



PhD thesis

By Kasper Aanæs

Bacterial sinusitis can be a focus for initial lung colonisation and chronic lung infection in patients with cystic fibrosis

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List of publications

- I. [Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response.](#) Johansen HK, **Aanaes K**, Pressler T, Nielsen KG, Fisker J, Skov M, Høiby N, von Buchwald C. J Cyst Fibros. 2012 May 15. [Epub ahead of print]
- II. [Secretory IgA as a diagnostic tool for Pseudomonas aeruginosa respiratory colonization.](#) **Aanaes K**, Johansen HK, Poulsen SS, Pressler T, von Buchwald C, Høiby N. J Cyst Fibros. 2012 Jul 19. [Epub ahead of print]
- III. [The effect of sinus surgery with intensive follow-up on pathogenic bacteria in patients with cystic fibrosis.](#) **Aanaes K**, von Buchwald C, Skov M, Hjuler T, Alanin M, Johansen HK accepted for publication by the American Journal of Rhinology and Allergy 6th September 2012.
- IV. [Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients – can chronic lung infections be postponed?](#) **Aanaes K**, Johansen HK, Skov M, Buchvald F, Hjuler T, Pressler T, Høiby N, Nielsen KG, von Buchwald C. Submitted to Respiration September 2012

List of abbreviations

BAL	Bronchoalveolar lavage
CF	Cystic fibrosis
CIE	Crossed immunoelectrophoresis
CFQ-R	Cystic fibrosis questionnaire-revised
CRS	Chronic rhinosinusitis
CT	Computed tomography scan
ELISA	Enzyme-linked immunosorbent assay
EPOS	European position paper on rhinosinusitis
FESS	Functional endoscopic sinus surgery
HRQOL	Health-related quality of life
Ig	Immunoglobulin
LTX	Lung transplanted
MRI	Magnetic resonance imaging
ORL	Oto-rhino-laryngologist
PFT	Pulmonary function test
PMNs	Polymorphonuclear leukocytes (neutrophil granulocytes)
RSOM-31	Rhinosinusitis outcome measure 31
SN-5	Sinus and nasal quality of life survey
SNOT-22	Sinonasal outcome test -22
St-Ag	Standard antigen

English summary

A major purpose of treating patients with cystic fibrosis (CF) is to prevent or delay chronic lung infections with CF-pathogenic Gram-negative bacteria. In the intermittent stage, bacteria can usually be eradicated from the lungs with antibiotics, but following eradication, the next lung colonisations often occur with bacteria of identical genotype (I). This may be due to re-colonisation from the patient's paranasal sinuses. In our study, we found that approximately two-thirds of CF patients having sinus surgery (FESS) had growth of CF-lung-pathogenic Gram-negative bacteria in their sinuses (*Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex) (IV).

The environment in the sinuses is in many ways similar to that of the lower respiratory tract, e.g. low oxygen concentration in secretions. Sinus bacteria are more difficult to eradicate than in the lungs, thus, having good conditions for adapting to the environment in the lungs. In the presence of bacteria, the environment of the sinuses differs from that of the lower respiratory tract by having a higher immunoglobulin A (IgA): IgG ratio, and reduced inflammation (I, II). We found a significant correlation between the concentration of IgA against *P. aeruginosa* (standard antigen and alginate) in nasal secretions and saliva and CF patients' infection status (not lung colonised, intermittently colonised or chronically lung-infected with *P. aeruginosa*) (II). This supports the hypothesis that infections often originate in the sinuses and can be a focus for initial lung colonisation or for maintaining lung infections in CF patients. We are confident that anti-*P. aeruginosa* IgA can be used as an early supplementary tool to diagnose *P. aeruginosa* colonisation; *P. aeruginosa* being the microorganism causing most morbidity and mortality in CF patients. This is important since urgent treatment reduces morbidity when CF

patients are early colonised with *P. aeruginosa*, however, there is a lack of diagnostic tools for detecting the early colonisation in the lungs and in the sinuses.

We initiated a treatment strategy for CF patients to prevent sino-nasal bacteria being seeded into the lower airways: we recommended extensive functional endoscopic FESS with creation of sufficient drainage from all involved sinuses with subsequent IV antibiotics and at least 6 months of twice daily nasal irrigation with saline and antibiotics. By this strategy, sinus bacteria could be eradicated in a large proportion of patients (III). Essentially, growth of CF-pathogenic bacteria from the lower respiratory tract was decreased following the treatment. Furthermore, a number of patients have been free from CF-pathogenic bacteria for more than one year after FESS, and thus re-classified as "not lung colonised". We also corroborated that CF patients obtain an improved quality of life and reduction in their symptoms of chronic rhinosinusitis after FESS (IV).

It is primarily intermittently lung colonised CF patients with CF-pathogenic bacteria in their sinuses that seem to benefit from the treatment strategy. This is in accordance with the fact that we did not see a significant increase in lung function and only a small decrease in specific antibodies after FESS; a high systemic immune and inflammatory response and a decreasing lung function is generally not present in patients who primarily have sinus CF-pathogenic bacteria.

It is important that guidelines are created for how CF patients with CF-pathogenic bacteria in the sinuses are to be treated, including criteria for who may likely benefit from FESS, and who may be treated exclusively with conservative therapy, e.g. saline and antibiotic irrigations.

Danish summary / Dansk resumé

Det vigtigste fokus-område inden for behandlingen af patienter med cystisk fibrose (CF), er at prøve at forhindre eller udskyde tidspunktet for en kronisk lungeinfektion med CF-patogene Gram-negative bakterier.

De første gange bakterierne dyrkes fra lungerne, kan de som regel udryddes med antibiotika-terapi, men efterfølgende sker de næste koloniseringer ofte med bakterier af samme genotype. Dette kan skyldes, at patienterne re-koloniseres fra egne bihuler, hvor bakterierne er vanskeligere at bekæmpe end i lungerne. Vi fandt, at cirka to-tredjedele af de CF patienter vi har bihule-opereret (FESS), havde CF-patogene Gram-negative bakterier i bihulerne (*Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex).

Miljøet i bihulerne minder om det, der findes i de nedre luftveje med f.eks. lav ilt-koncentration i sekretet, hvorved bakterierne kan adaptere til miljøet i lungerne. Når der er bakterier tilstede i bihulerne, ses en højere ratio af immunoglobulin A (IgA): IgG antistoffer og mindre inflammation end i lungerne. Vi fandt en signifikant sammenhæng mellem koncentrationen af IgA mod *P. aeruginosa* (standard antigen og alginate) i næsesekret og i spyt, med CF patienternes lunge-infektions-status (aldrig lunge-koloniseret, intermitterende koloniseret eller kronisk inficeret med *P. aeruginosa*). Disse fund støtter hypotesen om, at de alvorlige infektioner ofte starter i bihulerne, og kan være fokus for den initiale eller vedvarende lunge-kolonisering af CF patienter.

Vi er overbeviste om, at vores nye metode til at bestemme koncentrationen af anti-*P. aeruginosa*-IgA i fremtiden vil kunne bruges som en del af udredningen af de tidlige bihule og lunge *P. aeruginosa*-kolonisering. Dette vil have stor betydning, da tidlig

behandling af *P. aeruginosa* koloniseringer forbedrer morbiditeten, men er svær, da der mangler metoder til at diagnosticere tidlige *P. aeruginosa* kolonisationer i næse og lunger.

Vi har iværksat et behandlingsprogram for CF-patienter, hvis formål er at modvirke at bakterierne fra bihulerne bliver transporteret ned til lungerne og giver infektioner: vi anbefaler en ekstensiv FESS, hvor målet er at lave et godt afløb fra alle bihuler, efterfulgt af intravenøs antibiotika, samt nasal skylning med saltvand og antibiotika. Med denne behandling har vi vist, at en stor del af patienterne kan få fjernet bakterier fra deres bihuler. Vi fandt også en reduktion i frekvensen af sekret-prøver fra de nedre luftveje, som indeholdt bakterier der er farlige for CF patienter, og lige så vigtigt, blev flere patienter fri for de CF-patogene bakterier i mere end et år efter operationen, og kunne dermed re-kategoriseres som "ikke lunge-koloniseret". Ved den nævnte behandling opnåede patienterne også en øget livskvalitet med færre symptomer på kronisk bihulebetændelse.

