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Original Article

Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response ☆

Helle Krogh Johansen [a,*], Kasper Aanaes [b], Tania Pressler [c], Kim Gjerrum Nielsen [c], Jacob Fisker [b], Marianne Skov [c], Niels Høiby [a, d], Christian von Buchwald [c]

[a] *Department of Clinical Microbiology, afsnit 9301, Rigshospitalet, Juliane Maries Vej 22, DK-2100 Copenhagen Ø, Denmark*
[b] *Department of Otolaryngology, Head and Neck Surgery, afsnit 2072, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark*
[c] *CF Centre Copenhagen and Paediatric Pulmonary Service, Department of Paediatrics and Adolescent Medicine, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark*
[d] *Institute of International Health, Immunology and Microbiology, Panum Institute, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen N, Denmark*

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Abstract

Background: We studied whether the sinuses might be foci for *Pseudomonas aeruginosa* lung infection.
Methods: Endoscopic Sinus Surgery was performed in 78 CF patients; PFGE was used for bacterial genotyping. Material from sinuses and lungs were Gram-stained to detect biofilms. Immunoglobulins were measured in serum and saliva.
Results: When *P. aeruginosa* was cultured simultaneously from the sinuses and the lungs they were genetically identical in 38 of the 40 patients (95%). In the sinuses, *P. aeruginosa* formed biofilms with minimal cellular inflammation, probably because of a significantly higher local production of secretory IgA compared with IgG ($p<0.001$).
Conclusions: We have shown that *P. aeruginosa* form biofilm in the sinuses, which constitute an important bacterial reservoir for subsequent lung infection. The high amount of IgA in the upper airways probably protects *P. aeruginosa* from the inflammatory immune system, and they can proceed unnoticed into a permanent infectious focus that cannot be eradicated with antibiotics.
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Keywords: Rhinosinusitis; *P. aeruginosa*; Cystic fibrosis; Foci; Biofilm

1. Introduction

The most common manifestations of cystic fibrosis (CF) are pulmonary disease, radio-opaque sinuses, nasal polyposis and exocrine pancreatic insufficiency. Symptoms of chronic rhinosinusitis have been reported in 11–94% of CF patients [1]. Sinusitis is seldom recognised by the patients but nearly all CF patients have radiographic and clinical signs of infection [1,2].

Molecular epidemiology studies have shown that CF lung transplant recipients become re-colonised in their lung grafts with the same clones as those cultured before transplantation [3]. It is likely that *Pseudomonas aeruginosa* is drained into the lung allografts from a bacterial reservoir in the paranasal sinuses via the airways [3,4]. In lung transplanted CF patients sinus surgery aiming at eradication of bacteria, mostly *P. aeruginosa*, has

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* Corresponding author. Tel.: +45 3545 4973; fax: +45 3545 6412.
E-mail address: hkj@cochrane.dk (H.K. Johansen).

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lowered the incidence of bacterial colonisation and infection after transplantation [5].

We have previously genotyped initial and subsequent *P. aeruginosa* lung isolates from CF patients by using Array Tube biochip analysis (Clondiag®), a method based on single nucleotide polymorphisms (SNPs) [6] and by Pulsed Field Gel Electrophoresis (PFGE) [6]. We found that the initial colonising *P. aeruginosa* strains had different genotypes; i.e. every patient had a genotype different from all other patients. This suggests that the initial colonisation comes from environmental sources rather than from transmission between patients [7]. We also found that new lung colonising isolates had the same genotype as the initial colonising strain after antibiotic eradication of *P. aeruginosa*, suggesting a bacterial reservoir either in the patient or in the patient's close environment [6].

In the present prospective study, we examined whether the paranasal sinuses serve as a bacterial reservoir for CF pathogens, especially *P. aeruginosa*, leading to reinfections with the same genotype as the initial infection. We cultured and genotyped historical isolates as well as paired *P. aeruginosa* isolates from sputum and sinuses from children and adults with CF who underwent Functional Endoscopic Sinus Surgery (FESS) to see if bacteria from the two anatomical locations were identical.

2. Materials and methods

2.1. General care

300 CF patients are followed at the Danish CF centre, Copenhagen [8,9] and treated according to fixed guidelines [8].

Intermittent colonisation: Patients with at least one isolate of *P. aeruginosa*, but normal levels of precipitating antibodies against *P. aeruginosa* (0–1 precipitins) [8].

