *Staphylococcus aureus* enterotoxin B; SC, secretory component; S-IgA, secretory IgA.

At mucosal sites, plasma cells produce dimers of IgA, which are translocated across the epithelium from the lamina propria into mucosal secretions. This active transport is assumed by the polymeric immunoglobulin receptor (pIgR), expressed by epithelial cells, up to the apical pole where a proteolytic cleavage releases secretory IgA (S-IgA) consisting of d-IgA and the extracellular part of the pIgR known as secretory component (SC). This latter is thought to protect IgA from proteolysis and displays itself, independently from IgA, some anti-infectious properties (8). IgA is secreted via this route into the mucous lining fluid of the gastrointestinal, urogenital and respiratory tracts, as well as into tears, saliva and milk (9). Expression and function of pIgR are regulated through multiple pathogen-associated molecular patterns and pro-inflammatory cytokines such as IFN- γ , IL-4, TNF- α and IL-1 via transcriptional and post-transcriptional mechanisms (10, 11).

The expression of IgA and pIgR/SC has been mainly studied in the lung, where pIgR is decreased in severe chronic obstructive pulmonary disease (COPD) and in lung cancer (7, 12). In the upper airways, pIgR is downregulated in patients with nasopharyngeal cancer, but little is known regarding IgA and pIgR expression in chronic inflammatory sinonasal pathologies (13, 14). It has been shown that IgA is increased in tissue homogenates from patients with CRSwNP (15, 16), but it remains to determine whether this results in increased IgA in nasal secretions. We hypothesized that pIgR could be downregulated in CRSsNP, often seen as the upper airway equivalent of COPD, and could result in reduced secretion of pathogen-specific IgA antibodies.

The aim of this work was therefore to evaluate IgA synthesis and pIgR/SC expression in human nasal and sinus tissues from patients with CRSwNP, CRSsNP and AR, as compared with controls, and to assess in nasal secretions from these patients SC and IgA to relevant bacterial antigens (*S. pneumoniae* and *S. aureus*), as well as their relation to eosinophilic and Th1 (IL-12p35)- or Th2 (IL-13)-related inflammation.

Methods

For details, refer to the online Supporting Information section.

Subjects and surgical sampling

Patients with CRSsNP ($n = 13$), CRSwNP ($n = 10$), AR ($n = 13$) and controls ($n = 20$) were recruited at the outpatient clinic of our Department of Otorhinolaryngology (Cliniques Universitaires Saint-Luc, Belgium). The diagnosis of CRSwNP and CRSsNP was, according to EPOS criteria, based on history, clinical examination, nasal endoscopy and CT scanning (1). Allergic rhinitis was diagnosed based on symptoms and positive skin prick tests and/or serum-specific IgE (>0.35 kU/l) to common aeroallergens, following the ARIA guidelines (17). Clinical characteristics of the patients are described in Table 1. Controls and patients with AR underwent surgery for anatomical obstruction, whilst patients with CRS underwent surgery as indicated by the disease. All patients were weaned off oral and nasal corticosteroids or antibiotics for at least 3 weeks before surgery.

Sinonasal biopsies were taken from the inferior turbinate and anterior ethmoidal sinus. Nasal secretions were collected as previously described (18) for assessments of soluble factors including IgA and SC. Biopsies were processed for immunohistochemistry or PCR. Serum was also sampled to measure serum IgA.

All subjects gave signed informed consent, and the study was approved by the local ethical committee (Cliniques Universitaires Saint-Luc).

Staining for pIgR, IgA and eosinophils

Paraffin-embedded sections of turbinal and ethmoidal tissues were incubated with rabbit anti-human SC IgG prepared as previously reported (19) and mouse anti-human IgA (ThermoScientific, Rockford, IL, USA). To quantify pIgR and IgA staining, immunostained sections were scanned and analysed using the Framework for Image Dataset Analysis (FrIDA) (20). The percentage of stained area was calculated in 10 epithelial (pIgR) or subepithelial (IgA) fields, at $\times 400$ magnification, and reported to the surface of examined area. Eosinophils were counted after haematoxylin and eosin staining in 10 epithelial fields, at $\times 400$ magnification. The presented data consist of the mean number of eosinophils per high-power ($\times 400$) field.

Table 1 Patients' characteristics

	Controls	CRSsNP	CRSwNP	AR
<i>N</i>	20	13	10	13
Sex (M/F)	13/7	8/5	8/2	8/5
Age, years (range)	38.6 (18–62)	35.7 (18–51)	48.2 (28–70)	35.4 (20–48)
History of asthma	1	0	3	2
Atopy	0	0	5	13
Smoking	4 (20%)	4 (30%)	3 (30%)	3 (23%)
CT score (Lund–Mackay)	1 (0–2)	12 (7–21)*	18 (14–24)*	1 (0–2)
Serum IgA (mg/dl)	228 (89–633)	233 (113–485)	207 (97–367)	185 (173–289)

Patients were assigned to disease groups based on the criteria described in the *Methods* section, and associated features (asthma, atopic background, cigarette smoking) are stated.

* $P < 0.0001$ (Mann–Whitney *U*-test) for CT scores, as compared to control subjects.

