

Nasal polyps in patients with and without cystic fibrosis: a differentiation by innate markers and inflammatory mediators

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Summary

Background The dysfunction of the mucosal interface of the upper respiratory tract in cystic fibrosis (CF) patients is clinically visible by the development of nasal polyps (NP) at a young age. Innate defence markers and inflammatory mediators in NP from patients with CF were compared with non-cystic fibrosis nasal polyps (non-CF-NP) to determine a possible different immunological background in macroscopically similar tissue.

Methods Surgical samples were obtained from patients with non-CF-NP, cystic fibrosis patients with nasal polyps (CF-NP) and control patients (CO). With real time PCR, the mRNA expression of human β defensins (HBD) 2 and 3, toll-like receptors (TLR) 2 and 4 and the macrophage mannose receptor (MMR) were measured. On homogenates of the surgical samples eotaxin, myeloperoxidase (MPO), IL-5 and IL-8 protein content was measured using commercial ELISA kits; IgE and eosinophilic cationic protein (ECP) were measured by the Unicap system.

Results In CF-NP we found a statistically significant higher mRNA expression of HBD 2 compared with non-CF-NP and CO and of TLR 2 compared with non-CF-NP. In the non-CF-NP group, MMR mRNA expression was significantly elevated compared with CO and CF-NP. For TLR 4 mRNA expression no statistically significant differences were found between groups. IL-5 was below detection level in all CO and CF-NP, but was measurable in 80% of the non-CF-NP. MPO and IL-8 concentrations were significantly higher in CF-NP compared with CO and non-CF-NP, whereas ECP, eotaxin and IgE were significantly higher in the non-CF-NP group.

Conclusions We here demonstrate that CF-NP and non-CF-NP not only differ in terms of inflammatory mediator profile, but also in terms of innate markers.

Keywords cystic fibrosis, human β defensin, inflammatory mediators, macrophage mannose receptor, nasal polyps, toll-like receptor

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Introduction

The incidence of nasal polyps (NP) is much higher in cystic fibrosis (CF) patients than in the general population and chronic rhinosinusitis, with or without NP, is now recognized as one of the major otolaryngological manifestations of CF. The genetic base of CF is clearly established but no particular cystic fibrosis transmembrane conductance regulator (CFTR) mutation, correlating with the phenotype of nasal polyp formation in cystic fibrosis patients (CF-NP), has been found until now [1]. NP are also described in adult patients without CF (non-CF-NP), and although many clinical similarities exist, careful analysis of the literature points to differences in histopathology and inflammatory characteristics [2].

The respiratory mucosal surface of CF patients shows changes that undoubtedly contribute to an ideal environment

for microbial growth and consecutive chronic sinus inflammation.

Dysfunction of the CFTR protein, which acts as a chloride channel, causes changes in the mucous composition with production of thick viscous secretions and impaired mucociliary clearance [2]. Although bacterial infection is widely accepted to be a major factor in the pathogenesis of acute exacerbations and chronic progression of lung disease in CF [3], it remains unclear if the CF-specific sinonasal pathogens, of which *S. aureus*, *P. aeruginosa*, *H. influenzae* and anaerobes are the most common [1], play a particular role in the pathogenesis of CF-NP.

In patients without CF, however, bacterial colonization seems to play a pivotal role in NP formation. The pathogenesis of non-CF-NP is not yet fully understood, but recent insights emphasize a local eosinophilic tissue inflammation with increased prevalence of colonization with *S. aureus* and in severe cases associated with asthma and aspirin sensitivity [4]. Eotaxin and IL-5 are key mediators in this eosinophilic inflammation, because of their role in eosinophil chemotaxis,

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activation and survival, whereas an increased release of eosinophilic cationic protein (ECP) could induce exudation and polyp growth [5]. Furthermore, non-CF-NP are characterized by a prominent local IgE synthesis irrespective of skin test results, but related to eosinophilic inflammation [6]. Whether similar mechanisms hold true in CF is currently unclear. However, in CF patients, a predominant neutrophil infiltration of the lower respiratory tract seems to be present with increased myeloperoxidase (MPO) and IL-8 levels in sputum of CF patients compared with chronic bronchitis patients [7]. An increased expression of IL-8 is also found in sinonasal tissue of CF patients with chronic rhinosinusitis, compared with non-CF patients with chronic rhinosinusitis [8].