Det var primært CF patienter, som er tidligt koloniseret i deres lunger, og samtidig har CF-patogene bakterier i bihulerne, der så ud til at have gavn af behandlingen. Dette passer med, at vi ikke så en forbedring af lungefunktionen eller et stort fald i antistofferne efter FESS; de patienter, der primært har infektionen i øvre luftveje, har generelt ikke dannet et systemisk immunrespons og fået fald i lungefunktionen.

Vi finder det vigtigt at der, i nær fremtid, bliver lavet retningslinier for hvilke patienter med formodet CF-patogene bakterier i bihulerne, der skal tilbydes FESS, hvem der ikke vil have gavn af det, og hvem der måske kan klare sig med konservativ behandling, som f.eks. næseskylning og antibiotisk behandling.

1. Introduction

There has been little focus on the paranasal sinuses in patients with cystic fibrosis (CF). However, our multidisciplinary group has contributed to creating awareness about this important issue during recent years. Due to brevity, I have chosen to base this PhD thesis on four studies, which all have been submitted to or accepted by peer-reviewed journals. There is a common thread running through all four papers combining basic, paraclinical and clinical research. The four papers are enclosed at the end of this thesis. Other related CF projects that I have participated in will be discussed or cited when appropriate.

1.1 Aims of thesis

I have focused on elucidating whether the paranasal sinuses can be a focus for initial lung colonisations and whether it is plausible that they also may serve as a focus for re-infections in CF-patients. In detail, I will:

- a) Discuss the prevalence of bacteria found in the CF sinuses (I, II, III, IV).
- b) Put the sinus mucosal inflammation into perspective (I, II).
- c) Describe a potential new method of diagnosing CF pathogen sinusitis; this will be a platform to discuss how CF sinusitis may be identified and treated (II, III).
- d) Put forward a treatment strategy for CF patients with pathogen sinusitis and discuss pros and cons for different treatments of CF sinuses (III, IV).
- e) Present and discuss results on how our treatment addressing the sinuses influences lung colonisations and re-infections and relate these to further studies (III, IV).

2. Background

2.1 Cystic fibrosis (CF)

CF is a severe recessive genetic disease, which is common among the Caucasian population. In Denmark, the incidence is 1:4,700 (1); the Faroe Islands having one of the highest incidences in the world. Currently, approximately 450 patients with CF are living in Denmark; one-third of the patients are followed at the Cystic Fibrosis Centre in Århus while two-thirds are followed at the Cystic Fibrosis Centre in Copenhagen Rigshospitalet.

The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) located on chromosome 7 (2). The gene encodes the cAMP-dependent chloride channel, and as a consequence of the defect, abnormal transport of chloride and sodium across the cell epithelium is seen. Thus, all secretions contain a higher concentration of salt, which more than doubles the viscosity compared with non-CF individuals (3).

The main clinical characteristics of CF are increased salt loss in sweat, malabsorption, diabetes, male infertility, chronic rhinosinusitis and increased fungal and viral airway infections; most severe is the increased susceptibility to bacterial infections of the lower airways.

2.2 Lower airways

Due to the viscous secretions, the mucocilliary clearance of inhaled microbes is impaired making CF patients very susceptible to lower-airway infections (4;5). From

early childhood, the infections are mostly caused by *Haemophilus influenzae* and *Staphylococcus aureus*. When older, the CF-pathogenic Gram-negative bacteria *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex and especially *Pseudomonas aeruginosa* are more frequently seen. When reaching adulthood, the vast majority of patients have been colonised or infected with one or more of the three mentioned CF-pathogenic Gram-negative bacteria responsible for most of the morbidity and mortality in CF (6).

The initial stage of “never been lung colonised” with CF-pathogenic Gram-negative bacteria is often replaced by a stage of intermittent colonisation before entering the final stage of chronic infection. In spite of a frequently and regularly intensive antibiotic treatment (7), the bacteria are presumably constantly present in some pulmonary segments when chronically infected. The chronic stage is paraclinically characterised by constantly high serum levels of immunoglobulin G (IgG) antibodies and numerous polymorphonuclear leukocytes (PMNs) in the lower airways. Elevated serum levels of specific antibodies are also seen and are used as a supplementary diagnostic tool (mentioned in 2.2.1 and 2.5.1). Likewise, increased numbers of PMNs are strongly correlated to poor lung function; the imbalance between PMN-proteinases and their inhibitors leads to impaired phagocytosis, T-cell and B-cell imbalance, and lung tissue damage (8). Thus, the onset age of chronic lung infection with *P.aeruginosa* is correlated with the life expectancy in CF patients (9).

Clinically, CF-patients with chronic bacterial lung infections tend to have lower quality of life, lower body mass index (BMI) and declining lung function measured by FEV₁(forced expiratory volume in 1 sec. %-predicted) and FVC (forced vital capacity %-predicted). The major purposes of treating patients with CF are to prevent or delay chronic lung infections and keep the lung function at a steady state. This goal is difficult to achieve, consequently, CF is the second largest group of lung-transplanted recipients in Europe (10).

2.2.1 Grading of pulmonary infection

Years ago, there was no international consensus about the consequences of Gram-negative chronic lung infections for the progression and prognosis of CF lung disease. By an epidemiological study of the respiratory tract microbiology, the definition of different infection categories were introduced (11). It was shown that high serum levels of precipitating antibodies against *P.aeruginosa* was characteristic in chronically infected patients and in patients harbouring mucoid strains (12), and that high and rapidly increasing levels of antibodies correlated with poor prognosis (9). The antibody response was shown to have high sensitivity and specificity for the early detection of chronic *P.aeruginosa* lung infection and was included in the following definition of chronic infection: persistent presence of bacteria in six consecutive months, or less when combined with the presence of elevated precipitating antibodies (9;13).

Since 1974, our centre has used the Copenhagen criteria (9;12), which grades pulmonary infection into three categories based on having 10–12 lower airway samples

cultured a year: 1) never colonised, 2) intermittently colonised, and 3) chronically infected. These criteria cannot be applied in most centres, because the patients are not seen as regularly as in Copenhagen and as only a few centres have access to the antibody tests. Consequently, the Leeds criteria were developed (14) and were shown to correlate well with the Copenhagen criteria (15-17). The advantage of using the Copenhagen criteria when patients are seen on a monthly basis is that they allow an earlier initiation of eradication or maintenance therapy, which improves lung function in both intermittently colonised and chronically infected CF patients (16;18).

Based on the fact that most CF centres only see their patients every third month, the following Leeds criteria are used:

1. Never infected: there has never been growth of any CF related Gram-negative bacteria.
2. Non-infected: no growth of any CF related Gram-negative bacteria over 12 months.
3. Intermittent colonisation: growth in >0% and ≤ 50% of samples.
4. Chronic infection: growth in >50% of a patient's monthly lower-airway samples.

2.3 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod-shaped bacterium frequently found in soil, water and man-made environments (e.g. water pipes). It is an opportunistic pathogen of immune-compromised individuals. It thrives not only in normal atmospheres, but can adjust to hypoxic conditions as in the sputum and sinus secretions of CF patients (19;20).

Partly induced by oxygen radicals from the PMNs, some *P. aeruginosa* mutate during the initial colonisation making them more suitable for a chronic infection. The most important bacterial gene is *mucA*, which causes *P. aeruginosa* transition from a non-mucoid to a mucoid-phenotype producing alginate and biofilms. Other important mutations or changes of phenotypes cause: down-regulation of the cell-to-cell communication (quorum-sensing; *las* and *rhl* genes); increase antibiotic resistance; change colony morphology; reduce swimming, swarming and twitching motility; growth advantages; modify immune system tolerance; and increased protease production (21;22).