Chronic *P. aeruginosa* lung infection: Growth of *P. aeruginosa* in six consecutive monthly sputum samples, or less if there are two or more precipitating antibodies against *P. aeruginosa* [8].

2.2. Bacteria

Since 1973, we have collected and stored (−80 °C) *P. aeruginosa* sputum isolates sequentially [8,9].

2.3. Included patients

Seventy-eight CF patients >6 years (median 19 years, range 6–50), (29 males, 49 females) were treated with FESS between January 27, 2007 and April 1, 2010.

There are no guidelines for sinus surgery in CF patients [10]. Patients were selected based on one or more of the following criteria: 1) intermittent colonisation with declining lung function despite intensive antibiotic therapy and/or increasing antibodies against Gram-negative bacteria e.g. *P. aeruginosa, Achromobacter xylosoxidans* or *Burkholderia cepacia complex*, 2) lung transplantation within the last year, and 3) severe symptoms of rhinosinusitis according to the European Position Paper guidelines (EPOS) [10]. The majority fulfilled the first and third criterion.

2.4. Functional Endoscopic Sinus Surgery (FESS)

FESS created ventilation and drainage pathways to and from the sinuses, making the paranasal sinuses accessible for irrigations [11].

An average of six tissue or pus samples (range 1–14) was taken from each patient. The FESS was finalised by sinus irrigation applying 3 MIE polymyxin E (colistin).

A sputum sample was obtained on the same day as the surgery to compare bacteriology in the lungs with the sinuses.

After surgery, the majority of patients did nasal irrigations with colistin twice daily for at least 6 months. An ENT specialist performed postoperative follow-up five times during the first year after surgery.

2.5. Bacteriology and typing methods (PFGE)

Gram-stained smears for biofilm detection, and aerobic and anaerobic cultures at 37 °C on standard agar media for 5–7 days, were carried out on all tissue and pus samples [9,12]. PFGE was used for genotyping *P. aeruginosa* isolates from the sinuses and the lungs [9].

2.6. Serum and saliva

Serum and saliva were collected from 25 randomly chosen FESS patients who were either intermittently colonised or chronically infected with *P. aeruginosa*. Saliva was obtained by using four sterile 6 mm diameter paper discs (Antibiotica Testblættchen, Struers, Denmark) that were placed on the mouth mucosa for 30 sec. The saliva-soaked discs were stored at −80 °C until analysed.

2.7. IgG and IgA antibodies against P. aeruginosa in saliva and serum

Specific antibodies were measured using ELISA [13,14]. The volume of all reagents for the serum ELISA was 100 μl, but 50 μl for the saliva ELISA. Phosphate-buffered saline (PBS pH 7.2)+0.1% Tween-20 (Sigma)+NaCl 15 g/l (= dilution buffer) were used for all washing steps and the plates were washed three times.

Saliva-impregnated paper-discs were incubated on a shaker for one hour at 35 °C in 175 μl dilution buffer to elute IgG and IgA antibodies.

Antibodies against alginate, 96-well microtiter plates (Mikro-well, BiotechLine A/S, Denmark) were coated with alginate (10 μg/ml) purified from a mucoid CF *P. aeruginosa* strain (6680NH). The plates were coated over night at 35 °C and blocked for one hour at 35 °C in dilution buffer. Serum was diluted 1:4,000 and saliva was already diluted 1:8 in the elution procedure and used without further dilution for detection of IgA and IgG. After washing, horseradish peroxidase (HRP)-conjugate (Dako A/S, Glostrup, Denmark) detecting IgG and IgA antibodies were added for one hour. The detecting antibodies were rabbit anti-human IgG (γ-chain-specific) diluted 1:10,000 or rabbit anti-human IgA (α-chain-specific) diluted 1:10,000.

Antibodies against *P. aeruginosa* standard antigens (a sonicated cell extract of *P. aeruginosa* serogroups 1–17) were used as standard antigen (protein concentration 16 mg/ml). The antigen was coated onto irrigated 96-well polysterene plates (Maxisorb, BiotechLine A/S, Denmark) at a dilution of 1:2,000. The plates were incubated for one hour at 35 °C and blocked overnight with dilution buffer at 4 °C. Serum was diluted 1:100 and saliva 1:8 for detection of IgG and IgA and allowed to react for one hour at 35 °C. After washing, horseradish peroxidase (HRP)-conjugate diluted 1:20,000 (Dako A/S, Glostrup, Denmark) detecting IgG (P0214) and IgA (P0216) antibodies in serum and saliva were added for one hour. The peroxidase-conjugated second antibodies were as described above.