Quantitative RT-PCR for pIgR, IgA1, IgA2 and cytokines

Two biopsies per patient (one ethmoidal and one turbinal) were processed for gene expression by RT-qPCR. The expression levels of pIgR, IL-12p35 and IL-13 in sinonasal tissue were quantified by RT-qPCR using the iCycler IQ5 PCR thermocycler (Bio-Rad, Hercules, CA, USA) and reported to those of RPS18 housekeeping gene. Absolute quantification of IgA1 and IgA2 expression levels was determined with RT-qPCR using an ABI PRISM 7900 Sequence Detection System thermal cycler according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Immunoassays for IgA, SC and ECP

Concentrations of IgA, SC, IgA1, IgA2 and specific IgA antibodies to pneumococcal anticapsular and *S. aureus* enterotoxin B (SAEB) antigens were determined in nasal secretions by ELISA. Eosinophil cationic protein (ECP) was measured by ImmunoCAP (ThermoFisher, Rockford, IL, USA) following manufacturer's instructions.

Statistical analysis

Data are expressed in dot plots and presented as median and interquartile range. Statistical analysis was performed by the Kruskal–Wallis and Mann–Whitney two-tailed *U*-tests for unpaired comparisons. When multiple between-group comparisons were made, the Kruskal–Wallis test was used to establish the significant intergroup variability, and the Mann–Whitney *U*-test was then used for between-group comparisons. Data were correlated by the Spearman's rank correlation test. A *P* level < 0.05 was considered as significant.

Results

pIgR expression in the sinonasal mucosa

pIgR mRNA expression was decreased in the ethmoidal mucosa from patients with CRSwNP and AR, when compared with controls (Fig. 1A), whilst it was not reduced in CRSsNP. No significant change in sinonasal pIgR expression was observed in relation to cigarette smoking (not shown). This downregulation in pIgR transcription was only observed in the ethmoidal, and not in the turbinal mucosa from these patients.

At the protein level, pIgR immunodetection was confirmed in ciliated and seroglandular cells from the surface epithelium and submucosal glands (Fig. 1C). No staining was found in basal cells and in goblet cells. Quantification of pIgR protein confirmed a significant reduction in CRSwNP and AR (Fig. 1B).

Total IgA and SC in nasal secretions and sinonasal mucosa

We assessed whether pIgR downregulation resulted in decreased IgA levels in nasal secretions and sinonasal

mucosa. First, SC concentration in nasal secretions was accordingly reduced in CRSwNP (Fig. 1D) and correlated with tissue pIgR mRNA levels (Fig. 1E).

To assess each compartments of the airway mucosa, we evaluated IgA content in sinonasal tissue by IgA immunostaining. A significant accumulation of IgA was observed in subepithelial areas of ethmoidal tissue from patients CRSwNP (Fig. 2A).

IgA synthesis was then assessed by RT-qPCR for IgA1 and IgA2 transcripts in sinonasal tissues, which showed no significant changes among the different groups (Fig. 2B). Similarly, levels of IgA and IgA subclasses in nasal secretions were not significantly affected (Fig. 2C). Of note, serum IgA was not significantly different among the groups (Table 1).

Specific IgA antibodies in nasal secretions

We next assessed specific IgA antibodies to *S. pneumoniae* and *S. aureus* antigens. Whereas nonsignificant changes were observed for *S. pneumoniae*, SAEB-specific IgA was significantly decreased in nasal secretions from patients with CRSwNP (Fig. 3).

pIgR expression and eosinophilic inflammation of the sinonasal mucosa

As pIgR/SC and SAEB-specific IgA were decreased in CRSwNP, which is reportedly associated with eosinophilic inflammation, we addressed the relationship between pIgR downregulation and eosinophilic inflammation in our patients.

Increased numbers of eosinophils were observed, as expected, in subepithelial ethmoidal tissues from patients with CRSwNP (Fig. 4A). Eosinophils were also observed in a subset of patients with CRSsNP.

When dichotomizing patients according to the presence or not of high eosinophil numbers (i.e. ≥ 2 vs < 2 eos per h.p. field), pIgR downregulation was selectively observed in patients with eosinophilic upper airway disease (Fig. 4B). To further assess the correlation between eosinophils and pIgR expression, ECP was measured in nasal fluid. ECP was significantly increased in patients with AR, but not CRSwNP (Fig. 4C), and no correlation was observed with pIgR expression ($P = 0.5$, $r = -0.1$).

Cytokine expression in the sinonasal mucosa

IL-13 mRNA was significantly increased in the ethmoidal mucosa of CRSwNP, as well as to a lower extent in CRSsNP (Fig. 4D). IL-13 mRNA was correlated with eosinophil numbers in ethmoidal biopsies ($P = 0.001$, $r = 0.48$), indicating a Th2-related immune response.

In contrast, IL-12p35 expression was only increased in the ethmoidal mucosa of patients with CRSsNP (Fig. 4E). In addition, IL-12p35 mRNA was correlated with pIgR expression (Fig. 4E, right panel).