As the upper airway is continuously exposed to and challenged by external matter, innate and adaptive defence mechanisms have to cooperate to maintain immunological homeostasis. Upper airway mucosa expresses innate antimicrobial peptides, including human β defensins 2 and 3 (HBD 2 and 3) [9]; innate pattern recognition receptors (PRRs) such as toll-like receptors 2 and 4 (TLR 2 and 4) [9] and macrophage mannose receptor (MMR) [10]; and inflammatory mediators [11] that activate the innate and adaptive immune system. A previous study of our group showed a significant increase of MMR expression in non-CF-NP compared with CRS tissue, suggesting a local PAMP (Pathogen Associated Molecular Pattern)-PRR interaction with sustained inflammation in non-CF-NP [10].

To evaluate specific features of innate and adaptive defence mechanisms, associated with persistent therapy resistant NP formation in the upper airways, we measured expression of innate markers and inflammatory mediators in CF-NP and non-CF-NP, with normal nasal mucosa samples serving as controls (CO). For the assessment of innate defence in the upper airways, we measured tissue expression of HBD 2, HBD 3, TLR 2, TLR 4 and MMR, whereas inflammatory signalling was determined by the expression of IL-5, IL-8, eotaxin, ECP, MPO and IgE concentrations.

Materials

Sinonasal samples were obtained from patients with or without CF undergoing functional endoscopic sinus surgery for nasal polyposis. Control samples were obtained during septal surgery from the inferior turbinate of patients without sinonasal disease.

The reasons for surgical procedure were unrelated to the study. Tissue samples were immediately snapfrozen in liquid nitrogen. The ethical committee of Ghent University Hospital approved the study; an informed consent was obtained from all subjects prior to inclusion in the study.

The patients were divided into three groups. First: CO, second: non-CF-NP, third: CF-NP.

In patients with CF-NP and non-CF-NP, polyp formation was visualized in the nasal cavity and middle meatus by nasal endoscopy and in the sinuses by CT scan prior to surgery, whereas CO were free of polyps also demonstrated by nasal endoscopy and on CT scan.

We collected 39 sinonasal surgical samples, from 39 different patients, (10 CO: 8 male/2 female, 14–63 years old; 15

non-CF-NP: 10 male, 5 female, 23–71 years old; 14 CF-NP: 11 male, 3 female, 4–29 years old). A history of asthma was registered in 7/15 patients with non-CF-NP, with one patient also demonstrating a history of aspirin sensitivity. None of the patients of the CF-NP group and none of the patients of the CO group had a history of asthma or aspirin sensitivity. Skin prick test was positive for at least one inhalant allergen in 3/15 patients with non-CF-NP, in 2/14 patients with CF-NP and was negative in all CO patients. None of the patients were treated with oral corticosteroids within 4 weeks prior to surgery. 4/15 patients of the non-CF-NP group, 2/14 patients of the CF-NP group and none of the CO group used nasal corticosteroids within 4 weeks prior to surgery.

All 39 samples were analyzed for IL-5, IL-8, eotaxin and MPO with commercially available ELISA kits and for ECP and IgE by the Unicap system.

On 28 sinonasal surgical samples (6 CO, 9 non-CF-NP, 13 CF-NP), which were available for analysis, quantitative real time RT-PCR for HBD 2 and 3, TLR 2 and 4 and MMR mRNA expression was performed.