Lung infections with *P. aeruginosa* cause inflammation resulting in a systemic increase of IgG antibodies against polyvalent *P. aeruginosa* antigen (Standard Antigen (St-Ag)) (23) and the mucoid exopolysaccharide alginate (a biofilm-matrix component), which are highly characteristic of *P. aeruginosa* (24;25). In addition to serum, specific antibodies are present in tears and in the upper airways as saliva and sputum; IgA being the dominant antibody at mucosal surfaces (25;26). Prior to our studies (I, II), research on IgA in nasal secretions from CF patients has, to our knowledge, never been investigated.

2.4 *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex

In CF, most research has been done on *P. aeruginosa*, being the bacteria causing the majority of chronic infections (Figure 1). *A. xylosoxidans* and *B. cepacia* complex are less prevalent but have similar negative impact on pulmonary disease progression. They are expected to have similar adaptive mechanisms as *P. aeruginosa* causing similar inflammation and lung destruction (27). These bacteria are also Gram-negative rods. In

contrast to *P. aeruginosa*, *B. cepacia* complex is resistant to colistimethate sodium; *P.*

aeruginosa is seldom pan-resistant and can often be treated with oral antibiotics, which is in contrast to *A. xylosoxidans*, which is difficult to treat with oral antibiotics and rapidly develops multi-resistance (28;29). Patients can be infected with more than one Gram-negative bacteria but the outcome of bacterium-bacterium interactions are unknown.

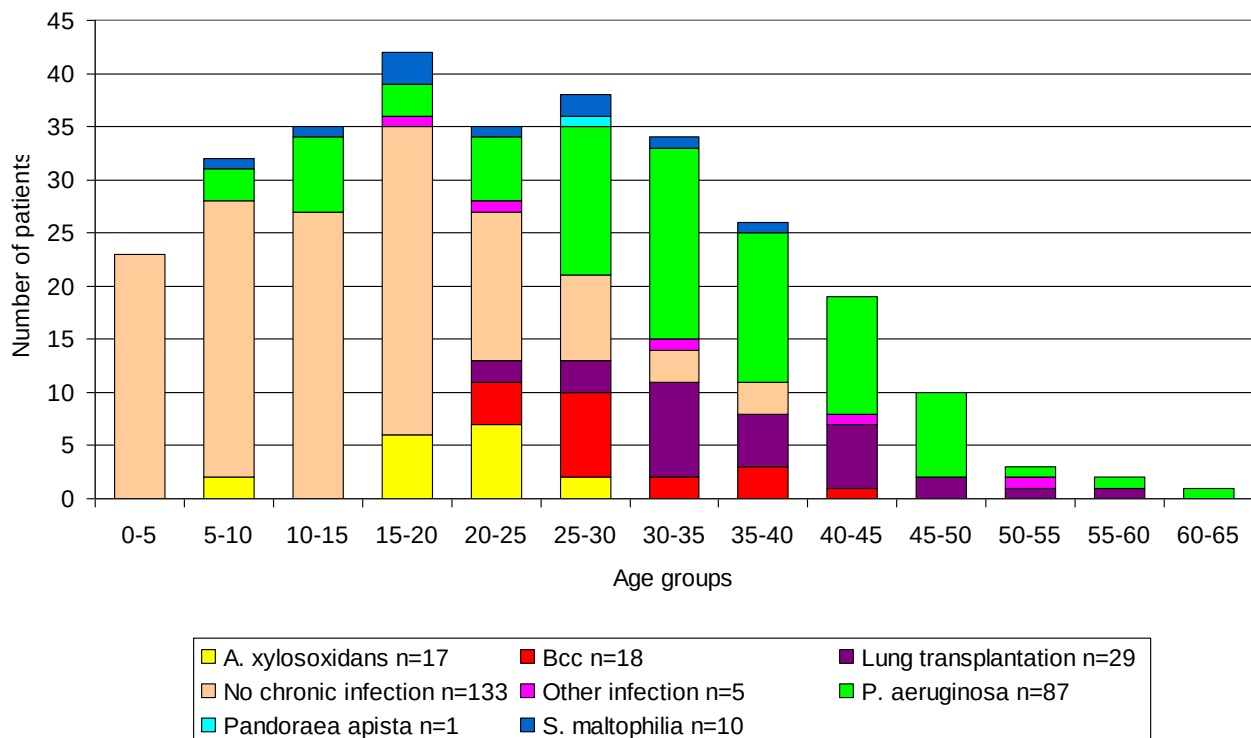


Figure 1: Bacteria causing chronic lung infection in CF patients at the Copenhagen CF Centre in 2008 (by permission from Christine Rønne Hansen).

2.5 Detection of CF-pathogenic Gram-negative bacteria

Early aggressive antibiotic treatment of the first *P. aeruginosa* colonisation is crucial in order to prevent or postpone chronic lung infections and is also cost-beneficial (7;30). Thus, this fact has increased the necessity of rapid and sensitive detection techniques (31;32). Serum antibody titres against alkaline protease, elastase and exotoxin A

are on average low when *P. aeruginosa* is isolated from the respiratory tract for the first time (33) and early diagnosis is challenging (34). In our clinic, specific IgG and precipitating serum antibodies are used as a supplementary tool for monitoring lung colonisations and infections. Clinical and paraclinical outcomes, e.g. pulmonary function tests, are also used in the detection of pulmonary bacteria. Culturing lower airway samples is the one of the most important tools in the detection. These samples are obtained by coughed sputum, endolaryngeal suction in non-sputum producers, induced sputum that increases the recovery rate of *P. aeruginosa* (35), or by bronchoalveolar lavage (BAL) having a lower degree of upper respiratory tract contamination (36). Detection of CF-pathogenic bacteria from the upper airways is discussed below.

2.5.1 Immune responses

Elevated levels of specific anti-Pseudomonas IgG antibodies, measured by enzyme-linked immunosorbent assay (ELISA), is a risk-indicator for developing chronic *P. aeruginosa* infection (37). Precipitating antibodies measured by crossed immunoelectrophoresis (CIE) is used as a supplementary tool for diagnosing and predicting the outcome of lung infections (38). Precipitating antibodies remain within the normal range (0–1) in most cases during intermittent lung colonisation but rise during chronic infection. The antibody response has previously been shown to be helpful in distinguishing between intermittently colonised and chronically infected patients using the Copenhagen criteria mentioned in 2.2.1 (13;37;38).

2.6 Upper airways

2.6.1 Sinus anatomy

The paranasal sinuses are a group of air-filled-spaces: the maxillary sinuses surrounding the nasal cavity, the frontal sinuses placed in the forehead above the eyes, the ethmoidal sinuses are many small sinuses between the orbits, and the sphenoid sinuses are deep and posterior to the ethmoids. The sinuses are lined by mucosa and produce mucus. The drainage pathways are shown in Figure 2. Unexplained, CF sino-nasal anatomy very often divagates from non-CF patients. Common findings are nasal congestion, polyposis, mucocoeles, mucopurulent material, medial bulging of the maxillary walls, otitis and hypoplasia or aplasia of the paranasal sinuses especially of the frontal sinus (39-42).