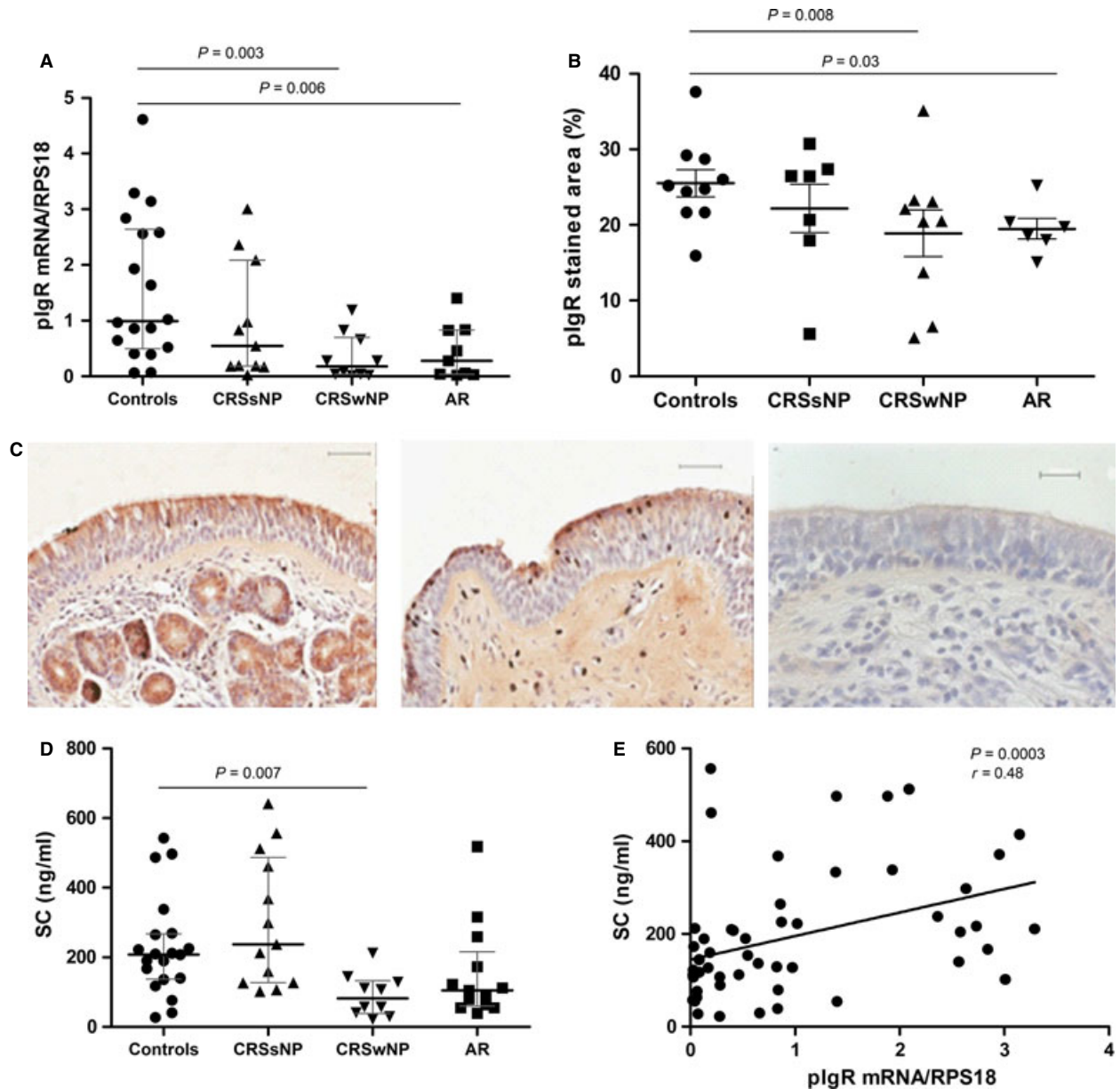


Figure 1 pIgR/SC expression in patients with upper airway disease. pIgR mRNA expression (A) in the ethmoidal mucosa from patients with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), CRS without nasal polyps (CRSsNP) and allergic rhinitis (AR), as compared to controls. pIgR protein detected by IHC was quantified in sinonasal tissue (B) and is shown (C) in tissue from one representative control and one representative patient with

CRSwNP, as compared to negative control with rabbit IgG (left, medium and right panels, respectively). Secretory component (D) production in nasal secretions. Correlation of pIgR mRNA expression in the ethmoidal mucosa with SC in nasal secretions according to Spearman's rank correlation method (E). Bar represents 50 μ m. P -values according to Kruskal–Wallis followed by Mann–Whitney U -tests.

Discussion

This study is, to our knowledge, the first to investigate epithelial pIgR in human chronic inflammatory disorders of the upper airways. It shows that the local expression of pIgR is downregulated in patients with CRSwNP and AR, but not in CRSsNP. This epithelial defect in CRSwNP resulted in the accumulation of IgA in subepithelial tissue (whilst active synthesis of IgA1

and IgA2 was unaffected) and in reduced SC and SAEb-specific IgA antibodies in nasal secretions. Moreover, correlation with immunoinflammatory features highlighted that whilst pIgR positively correlates with IL-12 expression as Th1 signature, pIgR downregulation was closely related to Th2-type eosinophilic inflammation of the ethmoidal mucosa.