Methods

RNA preparation and real-time reverse transcriptase-polymerase chain reaction

Snapfrozen tissue samples (50–100 mg) were placed in liquid nitrogen and thoroughly grounded with a mortar and pestle. One millilitre of TRI Reagent (Sigma, Bornem, Belgium) was added to the tissue and further homogenized by repeated pipetting. Total RNA was purified according to the instruction of the manufacture. One microgram of total RNA was first incubated with Dnase I (1 U, Invitrogen, Merelbeke, Belgium) and then reverse transcribed to generate cDNA with SuperScriptTMII (Invitrogen) in the presence of oligo(dT), random primer, DTT and dNTPs.

Primers and probes used for PCR amplification were purchased from Eurogentec (Liège, Belgium). Sequences were previously published [9, 10].

Real-time PCR was performed in a 25 μ L reactions mixture composed of 5 μ L cDNA (equivalent to 25 ng total RNA), 1 μ M (final concentration) oligonucleotide primers, 250 nM probe, 5 mM MgCl₂, 200 μ M dNTPs, and 0.025 U/ μ L Hot GoldStar enzyme (Eurogentec) and measured with a Perkin Elmer ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were incubated for 10 min at 95°C, followed by 45 cycles of a two-step amplification-procedure composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. In each run, dilution series of pooled cDNA from donor tonsils were amplified to serve as a standard curve for calculation of relative quantities, normalized for PBDG (porphobilinogen deaminase) house keeping gene expression.

Preparation of nasal tissue for enzyme-linked immunosorbent assay measurements

Nasal tissue specimens were weighed and 1 mL of 0.9% NaCl solution, supplemented with protease inhibitor cocktail (complete protease inhibitor cocktail, Roche Diagnostic, Vil-

voorde, Belgium) was added to every 0.1 g of tissue. The samples were then homogenized for 5 min at 1000 r.p.m. on ice with a mechanical homogenizer (B. Braun Melsungen, Melsungen, Germany) as described previously [4]. After homogenization, the suspensions were centrifuged at 1500 *g* for 10 min at 4 °C and supernatants were separated and stored at –20 °C until analysis.

The samples were analysed for IL-5, IL-8, MPO, eotaxin with commercially available ELISA kits according to the manufacture's instructions (all kits were purchased from R&D Systems, Minnesota, MN, USA, except MPO from Oxis Research, Immunosource, Zoersel, Belgium). ECP and IgE were measured by the Unicap system (Pharmacia, Uppsala, Sweden).

Statistical analysis

Data are expressed as median and inter-quartile ranges. When comparisons were made between groups, significant between-group variability was first established using the Kruskal–Wallis test. The Mann–Whitney *U*-test was then used for between-group (unpaired) comparison. Differences

between the paired data were calculated by using the Wilcoxon test. Spearman rank correlation coefficient (*r*) was used to assess the relationship between the parameters. *P*-values <0.05 were considered statistically significant.

Results

With real-time RT-PCR we measured HBD 2 and 3, TLR 2 and 4 and MMR mRNA in surgical samples from non-CF-NP, CF-NP and CO. Quantification of HBD 2, TLR 2, TLR 4 and MMR is presented in Figs 1a–d. HBD 3 was below detection level in all samples. We found a statistically significant higher mRNA expression for HBD 2 in CF-NP compared with non-CF-NP ($P = 0.001$) and CO ($P = 0.001$). TLR 2 mRNA expression was significantly elevated in the CF-NP group compared with the non-CF-NP group ($P = 0.045$). In non-CF-NP, we found a significantly higher gene expression of MMR compared with CF-NP ($P = 0.021$) and to CO ($P = 0.010$). For TLR 4, no statistically significant differences were found between groups.