TMB Plus was added (KemEnTec Diagnostics). The reaction was stopped after one hour by 1 M H_2SO_4. The absorbance was measured at 450 nm on a plate reader (Multiscan EX, Bie & Berntsen, Denmark). The results were expressed as optical density values (OD).

2.8. Ethics

The study was approved by the local ethics committee, Region Hovedstaden, (H-A-141) and all patients gave informed consent.

2.9. Statistics

Within-patients samples of serum and saliva antibodies were compared using the sign test.

3. Results

Thirty-two of the 78 patients were chronically infected; 21 with *P. aeruginosa* (median age 31 years, range 12–50 years), 5 with *A. xylosoxidans*, 4 with *B. multivorans complex* and 2 with *Stenotrophomonas maltophilia*. The remaining 46 patients were intermittently colonised; 31 with *P. aeruginosa* (median age 14 years, range 7–29 years), 6 with *A. xylosoxidans* and 9 with other CF pathogens such as *Haemophilus influenzae*, *Staphylococcus aureus* or *Streptococcus pneumoniae* in their lungs.

Of the 21 patients chronically infected with *P. aeruginosa*, 18 (86%) had simultaneous growth of *P. aeruginosa* in their sinuses and lower airways; this was also the case for all 5 patients with growth of *A. xylosoxidans*, all 4 with growth of *B. multivorans complex*, whereas none of the 2 patients with chronic *S. maltophilia* infection in their lungs had growth of this bacterium in their sinuses.

In 22 of the 31 (71%) intermittently colonised patients, we cultured *P. aeruginosa* from both the sinuses and lungs.

We did not detect any anaerobic microorganisms in any of the sinuses although the cultures were performed less than one hour after the sinus samples were taken and incubation was performed for 7 days.

PFGE patterns were used to compare the relatedness of sequentially collected *P. aeruginosa* lung isolates with *P. aeruginosa* cultured from the lungs and sinuses after FESS. In 18 of the 18 patients (100%) with chronic *P. aeruginosa* lung infection and from whom we had simultaneous growth of *P. aeruginosa* in their sinuses and lungs, *P. aeruginosa* was genetically identical. We found that chronically infected patients had the same *P. aeruginosa* genotype in the lungs for a median of 15 years similar to the *P. aeruginosa* that were cultured in the sinuses (range 1–29 years). We found that 5 of the 18 patients had identical genotypes in their lungs for more than 20 years and this was the same genotype as cultured from the sinuses after FESS.

Twenty of the 22 (91%) intermittently colonised patients with *P. aeruginosa* in their sinuses had PFGE identical isolates in their lungs, whereas a different genotype was found in two patients. Patients who had identical *P. aeruginosa* in their lungs and sinuses at the time of surgery had a similar genotype in their lungs for a median of 3.5 years (range 1–6 years).

Gram-stained smears from the sinuses showed that all patients chronically infected with *P. aeruginosa* had their bacteria organised in biofilm-like structures similar to what is seen in the lungs of the patients [15,16] (Fig. 1 a–f). When microscopically analysing Gram-stained smears from the lung and sinuses obtained on the same day in patients chronically infected with *P. aeruginosa*, we found that bacterial biofilms in the lung were surrounded by a large number of inflammatory cells, predominantly polymorphonuclear cells (PMNs), whereas only very few and scattered PMNs were seen in the surroundings of the sinus biofilms (Fig. 1 b, d, f). In addition, we found that patients with chronic *A. xylosoxidans* and *B. cepacia complex* infection also had their bacteria organised in aggregates in the sinuses [17].

None of the 21 patients chronically infected with *P. aeruginosa* had the bacteria eradicated from the lungs following FESS. In intermittently colonised patients (N=31) the median time from sinus surgery to regrowth of *P. aeruginosa* in sputum was 7.3 months. In patients who had *P. aeruginosa* cultured from their sinuses (N=22), *P. aeruginosa* was cultured again from the lungs 5.3 months after FESS.