In the upper airways, pIgR has been studied in nasopharyngeal carcinoma, where it has been shown to be decreased

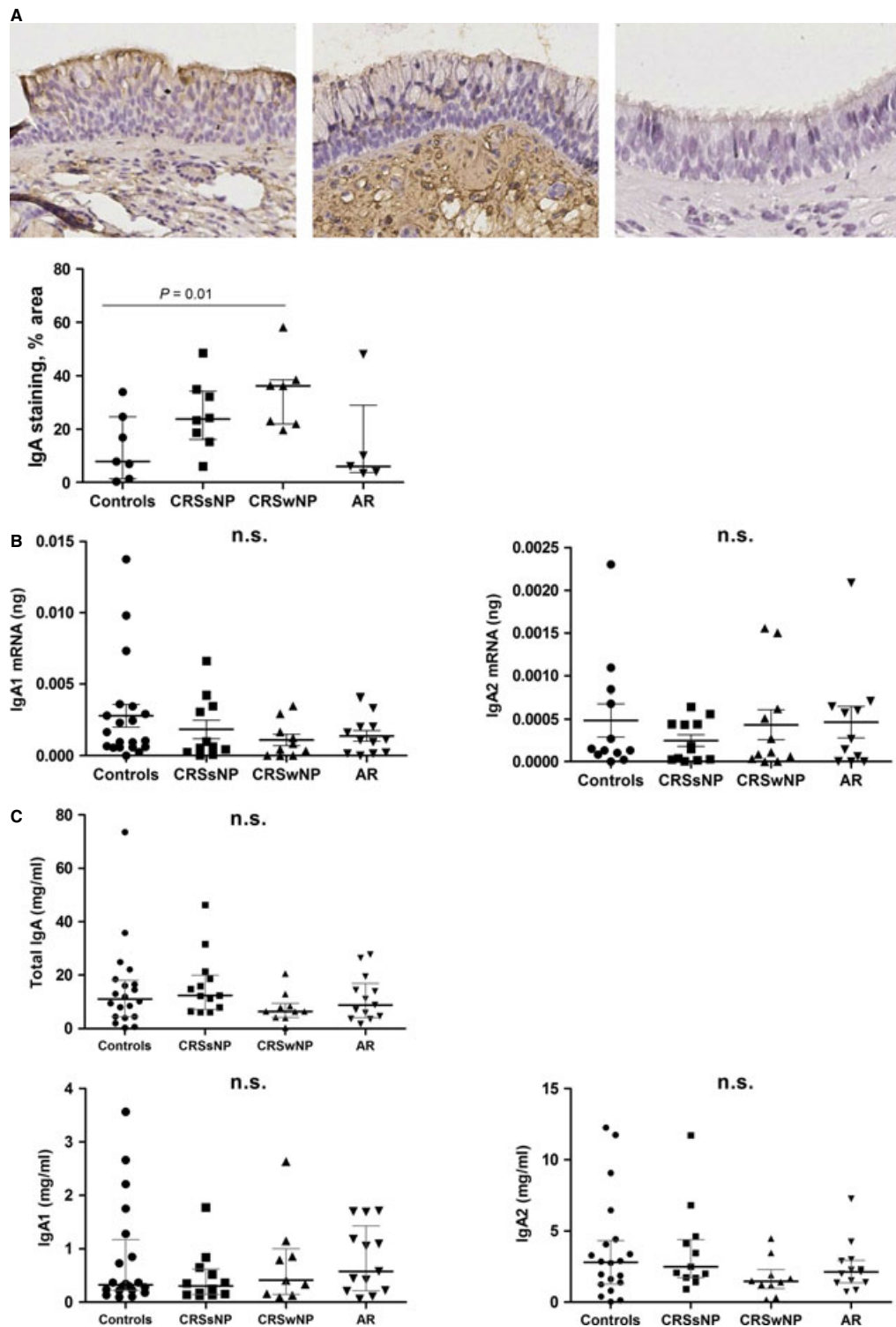


Figure 2 IgA expression in patients with upper airway disease. IgA detected by IHC is shown in tissue from one representative control and one representative patient with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), as compared to negative control with mouse IgG (upper left, right and lower left panels, respectively), and was quantified in subepithelial areas (A). IgA1 and IgA2

mRNA expression (B) in the ethmoidal mucosa from patients with CRSwNP, CRS without nasal polyps (CRSsNP) and allergic rhinitis (AR), as compared to controls. IgA1, IgA2 and total IgA (C) in nasal secretions from the same patient groups as shown in Fig. 1. *P*-values according to Kruskal–Wallis followed by Mann–Whitney *U*-tests.

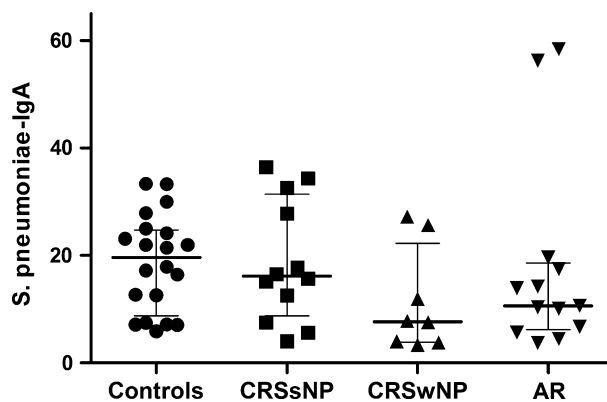
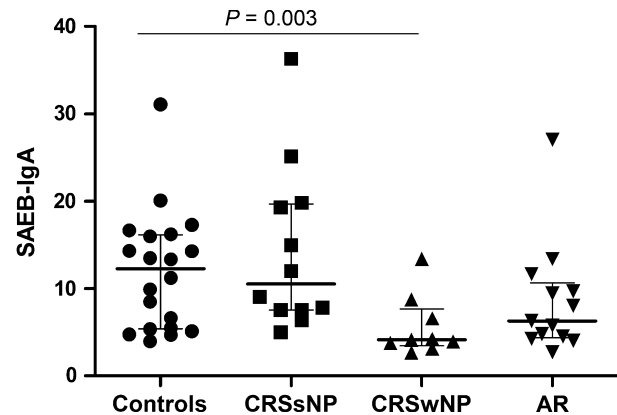