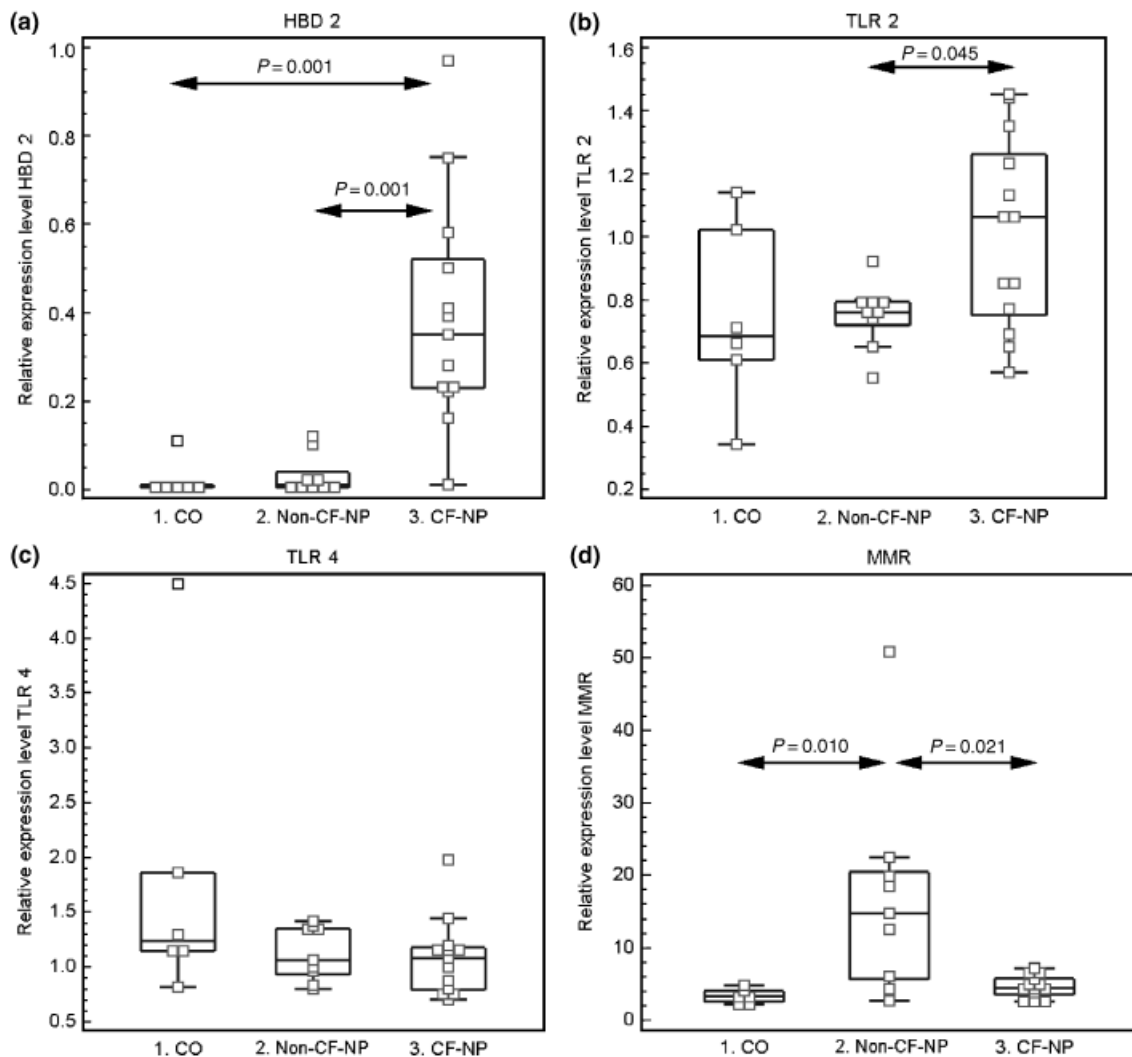


Fig. 1. Comparison of HBD 2 (a), TLR 2 (b), TLR 4 (c) and MMR (d) mRNA quantification between CO, non-CF-NP and CF-NP. *P* values for statistically significant differences between groups are represented. CO, controls; non-CF-NP, non-cystic fibrosis nasal polyps; CF-NP, cystic fibrosis nasal polyps; HBD, human β defensins; MMR, macrophage mannose receptor; TLR, toll-like receptors.