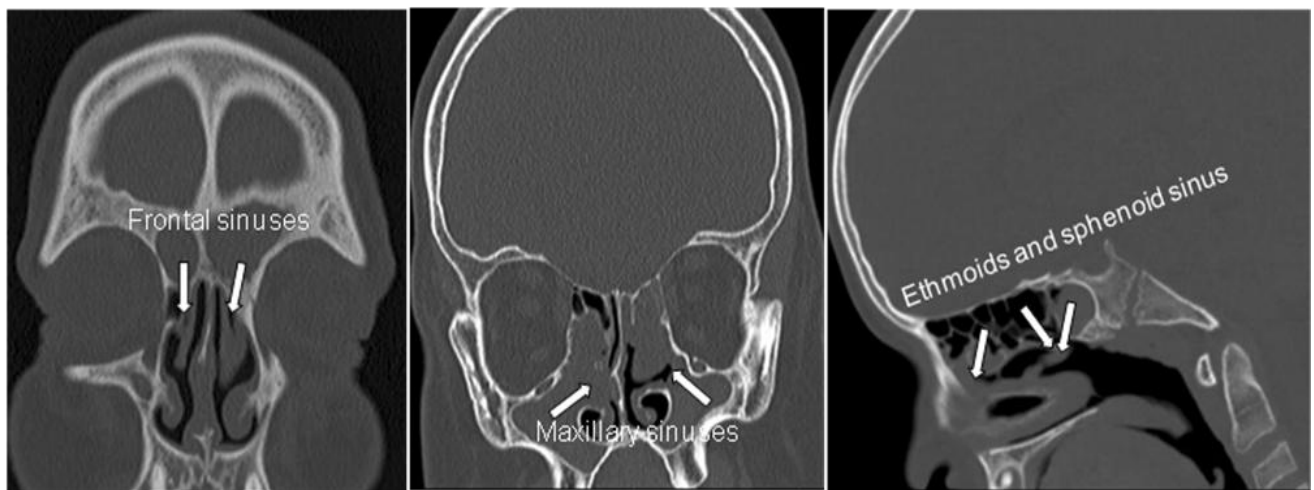


Figure 2: The arrows show the supposed drainage pathways of the paranasal sinuses. In these CF patients the drainage seems to be occluded and the sinuses are partly opacified.

2.6.2 Chronic rhinosinusitis

The hallmark of CF in the head and neck region is chronic rhinosinusitis (CRS) and nasal polyps. There is no specific definition on CRS in CF patients, so they follow the general definition stated in the European position paper on rhinosinusitis (EPOS) (43) shown in Table 1. However, nasal and sinus mucosal disease is by definition present in patients with CF because of defective CFTR-channels in the sinonasal mucosa, as found in the lower CF airways (43). The inflamed tissue and viscous mucus results in a mechanical obstruction of the sinus ostia (42;44). Further, the vast majority of CF patients have radiologic evidence of sinus disease (39;42;45-50), and nasal polyposis becomes more common with age that has been reported in varying prevalence with up to 50% of all CF patients (45;49;51;52). There are inconsistent results on whether CF patients with nasal polyps and symptoms of CRS can be correlated with a better lung function (53-56).

CF patients are likely to under-report their symptoms of CRS, giving a false low share of CF patients with CRS by the definitions in Table 1; approximately two-thirds of all CF patients have impaired olfactory function (57), and 81–86 % of CF patients fulfil the EPOS criteria for CRS ((58) (unpublished material by Berkhout *et al.*), which is in contrast to the low 10–15% who complain about CRS without specific questioning (42;45;59-61). It is unknown whether the CF patients who do not complain about CRS always were asymptomatic, if they have adapted to their symptoms, or if their CRS symptoms are overshadowed by more troublesome symptoms from e.g. the lungs (42).

In general, non-CF patients with nasal polyposis being otherwise healthy have been shown to score worse on quality of life than patients with chronic obstructive pulmonary disease and patients with coronary artery disease (62;63). This ought to give food for thought as to why symptoms of CRS should not be neglected in CF patients. RSOM-31 and SNOT-22 (62;64) are both questionnaires that are recommended outcome tools for adult CRS and can easily be used, while the SN-5 questionnaire (65) is recommended for paediatric CRS (43).

Table 1: CRS definition in non-CF patients adapted from the EPOS (43)	
• Inflammation of the nose and the paranasal sinuses with two or more symptoms for ≥ 12 weeks;	
one should be	
	• nasal blockage
	• obstruction
	• congestion
	• nasal discharge (anterior/posterior nasal drip)
others	
	• facial pain/pressure
	• reduction of smell
Furthermore, demonstrable disease; at least one of following:	
	• nasal polyps
	• mucopurulent discharge
	• oedema/mucosal obstruction
	• CT changes
	• mucosal changes within the ostiomeatal complex and/or sinuses

2.6.3 Bacteriology of the upper airways

In non-CF patients, the paranasal sinuses are regarded as sterile though they may be frequently and transiently contaminated by bacteria from neighbouring surfaces (66). CRS in otherwise healthy individuals predominantly have virus as a part of the aetiology. When bacteria are involved, the following species are most frequently cultured: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* gr.A, *Haemophilus influenzae* and *Moraxella catarrhalis* (67;68). In CF patients, the picture is somewhat different. Sinus bacteria are more frequently present, *P. aeruginosa* being the most common as in the lungs. Other frequently found bacteria are *S. aureus*, *H. influenzae* and coagulase-negative staphylococci; anaerobes and other bacteria found in the lower airways such as *A. xylosoxidans* and *B. cepacia* complex are also found in the CF sinuses (41;50;68-74). Presence of sinus bacteria is reported in 44–95% (60;71;74). Two articles have described fungal sinusitis among North American CF patients but disagree on the prevalence (0–33%) (72;74).

Though it has not been addressed, it is likely that the sinus bacteria in CF patients also produce biofilms that further increase antibiotic resistance in the same way as in the non-CF patients (21;68;75;76). As in the lungs, the sinus bacteria develop phenotypes that are resistant to the host immune response and antibiotic treatment.

2.7 United airways in CF patients

A marked association exists between upper and lower airway cultures in patients with CF (21;43;69;73;74;77-85) due to the paranasal sinuses often being colonised with concordant CF-lung-pathogenic Gram-negative bacteria of the same genotype (21;50;77). Varying predictive values of CF-pathogenic bacteria in the upper airways have been reported when diagnosing lower airway pathogens (60;69;73;82).

A CF patients' initial lung colonisation with *P. aeruginosa* reflects the great diversity of genotypes in the environment, being in contrast to some patients that, after antibiotic eradication, subsequently are re-colonised with bacteria that are clonally related (69;86-88). This indicates that the initial bacteria come from environmental sources rather from transmission between patients (89) and an existence of a bacterial reservoir in the patients' close environment after the initial colonisation. This reservoir is likely to be the sinuses where the bacteria can drain/migrate/be aspirated to the lower airways as seen with viruses (21;90).

The environment of the sinuses and the lower airways are similar in many ways (19;20;43), thus the sinuses may be colonised with bacteria before the lungs and be an evolutionary 'nest' in early airway colonisations, where the bacteria are diversifying, evolving antibiotic resistance and other phenotypes associated with adaptation to the CF airways in general; from there, the bacteria intermittently migrate and colonise the lungs and may ultimately cause chronic lung infections (20;21;69;74;77). When bacteria colonise the lungs they are then pre-adapted to the environment and are therefore less virulent and

more resistant compared with environmental *P. aeruginosa* isolates (21). This also accounts for lung transplanted (LTX) CF patients, where molecular epidemiology studies have shown that CF lung-transplant recipients become re-colonised in their lung grafts with the same bacterial clones as those cultured before transplantation (85).

One study has shown that CF-lung-pathogenic bacteria potentially can be eradicated from the sinuses with extensive functional endonasal endoscopic sinus surgery (FESS) and postoperative local antibiotic treatment (91). Nevertheless, large-scale prospective studies investigating the effects of FESS on lung colonisation and infection in CF are lacking (43), and data on surgical therapy for CF patients with CRS is primarily based on level III evidence (Table 2) (43;92).

Table 2: Category of evidence (93)	
Ia	Evidence from meta-analysis of randomised controlled trials.
Ib	Evidence from at least one randomised controlled trial.
IIa	Evidence from at least one controlled study without randomisation.
IIb	Evidence from at least one other type of quasi-experimental study.
III	Evidence from non-experimental descriptive studies, such as comparative studies, correlation studies and case control studies.
IV	Evidence from expert committees' reports or opinions and/or clinical experience of respected authorities.