Figure 3 Specific IgA antibodies to *S. aureus* and *S. pneumonia* antigens in patients with upper airway disease. Specific IgA antibodies to pneumococcal anticapsular antigens and to *Staphylococcus aureus* enterotoxin B (SAEB) in nasal secretions from the same



patient groups as shown in Fig. 1. Results are expressed in arbitrary units. *P*-values according to Kruskal–Wallis followed by Mann–Whitney *U*-tests.

or mutated, with resultant decreased transcytosis of IgA (13, 14). Our data show that pIgR is well detected in the human sinonasal epithelium. Contrary to our original hypothesis, pIgR was selectively downregulated in the ethmoidal tissue of patients with CRSwNP and AR and was not affected in the turbinal mucosa. We also showed that pIgR defect results in reduced secretion of SC in CRSwNP. In contrast, the tissue content of IgA was increased in subepithelial areas from patients with CRSwNP, whilst active synthesis was not affected. These findings indicate that increased IgA content reported in previous studies of tissue homogenates from patients with CRSwNP (15) does not relate to increased IgA production, but probably to the aborted IgA transport in secretions due to impaired epithelial pIgR expression.

The pIgR defect in CRSwNP resulted in very modest and not significant reduction in IgA(2) in nasal secretions. However, levels of IgA antibodies to SAEB were significantly reduced in nasal secretions from these patients. Thus, as the pIgR assures the transport of IgA irrespective of its specificity, its reduced expression in the ethmoidal epithelium from CRSwNP is not sufficient to drive significant decreases in 'total' IgA, although a trend was observed (Fig. 2C), in contrast to secretory component, which was clearly decreased. IgA in secretions may arise locally, from synthesis by local plasma cells and subsequent pIgR-mediated transport, but also from exudation from the systemic circulation. As CRSwNP is associated with intense local inflammation and oedema, we hypothesize that the latter element may 'restore' the IgA levels in secretions from these patients, but does not compensate for reduced transport of locally produced IgA antibodies to *S. aureus* enterotoxin. An alternative possibility for the apparent discrepancy between reduced levels of SAEB-specific IgA and preserved levels of total IgA could lie in a specific defect in the generation of IgA antibodies to SAEB, which appears less likely.

The finding of a defect in specific IgA immunity to this *S. aureus* superantigen is interesting, considering the probably

important role of SAEB in driving IgE-mediated inflammation in CRSwNP, where a pro-Th2 role of SAEB has been identified (21). Given the major role of IgA in frontline defence of the airways, including inhibition of the adherence of pathogens to the epithelium (so-called immune exclusion) and neutralization of pro-inflammatory cytokines such as CXCL8/IL-8, it may be speculated that reduced pIgR-mediated immunity could affect firstline and specific immunity in (eosinophilic) CRS and AR, favouring bacterial colonization and leading to a vicious circle of impaired antibacterial immunity and secondary chronic inflammation (8, 22). The higher colonization by *S. aureus* in CRSwNP could be partly explained by decreased specific IgA to SAEB, as IgA plays an important role as a local scavenger of inhaled antigens. Moreover, IgA antibodies may probably also act as scavengers for allergens, which are reportedly defective in patients with AR and upregulated following allergen-specific immunotherapy (23). This postimmunotherapy IgA response was shown to be selective for the IgA2 subclass and induced monocyte IL-10 secretion, whilst inhibiting IL-12 expression (23, 24). In addition to Fab-mediated antigen neutralization, a defect in S-IgA antibodies could also result in reduced (Fc-mediated) opsonisation of pathogens. As a defect in the phagocytosis capacity of macrophages from patients with CRSwNP has been observed (6), it remains to explore whether reduced cytophilic S-IgA antibodies could contribute to this observation. Moreover, interplay between *S. aureus* and ECP has been recently observed, that is, for the regulation of receptor for advanced glycation end products (25). Our data further support a relationship between eosinophilic inflammation and local immunity to pathogen-derived products. Although balanced by the known degranulating effect of secretory IgA on eosinophils, these mechanisms could further indicate that IgA contributes via several pathways to control mucosal inflammation (26).

In the lung, pIgR deficiency has been observed in the bronchial epithelium from smokers who developed severe