In sinonasal mucosa homogenates of patients with non-CF-NP, CF-NP and CO patients, the protein content of ECP, MPO, IL-5, IL-8, eotaxin and IgE was measured using ELISA and Unicap techniques. The results are presented in

Figs 2a–f. IL-5 was below detection level in all CO and CF-NP samples, but was measurable in 12/15 non-CF-NP samples. In the non-CF-NP group, IgE was significantly elevated compared with the CO group ($P < 0.001$). No signif-

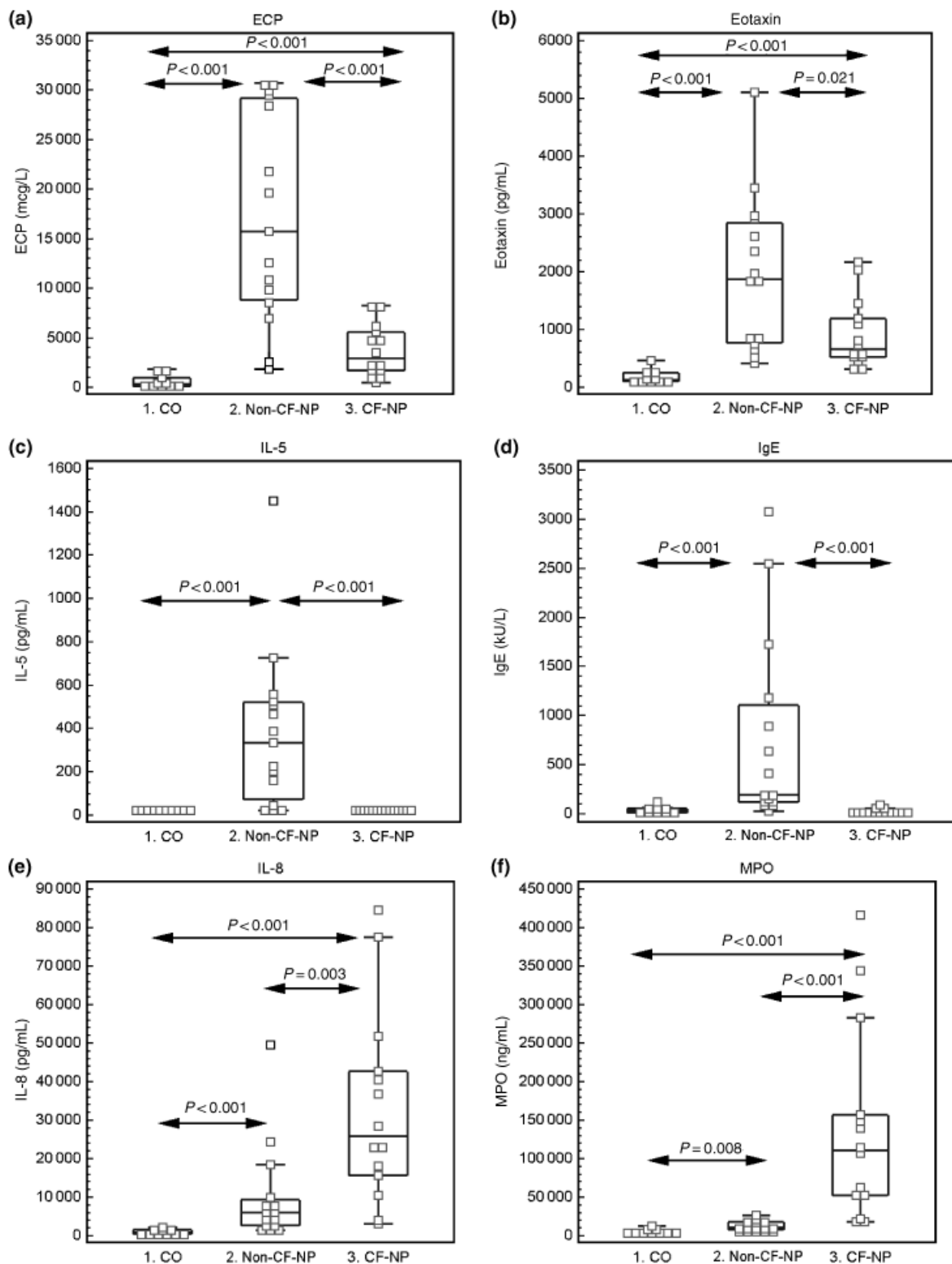


Fig. 2. Comparison of ECP (a), eotaxin (b), IL-5 (c), IgE (d), IL-8 (e) and MPO (f) concentrations between CO, non-CF-NP and CF-NP. P values for statistically significant differences between groups are represented. CO, controls; non-CF-NP, non-cystic fibrosis nasal polyps; CF-NP, cystic fibrosis nasal polyps; ECP, eosinophilic cationic protein; MPO, myeloperoxidase

ificant increase of IgE could be measured in CF-NP compared with CO. In both non-CF-NP and CF-NP samples ECP, MPO, IL-8 and eotaxin expression was significantly higher compared with CO tissue (P value for MPO in non-CF-NP vs. CO = 0.008, $P < 0.001$ in all other comparisons). Comparing the cytokine profile and IgE antibody levels in non-CF-NP and CF-NP, we found a statistically higher expression of ECP ($P < 0.001$), IgE ($P < 0.001$) and eotaxin ($P = 0.021$) in the non-CF-NP group, whereas MPO ($P < 0.001$) and IL-8 ($P = 0.003$) expression was significantly higher in the CF-NP group.

Furthermore, HBD 2 mRNA expression and IL-8 protein content showed a significant correlation in the CF-NP group ($r = 0.718$, $P = 0.0129$), whereas in the non-CF-NP group, a correlation between MMR gene expression and the protein content of IgE antibodies was found ($r = 0.770$, $P = 0.0209$). No other significant intra-group correlations were found between and among innate markers and inflammatory mediators.