Several studies have described the effect FESS on the lower airways using various parameters and treatment modalities, thus showing inconsistent results (Table 3). One prospective study has performed extensive FESS intending to eradicate sinus bacteria in a group of 82 LTX patients, which is described in three papers (91;94;95), showing that *P. aeruginosa* and *A. xylosoxidans* could be eradicated from the sinuses resulting in reduced lung allograft infections. Shatz (96) found decreased antibiotic use, a lower hospitalisation rate and an increase in FEV₁ six months after FESS among 15 CF non-LTX patients. Lewiston *et al.* (97) postoperatively installed tobramycin directly into the sinuses and reported a lower rate of *P. aeruginosa* in the lungs among 11 LTX patients. The other retrospective studies were all based on moderate sinus surgery, and did not focus on lung infection status or a protocol for postoperative treatment. These studies found inconsistent postoperative reduction in lung colonisation, lower hospitalisation rates, reduced use of antibiotics and improvement of the pulmonary function tests (PFT). Table 3 shows an overview of the published papers on sinus surgery in relation to the lungs; none of the studies are level I evidence (Table 2).

Table 2: Review of studies correlating sinus surgery to lower airway conditions.

Authors	No. of CF patients	Study design	Extend of FESS	Post-operative treatment	Outcome
Holzmann et al. (91;94;95)	82 LTX	Prospective	Fronto-spheno-ethmoidectomy and maxillary antrostomy	Nebulized colistin and irrigations; IV AB	Decrease in colonisation of the lower airways
Jarret et al. (98)	17 Non-LTX	Retrospective	Ethmoidal and maxillary sinuses	Nasal saline irrigation; oral AB	PFT and BMI – non-significant
Leung et al. (99)	87 LTX	Retrospective, case-control	Ethmoidal and maxillary sinuses	ND	Lung re-colonisation– no significant.
Lewiston et al. (97)	11 LTX	Retrospective	Ethmoidal and maxillary sinuses	Tobramycin in the sinuses	Low hospitalization rate after surgery. Reduced PA in the lungs
Madonna et al. (100)	14 Non-LTX	Retrospective	Ethmoidal and maxillary sinuses	IV AB	PFT – non-significant
Osborn et al. (101)	41 Non-LTX	Retrospective	Ethmoidal and maxillary sinuses	ND	Sparse improvement in FVC non in FEV1. No effect on microbes
Rosbe et al. (102)	66 Mixed	Retrospective	ND	Some had IV AB	Decrease in IHD. Steroid use and LFT –non-significant
Triglia et al. (103)	27 Non-LTX	Retrospective	ND	ND	Decrease in AB treatment, non in LFT
Umetsu et al. (104)	4 (ND)	Prospective	Ethmoidal and maxillary sinuses ; AB flushing	IV AB	IHD reduced postoperatively, non in PFT
Kempainen et al. (105)	32 Mixed	Retrospective	Fronto-spheno-ethmoidectomy and maxillary antrostomy	ND	PFT and IHD –non-significant
Shatz A (96)	15 Non-LTX	Retrospective	Frontal, ethmoidal and maxillary sinuses	Nasal irrigations	Decrease in AB and IHD. Increase in FEV1 after six months
Halvorson et al. (106)	8 Non-LTX	Retrospective, case-control	Ethmoidal and maxillary sinuses	ND	Increase exercise tolerance/ increased PFT after 3 months
Kovell et al (107)	21 Non-LTX	Retrospective, case-control	ND	ND	Increase in PFT

LTX: Lung transplanted; PFT: Pulmonary Function Test; IHD: In-hospital-days; AB: antibiotics; BMI: Body Mass

Index; FEV1: Forced expiratory volume in one sec.; FVC: Forced Vital Capacity; ND: not described

2.8 Assessment of the upper CF airways

Imaging: The radiation dose of one CT scan of the paranasal sinuses is now reduced to only 0.5–1.0 mSv (in comparison, the Danish annual radiation dose varies from 2–20 mSv). Imaging of the sinuses is mandatory for planning surgical interventions but should not be performed abundantly, thus, CT scans have a low diagnostic value in CF patients (50;59;108). Magnetic resonance imaging (MRI) allows better differentiation of mucosa, polyps and retained secretions but does not display osseous structures bordering the orbit and brain (47;109). Imaging is mandatory prior to FESS due to the altered and varying anatomy of the sinuses with relation to the orbit, brain and major vessels (2.6.1). The capacity for doing MRI is restricted at our institution, which is why solely CT scans are used.

Culture: Although sinus aspiration is the gold standard for the diagnosis of bacterial sinusitis, it is an invasive, time-consuming and potentially painful procedure (66;110). The diagnostic accuracy of oropharyngeal swab cultures is low in predicting *P. aeruginosa* sinusitis, particularly at younger ages (positive and negative predictive values: 73% and 72 %) (60). Cultures of endoscopically collected middle meatus secretions is reported as effective in identifying microorganisms in non-CF CRS patients and in CF patients (111). However, nasal irrigations are also suggested as a preferable technique over nasal swabs to obtain samples from the upper airways in CF patients (69).

Steroids: A Cochrane review states that oral corticosteroids appear to slow progression of lung disease in CF (112). However, no research is published on oral steroids' effect on CRS symptoms in CF patients, while one study recommends intrapolymp steroid injection (113). Another Cochrane review states that: "Overall, there is no clear evidence for using topical steroids in people with CF and nasal polyposis." This is due to the neutrophilic domination in CF polyposis compared with the eosinophil domination in non-CF patients (114). However, it should be mentioned that some studies report a positive effect of using nasal steroids on CRS and nasal polyps in CF (115-117).

Surgery: In 2006, a Cochrane review concluded that more randomize controlled trials comparing FESS with other treatments were required, thus it could not be confirmed that CF patients with CRS symptoms could benefit from FESS (92). The newest edition of EPOS 2012 and other recent studies state that symptoms of nasal airway obstruction, nasal discharge, facial pain, snoring, olfactory dysfunction, frequency of sinus infections and activity level are parameters that can significantly be improved after FESS in CF patients (43;106;118-123). When evaluating the different studies, it is important to note the criteria for FESS and what FESS and postoperative treatment comprise. In spite of postoperative instrumental debridement and saline irrigations (120;124), it is accepted that the effect of FESS on CRS symptoms, in general, last a shorter time in CF patients than in non-CF patients (54;103;106;120;121;123), which is why a more extensive approach has been suggested (81;125-129) combined with antibiotic sinus irrigations (70). As in any

other surgery, FESS involves risks. Though they are rare, situations where the optic nerve or brain is damaged and extensive bleeding can occur. Nevertheless, when surgeons are aware of the altered anatomy in CF patients (described in 2.6.1), reports show that FESS is well tolerated and that the complication rate in CF patients is similar to that of the non-CF population (43;103;130).

Local treatment: Nasal saline irrigations are well tolerated and the beneficial effect appears to outweigh the minor side effects, thus they can be included as a treatment adjunct for the symptoms of CRS in CF-patients (43;131). Hypertonic saline 7% may have mucolytic effects and improve mucociliary clearance in the sinuses as seen in the lungs of CF patients and may be used for nasal irrigations (132-134). Baby shampoo is also introduced as a supplement to the saline (135;136), as is nasal inhalation of dornase alfa used in the treatment of CRS in CF patients (137-139). Studies of nasal irrigations with antibiotics (tobramycin or aminoglycosides) decrease bacterial colonisation and nasal inflammation and show a positive effect on recurrence rate of CRS in non-CF patients (140). However, there is low-level evidence for the use of topical anti-bacterials in CF patients (43;141). Several devices including nebulizers have been developed for nasal irrigations and distribution of medicine (138;142), which are all better than delivering it by nasal spray (43). Finally, low-frequency ultrasound has recently been suggested as a supplementary method for biofilm disruption in patients with CRS (143).

3. Material

3.1 Study population

In all studies in this thesis (I–IV), patients were recruited among the 300 CF patients treated at the CF Centre in Copenhagen. The diagnosis of CF was based on clinical characteristics, abnormal sweat electrolytes, and genotype. CF patients followed a routine protocol with monthly medical examinations including lung function tests and lower airway samples taken for microbiological culture. Additional lower airway samples were taken whenever patients were hospitalised or when clinical and/or paraclinical parameters indicated a risk of lung colonisation or infection. Approximately every third month, blood samples were taken for analyses including specific antibodies against relevant Gram-negative bacteria (38). LTX patients followed a different outpatient setting with fewer routine samples taken.