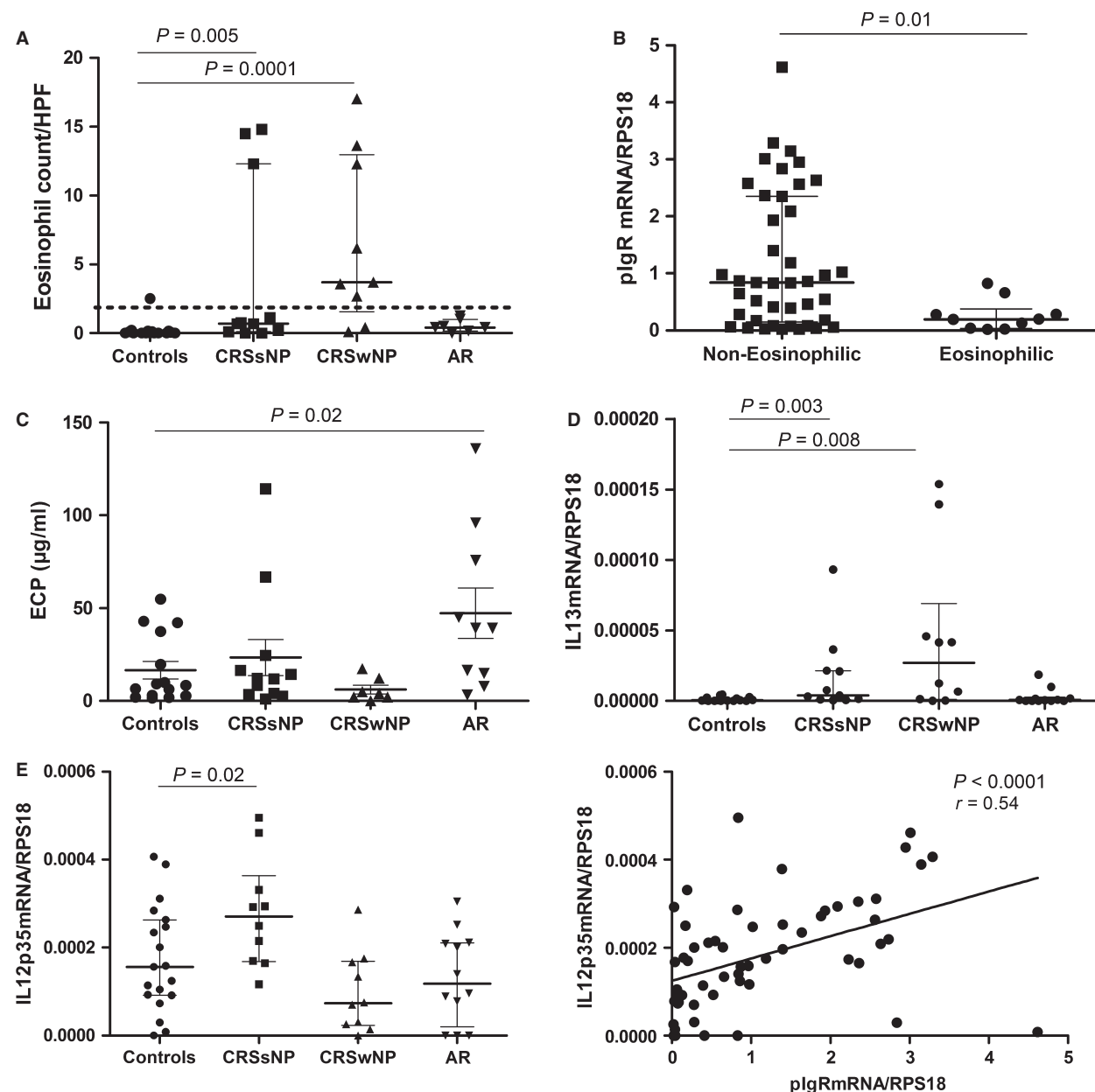


Figure 4 Eosinophils, ECP and expression of IL-13 and IL-12 in patients with upper airway disease. Eosinophils (expressed as n cells per h.p. field) in the ethmoidal mucosa from patients with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), CRS without nasal polyps (CRSsNP) and allergic rhinitis (AR), as compared to controls (A), and pIgR expression is shown in these patients plotted as 'eosinophilic' (≥ 2 eos per h.p. field) vs low/noneosinophilic

(<2; B). Production of ECP in nasal fluid (C), and expression of IL-13 mRNA (D) and IL-12p35 mRNA (E) in the ethmoidal mucosa from patients with CRSwNP, CRSsNP and AR, as compared to controls, and correlation with pIgR mRNA (right panel) according to Spearman's rank correlation method. P-values according to Kruskal–Wallis followed by Mann–Whitney U -tests.

COPD and linked to neutrophil infiltration of submucosal glands (7, 12). In contrast, our data show that pIgR downregulation is not clearly related to smoking, but rather to eosinophilic inflammation. Eosinophilia was as expected a common feature in CRSwNP, but was also observed in some patients with CRSsNP (27–29). We

observed that tissue eosinophils were more discriminant than the upper airway disease group, in identifying patients with pIgR downregulation, suggesting that this pIgR defect closely relates to eosinophilic inflammation. In contrast, a significant correlation was found between pIgR and IL-12p35 expression. As IL-12 is a key cytokine for the