Discussion

In bilateral eosinophilic NP in non-CF patients, IL-5 and eotaxin, belonging to the Th2 cytokine repertoire, have been described as key mediators for the accumulation and activation of eosinophils, and an increased release of ECP in non-CF-NP could possibly induce plasma exudation and polyp growth [5]. Furthermore, nasal polyposis is characterized by a prominent local polyclonal IgE synthesis related to the eosinophilic inflammation [6]. We recently also described an up-regulation of the MMR mRNA in non-CF-NP [10], a finding which was confirmed in this study, and extended with respect to the correlation between MMR gene expression and total IgE content. MMR is a 175 kDa transmembrane glycoprotein which facilitates the binding to bacteria and mediates phagocytosis of non-self and antigen delivery to MHC class II compartments [12]. MMR also functions as a signal transducing receptor, triggering production of cytokines IL-1, IL-6 and IL-12 [13, 14] and modulating cell surface receptors [15, 16]. The correlation of MMR mRNA expression and local IgE levels in non-CF-NP leaves us to speculate about a possible role of MMR in the uptake of bacterial matter and the initiation or/and maintenance of the IgE formation and eosinophilic inflammation in this disease. Furthermore, we here show that neither MMR mRNA expression nor polyclonal IgE formation, nor IL-5 and eotaxin release, are typical features of CF-NP. In contrast, the innate PRR TLR 2 gene was expressed significantly higher in CF-NP compared with non-CF-NP and CO. For TLR 4 no differences in gene expression were found between groups.