Saliva IgA against *P. aeruginosa* sonicate (median optical density (OD) 83) and alginate (median OD 186) was 15 and 39 times higher than serum IgA (median OD 5 in both cases) (p<0.001 in both cases), indicating a local production of IgA in the upper airways. The IgA antibody levels in saliva against *P. aeruginosa* sonicate and alginate were also significantly higher than the saliva IgG production (median OD 2 and 0, respectively) (p<0.001 in both cases). The IgG levels in serum against *P. aeruginosa* sonicate and alginate were also low (median OD 3 in both cases).

4. Discussion

We have performed the largest published study till now of invasive sinus surgery investigating the possible role of the sinuses as a reservoir for bacterial adaptation and repeated colonisation and infection of the lungs [3,18,19]. The group of intermittently colonised patients, which we included in this study, is a selected subgroup among our children population since most of them have been intermittently colonised in their lungs with the

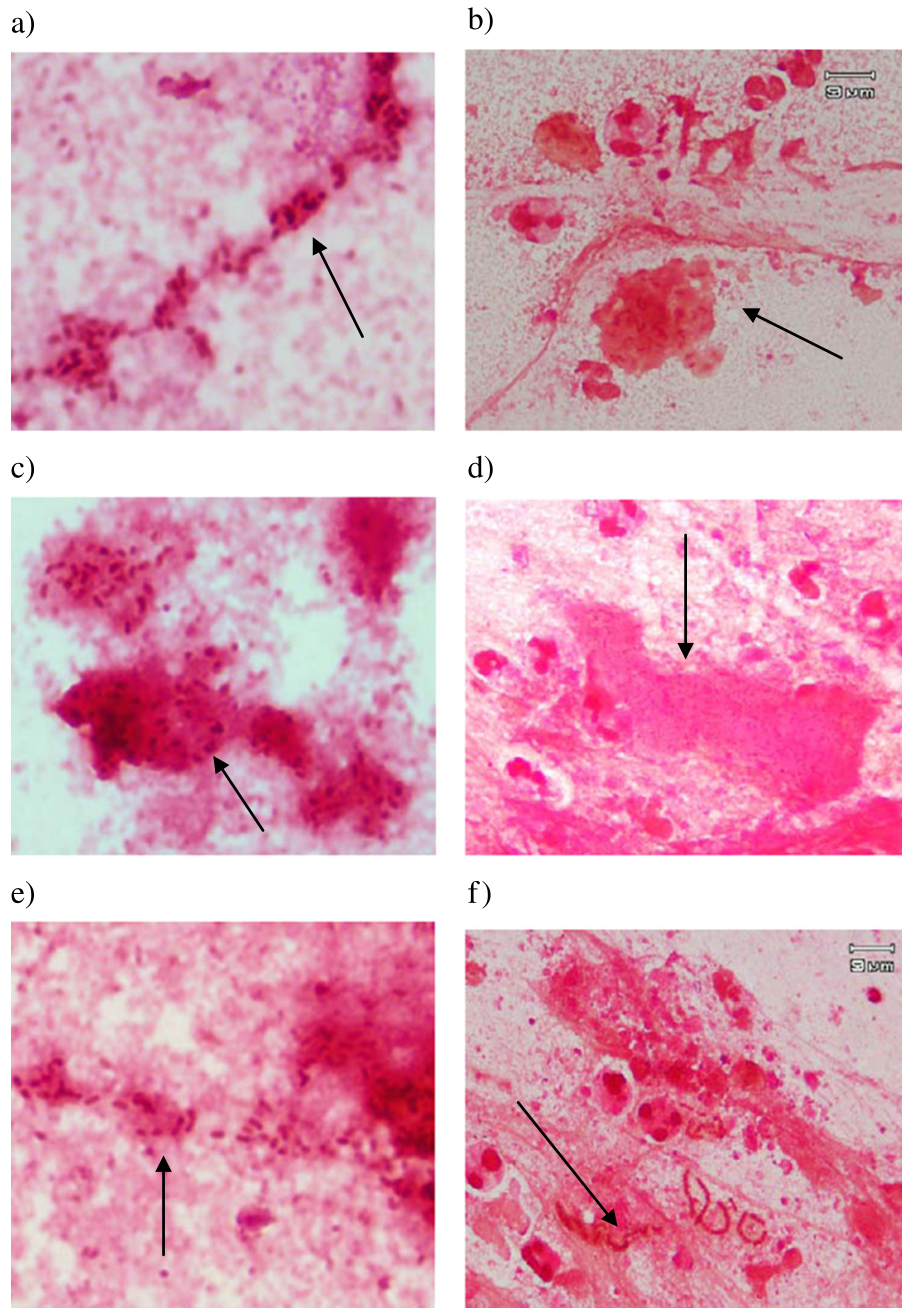

Fig. 1. **a–f**. Microscopic investigation of Gram-stained smears of pus from the sinuses (a, c and e) and corresponding sputum (b, d and f) obtained from three patients chronically infected with *P. aeruginosa* at the time of sinus surgery, magnification ×1000. Arrows indicate biofilms.

same *P. aeruginosa* genotype for more than 3 years. This group constitutes 24% of the overall intermittently colonised children population [20] whereas most of our children have been recolonised with a different genotype after successful eradication [20]. This is in agreement with the data reported by Taccetti et al. [21] and Munck et al. [22], who found that 73% and 74% of their patients had a different *P. aeruginosa* genotype in their lungs when recolonised.

In chronically infected patients we found 100% genetic identity between *P. aeruginosa* in the sinuses and lungs. In intermittently colonised patients, we found concordant bacteriology in the sinuses and lungs and in 91% of these patients we found identical *P. aeruginosa* genotypes. We also found that the same genotype had been colonising the lungs intermittently for up to 6 years prior to the FESS, although the bacteria apparently had been eradicated by antibiotic therapy every time they were cultured from the patients' sputum. This paradox challenges the current definition of intermittent colonisation versus chronic infection. We define chronic *P. aeruginosa* infection as continued presence of the bacteria in the lungs over a period of at least 6 months and/or

serum content of 2 or more precipitating antibodies against *P. aeruginosa*, whereas intermittent colonisation is defined as isolation of *P. aeruginosa* in a patient with no measurable antibodies in the blood (normal: 0–1 precipitins) and no clinical symptoms [8]. The intermittently colonised patients that have had sinus surgery in this article have been colonised with the same genotype for a median of 3.5 years with no measurable antibody responses and therefore need to be classified into a new group of chronically colonised patients. If the same clone of *P. aeruginosa* persists for such long periods of time it should be considered chronic despite the lack of precipitating antibodies. We therefore need to modify our operational definition of chronic infection.