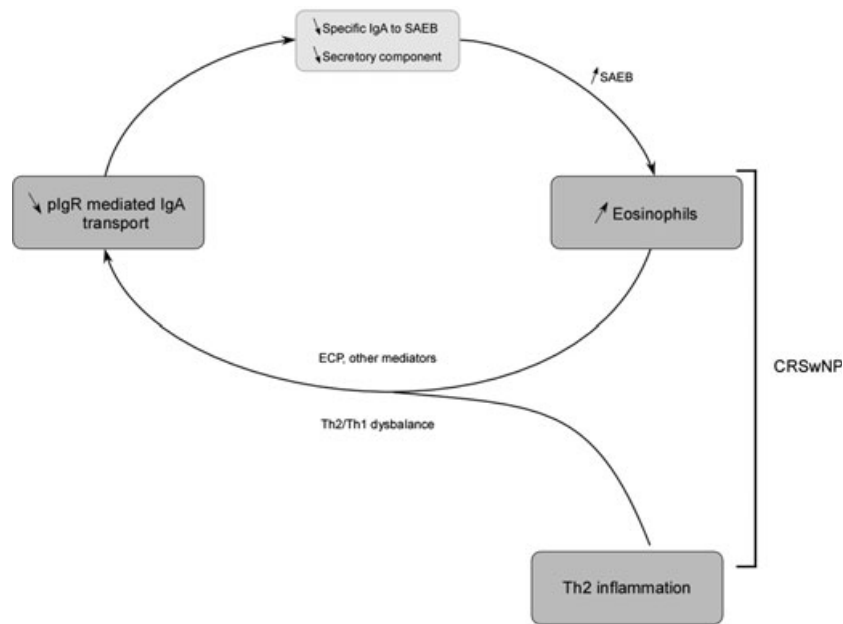


Figure 5 Putative pathways linking decreased pIgR expression and eosinophilic inflammation in chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP). The proposed mechanisms underlying the relationship between pIgR-dependent IgA immunity and eosinophilic inflammation of the upper airways include decreased pIgR expression resulting in reduced IgA-mediated neutralization of some

pathogens and/or antigens (e.g. *S. aureus* enterotoxin B) and in turn favouring eosinophilic inflammation (e.g. favoured by SAEB-IgE). Conversely, it may also result from the effect of local mediators of eosinophil/Th2-related airway inflammation on the sinonasal epithelium.

induction of IFN- γ -producing Th1 cells, this observation could be consistent with the known stimulatory role of IFN- γ on pIgR synthesis (30). However, it has been shown that IL-4 may also upregulate pIgR transcription, in synergy with IFN- γ (31). As eosinophilic upper airway disorders are associated with Th2-type immune responses, it could be speculated that besides IL-4, other Th2-related factors could drive pIgR downregulation. Thus, our positive correlation between pIgR expression and IL-12 may suggest that, in addition or alternative to favouring eosinophilic inflammation by limiting the scavenging effect of IgA antibodies to bacterial (super) antigens, pIgR downregulation could also result from the effect of mediators of Th2-type eosinophilic inflammation. Although this needs to be confirmed, notably using nasal epithelial cells, the putative pathways linking pIgR downregulation and eosinophilic upper airway disease are summarized in Fig. 5.

Altogether this study shows for the first time that pIgR is well expressed in the human sinonasal mucosa, especially in seroglandular and ciliated epithelial cells, and that its expression is decreased in patients with CRSwNP and AR. This defect results in reduced secretion of SC and specific IgA antibodies to SAEB in nasal fluid from patients with CRSwNP, as well as in subepithelial accumulation of IgA. These data also indicate a previously unrecognized link between eosinophilic inflammation and pIgR-mediated secretory immunity, suggesting that pIgR impairment is observed in the eosinophilic phenotype of upper airway disease.

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Author contributions

C.P., C.H. and P.R. contributed to conception and design of the study. C.H., M.L., H.B. and H.G. performed the experiments, data analysis and interpretation of the results. C.H. and C.P. wrote the manuscript. C.P. and P.R. reviewed the manuscript.

Conflicts of interest

The authors have no conflicting financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

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Supporting information

Downregulation of polymeric immunoglobulin receptor and secretory IgA antibodies in eosinophilic upper airways diseases

Original Article

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Methods

Immunohistochemistry for pIgR and IgA

After surgical removal, the turbinal and ethmoidal biopsies were immediately immersed in 4% formaldehyde in phosphate-buffered saline at pH 7.4 for at least 24 h. Samples for immunohistology were embedded under vacuum in paraffin. Serial sections of 5 μ m thickness were cut from paraffin blocks, spread on polylysine-coated glass slides, and dried at 40° C for at least 24 h.

The slides were then processed for immunostaining, each step of the procedure being followed by washing with Tris-buffered saline (pH 7.4). After disembedding and rehydration of the specimen, endogenous peroxidases were inhibited by incubation in 0.03% (vol/vol) H₂O₂ in water for 30 min, and the slides were treated with 1% (wt/vol) BSA in Tris-buffered saline for 30 min to neutralize remnant reactive aldehyde groups originating from fixation. Slides were then incubated overnight at 4° C with rabbit anti-human SC IgG prepared as previously reported¹ and mouse anti-human IgA (ThermoScientific). Control sections were treated with rabbit anti-chicken IgY/mouse IgG at the same dilution. The secondary antibody, (biotinylated goat anti-rabbit IgG (Sigma, 1/1000), biotinylated rabbit anti-mouse IgG (Sigma, 1/3000) was applied in 10% (wt/vol) defatted dry milk for 30 min. The reaction was amplified with streptavidin–peroxidase conjugate (BD Pharmingen, 1/500) in Tris-buffered saline containing 1% BSA for 30 min, and color was developed by incubation with diaminobenzidine in 0.03% H₂O₂ for 10 min. After the reaction was stopped by washing in water, slides were counterstained with Mayer's hemalum and mounted with coverslips in Eukitt's medium. Images of each section were captured by using an Aperio ScanscopeAT scanner.