All CF pathogens were treated with antibiotics regardless of clinical symptoms according to the Copenhagen CF centre's treatment protocols (7).

3.2 Usage of different grading of pulmonary infections

As mentioned in 2.2.1, there are at least two different ways of grading pulmonary infections. In papers I and II, we applied our standard Copenhagen criteria for defining lung infection status and LTX patients were categorised as chronically infected.

Modified Leeds criteria (14) were used for defining lung infection status in paper III and IV; as the main outcome was the lung infection status in paper IV, it was important to use simple criteria here that are known and can be used among other CF

centres. This facilitates an international comparison in the future. Secondly, the use of the Leeds criteria allowed us to put our findings into perspective because intermittently colonised patients could be re-classified as non-infected. Thirdly, a rise in antibodies was, in some cases, a part of the reason for setting patients up for surgery (described in section 4.1.1), and so it would be a circular argument if antibodies were also used to define the outcome of lung infection status.

4. Methods

4. 1. Functional endoscopic sinus surgery (FESS)

4.1.1 Criteria for FESS

In paper I, III, and IV FESS was a part of the study. CF patients were selected for FESS based on following criteria:

- 1: Search for an infectious focus: Intermittently colonised patients with increasing frequency of positive lower airway cultures or repeatedly declining lung function (> 10%), despite intensive antibiotic chemotherapy. Patients with an unknown infectious focus and increasing antibodies against *P. aeruginosa*, *A. xylosoxidans* or *B. cepacia* complex were given the highest priority.
- 2: Patients who had recently been LTX. The ambition was to perform FESS within the first postoperative year.
- 3: Patients with severe symptoms of chronic rhinosinusitis (CRS) according to the EPOS (43).

4.1.2 FESS procedure

A BAL was performed under general anaesthetic. The subsequent FESS was to ventilate and drain the paranasal sinuses and to make these accessible for postoperative instrumental cleansing and irrigation with saline and topical antibiotics. Each patient was evaluated for symptoms of chronic rhinosinusitis (43) followed by a clinical examination. The extension of surgery (e.g., exploration of the frontal or sphenoid sinuses) was undertaken based on the preoperative CT scan and perioperative findings. As a standard, we applied FESS with an uncinectomy, an anterior ethmoidectomy and a medial antrostomy, leaving a significantly enlarged maxillary ostium comprising more than half the medial maxillary wall as recommended (43). Visible intramucosal abscess-like structures (especially found in the maxillary sinuses) were resected along with other inflamed mucosal tissue when accessible. Finally, the opened and now accessible sinuses were irrigated with saline and colistimethate sodium.

To optimize culture results, no patients received IV antibiotics within two weeks prior to FESS, and different anatomic sampling locations and multiple samples for culture were prioritized during surgery, including: nasal secretions, pus, mucosa, polyps, and bone (Figure 4–6). Samples taken for culture were collected with sharp instruments or by suction tubes. The material obtained was immediately cultured at the Department of Clinical Microbiology at Rigshospitalet.



Figure 3:
Set-up doing
FESS

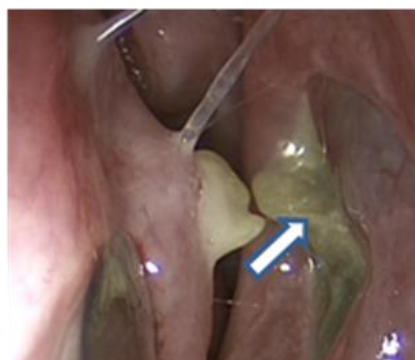


Figure 4: Pus containing
P. aeruginosa exiting the
left maxillary sinus before FESS

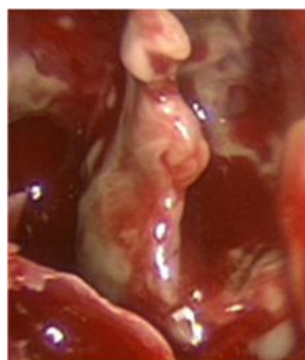


Figure 5: A view in the
maxillary sinus

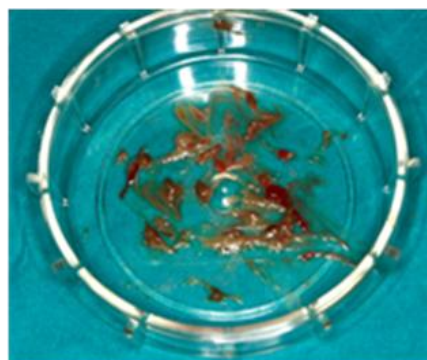


Figure 6: Obtained
material for culture

4.1.2 Postoperative treatment

Postoperative adjuvant therapy included: two weeks of IV antibiotics if there was the slightest suspicion that the lungs or sinuses contained CF-pathogens (7), at least 6 months of twice daily nasal irrigation with saline and antibiotics (starting Day 1 with colistimethate sodium but could be adjusted according to susceptibility), and 12 months of topical nasal steroids (mometasonfuroate). As a standard each patient had four postoperative visits to the oto-rhino-laryngologist (ORL) outpatient clinic: one week and one, three and twelve months postoperatively, where crusts and secretions were endoscopically cleansed from the nasal cavities and sinuses (Figure 7). At each follow-up, under endoscopic guidance, the patients were bilaterally cultured.



Figure 7: Postoperative cleansing and culturing without use of local anaesthesia.
(Thanks to 7 year-old Jonas; a fantastic young man)

4.2 Culture methods

In all four papers (I–IV) the bacteriology of the lungs and sinuses play a major role, thus the method of culture is described in detail:

Gram-stained smears and aerobic cultures on selective media were performed on all samples (Figure 8–9). These media included a Sabouraud plate (for fungal growth), a 7% NaCl plate, a *B. cepacia* plate containing Colistin and Gentamicin, a “blue plate” (modified Conradi Drigalski’s medium) selective for Gram-negative rods, and a non-selective media including 5% Danish blood agar and chocolate agar (Figure 8–11). In order to avoid sampling bias, bacteria with different susceptibility patterns and different colony morphologies were chosen and identified as previously described (144;145). In paper I, Gram-stained smears were used for biofilm detection and Pulsed Field Gel Electrophoresis (PFGE) was used for genotyping *P.aeruginosa* isolates from the sinuses and the lungs (145).

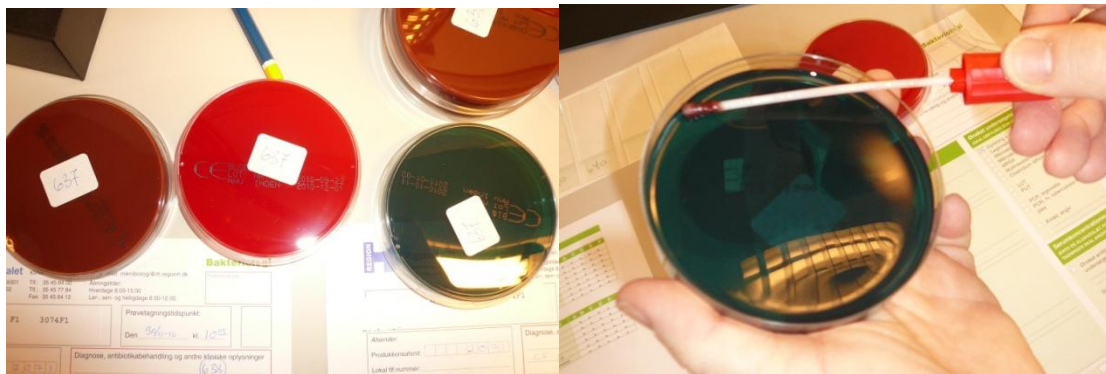


Figure 8–9: Smears on selective media for culturing.