All patients chronically infected with *P. aeruginosa* in their lungs and from whom we cultured *P. aeruginosa* in the sinuses harbored identical clones. A small subgroup of patients who underwent FESS had the same genotype in their sinuses and lungs for more than two decades. This is in accordance with the findings by Mainz et al. [23] who in a cross-sectional study found that 95% of the *P. aeruginosa* isolates from 24 patients had identical SNP-genotypes in both compartments, indicating that the upper airways play a role as a reservoir of *P. aeruginosa* in CF, and by Muhlenbach et al. [24] who demonstrated identical genotypes in the upper and lower airways in 83% of samples obtained from twelve patients.

Our study shows that the adaptation of *P. aeruginosa* to the sinuses is different from the adaptation in the lungs. In the lungs there is a PMN dominated inflammation with release of oxygen radicals [25], probably because the lungs, especially the respiratory zone, contain high levels of IgG against *P. aeruginosa*, which promotes an inflammation dominated by PMNs. In the sinuses, we did not find a similar PMN-dominated inflammation but a high level of IgA compared to IgG, and IgA has non-inflammatory properties and inhibit the PMN recruitment [26]. The immunoglobulin distributions fit well with a recent study by Schraven et al. showing that the number of plasma cells in CF patients with chronic polypoid sinusitis was significantly elevated compared to non-CF patients and that the number of neutrophils were low in both groups [27]. We have previously found that there is a common mucosal secretory IgA response to *P. aeruginosa* in CF patients, whereas the systemic response is dominated by IgG [28]. The secretory IgA response, both to the biofilm matrix-component alginate and to the *P. aeruginosa* sonicate, including proteins and LPS, implies that the *P. aeruginosa* sinusitis is rather silent because of the lack of PMNs compared with the chronic lung infection [16] and that *P. aeruginosa* may adapt to the chronic life-style and be well suited for causing lung infections especially if adaptation and biofilm formation has begun in the sinuses [11,20]. Although we cannot totally exclude that specific subpopulations of bacteria from the lungs are transmitted to the sinuses from time to time our previous results suggest that the direction of migration is mainly downwards at the early stages of infection [20]. None of the intermittently colonised patients had elevated precipitating antibodies against *P. aeruginosa* before surgery, which supports our hypothesis that the bacteria in the sinuses are not being recognised by the systemic immune system (IgG antibodies) but by the mucosal immune system (secretory IgA) [28]. In chronically infected patients we found that *P. aeruginosa* also forms biofilms in the sinuses similar to the biofilms in the lungs. However, there is an important difference. There are only very few PMNs around the biofilm in the sinuses in contrast to the significant amount of PMNs surrounding the biofilms in the lungs and this is, probably due to the non-inflammatory secretory IgA response (Fig. 1a–f) [16].