Recently, research has focussed on the regulation TLR expression, however, the physiological relevance of TLR up-regulation is not yet determined. TLR expression has been studied in relation to glucocorticosteroids, lipopolysaccharide, TNF- α , IL-1, IFN γ , IL-2 and IL-15 [17–19]. Additionally, a clear difference in TLR 2 and 4 mRNA expression, both *in vivo* [20] and *in vitro* [17, 18], has been shown and can be explained by their different promotor sequences (NF- κ B-binding site) [21–23]. However, in a previous study of our

group we could not demonstrate a significant difference for TLR 2 and TLR 4 mRNA expression in tonsils, adenoids and nasal mucosa of healthy subjects and patients with chronic upper airway inflammation [9]. The singular up-regulation of TLR 2 (and not TLR 4) in CF-NP suggests an important role for TLR 2 in the orchestration of host-defence in CF-NP. This observation enhances the necessity for further evaluation of the influence of bacterial infection, cytokine biology and therapeutic measures (corticosteroids) on the sentinel function of PRRs in the upper airway. The different PRR pattern (MMR and TLR) in NP of both patients groups might also depend on the 'host genetics' and/or the maturation status of the immune cells.

The hypothesis that TLR 2 may have an important sentinel function in the CF upper airway epithelium and its possible role in modulating the immune response can be supported by the capacity of TLR 2 to mediate induction of HBD 2 [24] and IL-8 [25] in response to Gram positive or Gram negative bacterial components. In this study we found the mRNA expression of the human β -defensin HBD 2 to be up-regulated in CF-NP, but not in non-CF-NP subjects. We furthermore describe a correlation between HBD 2 expression and IL-8 levels, which illustrates parallel regulation of HBD 2 and IL-8, also shown by other groups [26, 27]. The influence of microbial factors or inflammatory cytokines on HBD 2 [26, 28] and HBD 3 [26, 28] expression has been demonstrated previously. In this study HBD 3 expression was below detection level in all samples, also in CO, indicating a difference in constitutive expression and illustrating a different regulation for HBD 2 and HBD 3, which has already been described *in vitro* [29]. The finding that HBD 2 expression is suppressed by Th2 cytokines could explain why the cytokine milieu in non-CF-NP is probably less favourable for the induction of HBD 2 (lower pro-inflammatory molecules, more Th2 related cytokines [5, 6]). The lack of up-regulation of the inducible HBD 2 in non-CF-NP, previously also described in atopic dermatitis [26], a disease sharing many features with non-CF-NP, could induce an increased susceptibility to *S. aureus*, a pathogen related to the severity of non-CF-NP [4].

To confirm interaction between IL-8 synthesis, HBD 2 expression, and exposure to *P. aeruginosa* in CF-NP, further study will be needed. However, the up-regulation of TLR 2, HBD 2 and IL-8 in CF-NP illustrates the induction of innate immunity defence mechanisms, consisting of an antimicrobial activity against Gram positive and Gram negative bacteria (HBD 2), and the signal transduction through TLR 2 with consecutive release of IL-8 [30], leading to the attraction of neutrophils for phagocytosis.

On the other hand, we hypothesize that in non-CF-NP, a dysregulated eosinophilic inflammation, with IgE formation, is triggered by products of colonizing bacteria (e.g. enterotoxins of *S. aureus*), while the innate immune system increases phagocytosis of germs by MMR-positive macrophages. In contrast, in CF, nose and sinus mucosa, colonized with *S. aureus* and *P. aeruginosa*, responds with the up-regulation of TLR 2 and HBD 2, which in turn induces a dysregulated, insufficient, neutrophilic inflammation. This hypothesis needs to be further investigated, especially with focus on the impairment of non-CF nasal polyp tissue to respond by an adequate TLR and HBD response, and the effectiveness of such a response in CF.

The significant differences between innate markers and inflammatory mediators in CF-NP and non-CF-NP provide further arguments to understand clinically similar disease manifestations (NP) as different diseases of the upper airways.

Conclusion

We demonstrate here that CF-NP and non-CF-NP not only differ in terms of inflammatory cell and cytokine patterns, but also in terms of the expression of innate markers. CF-NP is associated with the up-regulation of both HBD 2 and TLR 2, while the expression of MMR dominates innate defence in non-CF-NP. Distinct correlations between innate markers and inflammatory markers in both diseases possibly indicate links, which are currently only partially understood and require further research.

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