Figure 10–11: Growth of mucoid *P.aeruginosa* on a blue plate; antibiotic susceptibility testing of *P.aeruginosa*

4.3 IgA and IgG antibodies against *P.aeruginosa*

In paper I and II we present and use a new method to diagnose antibodies against *P. aeruginosa* St-Ag and alginate:

Twelve 6-mm in diameter paper discs (Figure 12) with obtained serum or eluates of saliva or nasal secretions from each patient were examined for IgA and IgG antibodies against *P. aeruginosa* alginate and *P. aeruginosa* sonicate (St-Ag) (serogroups 1–17)) using enzyme-linked immunosorbent assays (ELISA) as reported previously by our group (25;26): Saliva and nasal secretion impregnated paper-discs were incubated on a shaker in dilution buffer to elute IgG and IgA antibodies. Phosphate-buffered saline + 0.1% Tween-20 + NaCl 15 g/l was used for dilution, and the plates were washed three times with it.



Figure 12: A paper disc to collect secretions inserted in the nasal cavity

4.3.1 Antibodies against *P. aeruginosa* alginate

Microtiter plates were coated with alginate purified from a mucoid CF *P. aeruginosa* strain as previously reported by our group (146). The plates were coated and blocked in dilution buffer. Diluted serum, saliva and nasal secretions (see above) were added and allowed to react. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA (P0216) and anti-human IgG (P0214) were added and reacted.

4.3.2 Antibodies against *P. aeruginosa* St-Ag

A sonicated cell extract of *P. aeruginosa* serogroups 1–17 was used as standard St-Ag (25;26) and coated onto irrigated 96-well polystyrene plates. The plates were incubated and blocked with dilution buffer. Serum, saliva, and nasal secretion were diluted and allowed to react. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA (P0216) and anti-human IgG were added and left to react.

4.3.3 ELISA

For all ELISAs, TMB-Plus media was added. The reactions were stopped after one hour at room temperature by adding 1 M H₂SO₄. The absorbance was measured at 450 nm on a plate reader. The results were expressed as optical density values (OD) (Figure 14).



Figure 13–14: ELISA procedures quantifying IgA and IgG in nasal and saliva secretions as well as serum.

4.4 Additional methods

The following well-established methods are used in the papers: traditional immunohistochemistry is used in paper II; lung function test (147;148), body mass index standard deviation scores (149), specific anti-*Pseudomonas* IgG antibodies measured by ELISA (37) and precipitating antibodies measured by CIE (38) are all regularly used when evaluating the CF patients conditions and are used in paper IV. The CFQ-R (Cystic Fibrosis Questionnaire-Revised) has also recently been initiated in the CF centre to estimate the disease-specific health-related quality of life (150), thus was logical to use in paper IV.

The sinonasal outcome test (SNOT-22) used in paper IV (inserted in section 10) deals with sinonasal conditions (64), but also includes health-related questions that can be influenced by other CF-related conditions, e.g. cough; the SNOT-22 questionnaire is used worldwide when evaluating CRS.

4.5 Statistics

In all four papers (I–IV), we tested whether data were continuous and if the comparisons fulfilled the criteria for normality and equal variance. The level of significance was set to < 0.05 (two-tailed). SAS 9.1.3 was used for calculations.

In paper I, the non-parametric sign test was used to compare within patient samples of antibodies, while the data of the antibodies in paper II were unpaired, continuous and positively skewed distributed why Log_{10} transformations were made. The transformed data had an approximately normal distribution justifying an unpaired two-sample t-test for the means and a one way analysis of variance (ANOVA).

Receiver operating characteristic (ROC) curves was used to find the best cut-off values between the three lung infection groups if IgA was to be used as a diagnostic test (paper II). A Spearman rank coefficient test was used to correlate nasal secretions and saliva in paper II as well.

A McNemar's test was used to compare the nominal data of postoperative frequencies of growth with the perioperative frequencies in paper III and to compare the

change in lung infection status after FESS (paper IV). In paper IV, a paired two-sample t-test for the means and an ANOVA was used for the rest of the comparisons.

The biggest statistical challenge was in paper IV. When planning the study, we received statistical advice from Professor Torben Martinussen at the Department of Biostatistics in how to quantify the frequencies of positive cultures. The conclusion was that every lower-airway sample was registered and given the same weight regardless of the interval between the samples. Using a Spearman rank coefficient test, these results were then compared to the results of lower-airways samples where each culture was given weight according to the period until the next culture.

4.6 Ethics

The study was approved by the local ethics committee (H-A-2008-141), and all patients gave informed consent. In patients <18 years of age, consent was also obtained from their parents.

The inclusion for FESS was not a part of the study, solely the outcome. We also obtained consent for doing additional analyses on the bacteria/material obtained during FESS and BAL, the postoperative treatment and culturing, as well as for using questionnaires and data from the patient files. In paper II, consent was used in order to obtain and analyze secretions and blood and for culturing; no change in treatment modality was made on behalf of these results.

5. Review of results

We found that the vast majority of CF patients have bacteria in their paranasal sinuses (paper I–IV). They are often colonised with CF-lung pathogens, especially *P. aeruginosa*, and there is a close correlation between the bacteriology of the sinuses and the lungs, including identical genotypes in the sinuses and lungs (paper I, II, IV). Importantly, the genotype remains unchanged over time. The chronically infected patients had the same *P. aeruginosa* genotype in their lungs for a median of 15 years as found in their sinuses, and up to 6 years in intermittently colonised patients, although the bacteria apparently had been eradicated from the lungs (paper I).

Though the environment of the sinuses in many ways is similar to that of the lower airways, including anaerobic niches and biofilm formation, it differs by excessive presence of the non-phlogistic (does not induce inflammation) secretory-immunoglobulin A (s-IgA) (paper I, II). Failure to eradicate CF-pathogens from the sinuses is probably a result of an inefficient local immune response: locally produced specific s-IgA binds Gram-negative bacteria on the mucosal surface, thereby reducing the inflammatory response by preventing antigen presentation inhibiting complement activation, inhibiting the recruitment of PMNs and thereby diminishing the oxidative burst (paper I). This was visualized by immunohistochemistry showing excessive amounts of IgA-producing plasma cells in the sino-nasal tissue and IgA in the excretory ducts. It was also visualized by Gram-stained smears from the sinuses, where the bacterial biofilms were surrounded

by very few and scattered PMNs in marked contrast to the pulmonary findings (paper I, II).

With this background information, our new method to quantify IgA and IgG against *P. aeruginosa* antigen and against the *P. aeruginosa*-specific extracellular polysaccharide alginate, was used to compare nasal, saliva and serum concentrations with the patients lung infection status (described in 2.2.1) in a cross-sectional study (paper II). A significant correlation ($p < 0.01$) was found between the *P. aeruginosa* lung infection status and the quantity of specific IgA in the nasal secretions and saliva; the intermittently colonised patients had the higher IgA concentrations than the non-infected patients (Table 4). This test may then be used as a supplementary tool for detecting CF patients with early lung colonisation. The theory background and our results indicate that the test actually reveals a *P. aeruginosa*-sinusitis, which again is a surrogate marker for lung infections due to the concordant bacteria in the upper and lower airways. In an upcoming prospective study we hope that the usefulness of the IgA test as a marker of *P. aeruginosa* sinusitis can be verified and that it will show a similar good sensitivity and negative predictive value as was the case when related to the lung infection status (Table 5).