To assess pIgR and IgA quantification, unmodified images of scanned turbinal and ethmoidal sections were analyzed using the Framework for Image Dataset Analysis (FrIDA), a custom open source image analysis tool from the Johns Hopkins University, as previously described.¹⁶ Briefly, color masks were generated to identify DAB brown. A meta mask created to include all brown pixels in the epithelial (pIgR)/subepithelial (IgA) zone was used to analyze each image. We calculated the percentage of positive area fraction in ten epithelial/subepithelial fields per section, at x400 magnification.

RT-PCR for pIgR, IL12p35, IL-13 and IgA1/IgA2 mRNA expression

Total RNA was isolated using the Rneasy® Plus Mini kit (Qiagen). Each 500 ng of total RNA was reverse-transcribed with RevertAid™ Reverse transcriptase kit (Fermentas) with 0.3µg of random hexamer (Invitrogen) and 1mM of each dNTP (Invitrogen) following to the manufacturer's protocol in a thermocycler (Applied Biosystem). The expression levels were quantified by real time quantitative PCR with the iCycler IQ5 PCR (Bio-Rad). The reaction mix contained 5 µl of cDNA diluted 10-fold, 400nM of each primer (pIgR reverse: CAG CCG TGA CAT TCC CTG ; pIgR forward: CTC TCT GGA GGA CCA CCG T ; RPS18 reverse: TGA TCA CAC GTT CCA CCT CAT ; RPS18 forward: TGT GGG CCG AAG ATA TGC T; IL-13 forward: CTG GAA TCC CTG ATC AAC GT . IL-13 reverse: GAA AAC TGC CCA GCT GAG AC) and 2x iQTM SYBR®Green Supermix (Bio-Rad) in a final volume of 20µl. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15s and 60°C for 30s. To control the specificity of the amplification products, a melting curve analysis was performed. Moreover, the negative controls (water and –RT) showed no amplification. Samples were run in duplicate and the copy number was calculated from a standard curve. The standard curve and data analysis were produced using Bio-Rad iQ5 Software.

For IgA1 and IgA2, PCR was performed by using an ABI PRISM 7900 Sequence Detection System thermal cycler according to manufacturer's instructions (Applied Biosystems, Life Technologies Corporation, California, USA) using the equivalent of 1.25ng reverse transcribed RNA per reaction. Gene specific primer/probe sets were designed using the on-line Universal ProbeLibrary Assay Design Centre (Roche Applied Science, Burgess Hill, UK). Universal ProbeLibrary probes were purchased from Roche Applied Science and corresponding forward and reverse primers synthesized by Sigma (Sigma-Aldrich Company Ltd, Dorset, UK). The primer/probes sets were as follows: IgA1, Forward-CATGCCACGTGAAGCACTAC, Reverse-GGTAGGTGGAGTTGAGGGAAC, Probe-UPL Probe 20; IgA2, Forward-CGCCAACATCACAAAATCC, Reverse-CGTCACCAGCTCGTTCAG, Probe-UPL Probe-17. SDS software was used to determine the absolute quantification of the target cDNA. Standard curves were created using plasmids containing cDNA for IgA1, I.M.A.G.E cDNA clone 4701069 and IgA2, I.M.A.G.E cDNA clone 4765168 (Source Bioscience UK Limited, Nottingham, UK).

ELISA for SC and IgA

SC, IgA, IgA1 and IgA2 concentration were determined in nasal secretions by a specific enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates were coated with 1 µg/ml affinity-purified goat anti-SC antibody (developed in our laboratory, recognizing both soluble SC and membrane pIgR/SC) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. After washings with 1/1.000 vol/vol Tween 20-PBS (PBST) and blockade with 1% wt/vol BSA in PBST for 1h at 37°C, samples and serial dilutions of purified human soluble SC/IgA1/IgA2 were incubated for 2 h at 37°C. After washings with PBST, plates were then incubated with biotinylated goat anti-SC/ biotinylated mouse anti-IgA1/ biotinylated mouse anti-IgA2 for 2 h and washed in TBST.

A volume of 100 µl streptavidin–polyHRP in a 1:10.000 dilution in HPE buffer was added to each well and incubated for 30 min at room temperature. The plates were washed three times using PBST, and 100 µl of (3,5,3',5')-Tetramethylbenzidine substrate was added to each well. After incubation, the reaction was stopped by the addition of 100 µl of 2 M H₂SO₄. The absorbance was measured at 450 nm.

Total IgA concentration was determined by the same method (coating with 1 µg/ml affinity-purified goat anti-SC antibody, blockade with 1% wt/vol BSA in PBST, samples and serial dilutions purified total IgA) except for the secondary antibody which was anti-human IgA (α-chain specific)–peroxidase antibody produced in goat. As it was peroxidase-conjugated, use of streptavidin–polyHRP was not necessary.

To assess specific IgA to pneumococcal anticapsular antigens and *Staphylococcus aureus* enterotoxin B, the same method was used, adapted from a previous report.² Microplates were coated with the whole 23-valent pneumococcal polysaccharide vaccine (Sanofi-Pasteur MSD) or with *Staphylococcal* Enterotoxin B from *Staphylococcus aureus* (Sigma-Aldrich). The detection antibody was HRP-conjugated goat anti-human IgA (α-chain specific). Results were expressed as arbitrary units, by reference to serial dilutions of a highly positive sample (OD given by 1:2 dilution set as 1 unit, and corrected to the dilution factor).

Immunoassay for ECP

Quantification of ECP was carried out on nasal fluid samples by ImmunoCAP assay (ThermoFisher), according to the manufacturer's instructions.

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