We have previously demonstrated that many CF patients in our clinic contracted their initial *P. aeruginosa* colonisation and chronic lung infection during the viral season in the winter months (October to March) [29]. When patients have a viral infection, the excessive secretions formed in the upper respiratory tract become liquid, and will easily find its way into the deepest portion of the lungs within minutes [30]. When *P. aeruginosa* containing secretions in the sinuses of CF patients become liquefied, they may easily enter the lungs by aspiration and become difficult to clear. Accumulation of bacteria-laden liquid in the supralaryngeal portion during viral infection may by aspiration overwhelm the mucociliary defense mechanisms of the sub-laryngeal portion, and the secretions are aspirated especially during sleep [30]. When bacteria from the sinuses are aspirated into the lungs they might be pre-adapted and therefore less virulent compared with environmental *P. aeruginosa* isolates, since they have had the opportunity to evolve to the lifestyle in the lungs [20,31]. The sinuses can be seen as an “evolutionary nest” where bacteria are diversifying, develop antibiotic resistance and other phenotypes associated with adaptation to the CF airways in general [20].

In intermittently colonised patients the overall duration until regrowth of *P. aeruginosa* in the sputum after FESS was 7 months whereas the median time to recurrence of *P. aeruginosa* in sputum in patients from whom we cultured *P. aeruginosa* in their sinuses was less than half a year. We believe that the FESS procedure itself cannot keep the children free of *P. aeruginosa* for a prolonged period of time but has to be carried out together with other postoperative treatment such as nasal irrigation with colistin and inhaled intravenous antibiotics. This is in agreement with a recent trial in CF patients assessing 0.9% NaCl versus dornase alpha delivered by vibrating aerosols into the paranasal sinuses [32]. Dornase alpha inhalation was associated with a significantly improved quality of life score. In the future such therapeutic approaches with simultaneously dornase alpha and sinonasal inhalation of antibiotics might benefit patients where FESS is not an option.

There are some limitations in our study that should be considered when interpreting the results. We could not culture any anaerobic microorganisms in the sinuses although this was expected based on our previous findings [11]. It could be due to the intensive antibiotic treatment of these patients or insufficient handling of the samples, although they were cultured within one hour after surgery [8,12]. In addition, we expected to find an even higher number of patients with matching *P. aeruginosa* in their sinuses and lungs, but there might have been too few bacteria in our samples. Molecular based methods may have been appropriate, but this would not have given any information on genotypes.

In conclusion, we have shown that the sinuses are bacterial foci for *P. aeruginosa* in CF patients. In the sinuses, *P. aeruginosa*

grow in biofilms that are similar to the biofilms seen in the lungs of chronically infected patients. In contrast to the situation in the lungs, a high concentration of non-inflammatory secretory IgA in the sinuses probably impedes the PMNs from being recruited, which may prevent local and systemic inflammation and recognition of microorganisms, contributing to adaptation and persistence of *P. aeruginosa* ready to colonise and infect the lungs.

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Competing interests

None.

Financial disclosures

None.

Contributors

HKJ was principal investigator. HKJ contributed to the conception and study design together with KA, NH and CvB. TP, KGN and MS included the patients and KA, JF and CvB did the surgery. HKJ and NH performed the microbiological analyses including genotyping of *P. aeruginosa* strains. HKJ, KA, NH and CvB contributed to the analysis and interpretation of data. HKJ drafted the manuscript and all authors commented on the manuscript prior to submission and approved the submitted version.

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