	Nasal IgA	Serum IgA	Nasal IgG	Serum IgG	Nasal IgA Serum IgA	Nasal IgG Serum IgG	Nasal IgA IgG	Serum IgA IgG
Non-CF Control group, n=9	0.17 0.63	0.05 0.51	0.09 0.28	0.12 0.96	3.29 1.74	0.92 0.24	2.19 2.93	0.57 0.56
Free from pathogenic bacteria n=32	0.28* (0.20) 0.77* (0.28)	0.13 (0.08) 0.73* (0.57)	0.09* (0.05) 0.19* (0.13)	0.12* (0.09) 1.01* (0.45)	2.57* (1.83) 1.99 (2.28)	0.84* (0.40) 0.19* (0.13)	3.72 (2.71) 5.94* (4.85)	1.24* (0.75) 0.66 (0.34)
Intermittent PA n=24	1.03 (0.68) 1.33 (0.35)	0.14 (0.14) 1.21 (0.87)	0.50 (0.50) 0.64 (0.59)	0.30 (0.26) 1.68 (0.64)	27.92 (83.42) 1.99 (1.87)	2.80 (3.64) 0.38 (0.27)	4.54 (5.24) 5.02 (6.58)	0.68 (0.67) 0.67 (0.34)
Chronic PA n=25	1.70* (0.60) 1.46 (0.47)	0.45* (0.31) 1.65 (0.68)	0.63* (0.50) 0.96* (0.41)	0.85* (0.71) 2.52* (0.77)	4.84* (2.79) 1.06* (0.58)	1.16 (1.41) 0.38 (0.16)	4.35 (3.76) 1.81* (1.07)	0.82 (0.78) 0.66 (0.26)

Table 4: Mean nasal and serum antibodies against *P.aeruginosa*. The Standard Deviations are shown in brackets. The ratios were first calculated for each individual and following the mean values were calculated. The white marked numbers refers to *P.aeruginosa* alginate; the grey marked numbers refers to *P.aeruginosa* St-Ag.

Table 5: combined nasal IgA	96 %	81%
St-Ag and alginate used for diagnostics	Sensitivity	Specificity
79%	23 patients	6 patients
Positive predictive value	True positive	False positive
96%	1 patients	26 patients
Negative predictive value	False negative	True negative

Sinus infections with CF-pathogens do not seem to be eradicated by the frequent oral and intravenous antibiotic therapies that CF patients receive. Conversely, in a prospective follow-up study (paper III) *P. aeruginosa*, *A. xylosoxidans* and *B. cepacia* complex could, in several cases, be eradicated from the sinuses or the quantity of colony-forming units were at least reduced, so the bacteria could not be re-detected by thorough sinus cultures for several months (Table 6). This was achieved by extensive sinus surgery and postoperative treatment (described in 4.1.2). Achieving these results was a prerequisite for doing the research in paper IV.

Lung status at surgery	Perioperative	One month	Three months	Six months	Twelve months
LTX	24 of 24 (100%)	8 of 24 (33%)	9 of 20 (45%)	11 of 22 (50%)	9 of 20 (45%)
Chronically infected	25 of 26 (96%)	8 of 24 (33%)	12 of 24 (50%)	13 of 26 (50%)	8 of 18 (44%)
Intermittently colonised	55 of 66 (83%)	5 of 60 (8%)	11 of 60 (18%)	8 of 50 (16%)	12 of 48 (25%)

Table 6: The table shows cultures from the left and right side of the middle meatus and maxillary sinus perioperative and at follow-up. In conclusion, 21 patients had no re-growth at any time at any sinus during six months of follow-up

By the same procedure, probably as a consequence of the sinus bacteria being eradicated, a significant reduction in frequencies of lower-airway cultures with CF-pathogens was accomplished (paper IV). In particular, intermittently colonised CF patients with concordant CF-pathogens in the sinuses seem to benefit from the treatment strategy. As a consequence, the one-year prevalence of intermittent colonisation decreased by 38% after FESS and the one-year prevalence of non-colonised patients increased by 150% (Table 7). In addition, specific IgG for *P. aeruginosa* decreased and quality of life including sinonasal symptoms also improved. This was shown by a prospective, non-randomised, uncontrolled, intervention cohort study (paper IV).

Main lung bacteria	Non-infected	Intermittently colonised	Chronically infected
	Before \after FESS	Before \after FESS	Before \after FESS
<i>P. aeruginosa</i>		50 \31	20 \20
<i>A. xylosoxidans</i>		9 \6	7 \5
<i>B. cepacia</i> complex		2 \1	2 \3
Total	16 \40	61 \38	29 \28

Table 7: The table shows the lung infection status (as described in 2.2.1) in the CF patients at FESS (the digit in front of the backslash) and in the same patients a year after FESS and adjuvant therapy (the digit behind the backslash).

6. Discussion

Bacterial sinusitis being a focus for lung colonisation and infection is supported by our finding of nearly all CF patients having bacteria in their sinuses and by frequent lower-airway cultures with CF-pathogens (*P. aeruginosa*, *A. xylosoxidans*, *B. cepacia* complex) correlating with a high frequency of concordant bacteriology in the sinuses. In our patients selected for FESS, 67% had concordant CF-pathogenic bacteria in the sinuses and lungs and additionally 5% had CF-pathogens in the sinuses that were not found in the lungs (paper IV). The high prevalence of bacterial sinusitis is despite massive intravenous and oral antibiotic treatment. Additionally, we often did not get any positive lower-airway cultures during BAL in intermittently lung-colonised patients even when we found CF-pathogens in the sinuses; this indicates that the sinuses are a more permanent focus than the lower airways. Our described prevalence of bacterial sinusitis is at the high end compared with other studies describing CF-sinus bacteriology (described in section 2.6.3), which is probably a result of our invasive and multiple-sample selection. Above all, we consider the risk of false positive results very small; the only way that samples can be cross-contaminated is via the anterior nasal cavity.

Few anaerobes and few fungus isolates were found in the sinuses. Anaerobes were found when doing molecular studies (unpublished material) but not even here were fungus frequently found. This is in contrast to our expectations, as CF-patients experience pulmonary problems with fungus and one study cited by EPOS found a high prevalence (43;72;151). However, our findings are in accordance with the general low prevalence of

fungus-sinusitis in Danish non-CF patients compared with the USA where the study was carried out.

In the early stage of lung colonisation, migration of CF-pathogens mainly occurs in a downward direction from the more permanent focus in the sinuses towards the lungs (21). This migration occurs more frequently during the viral season where the nasal secretions are more liquefied (90). The results in paper IV further support this theory, as it may be concluded that if sinus surgery and adjuvant therapy reduce the frequencies of lower-airway bacteria, the sinuses are bound to influence the lower airways by downward migration of bacteria. Moreover, when evaluating the literature on this subject, including the previous papers from our group, I find it unquestionable that the sinuses play an important role in causing pulmonary colonisations and infections. It is more debateable what can be done to eliminate this risk of colonisation.

There is empirical evidence that the persistent sinus bacteria are facilitated by inflamed tissue obstructing the sinus ostia, lower antibiotic concentration than in the lungs due to lower blood perfusion in the sinus mucosa, that the infection is localised as an empyema in the sinuses and maybe also as intramucosal abscesses. Furthermore, our previous research (21) has shown that the bacteria develop resistant genotypes and phenotypes in the sinuses. In contrast to the nasal environment, where CF-polyps show various patterns of neutrophil-dominated acute and chronic inflammation (152), we found a reduced number of PMNs surrounding the biofilms on the sinus mucosa compared with the lungs. All these points taken together with our results that the upper airways are

dominated by the non-phlogistic IgA (paper I, II), may explain the mechanism of why sinus bacteria are more persistent than in the lungs. In essence, what is important for the clearance of intermittent *P. aeruginosa* colonisation in the CF lungs is only partially functional in the sinuses, providing opportunities for the bacteria to adapt through evolution of resistance mechanisms.

Some non-infected CF patients were solely colonised with CF-pathogens in the sinuses (IV). It is likely that this represented their initial colonisation. Nevertheless, we cannot prove that these patients benefited from the treatment, and it is challenging to determine the prevalence of how often the colonisations initiates in the sinuses. We are confident that our prospective study on specific IgA in sputum and nasal secretions will prove useful in diagnosing *P. aeruginosa* sinusitis and thereby come closer to a conclusion. In fact, after we ended our study (paper II), two of the four patients from the non-infected group with the highest IgA levels have now become intermittently lung-colonised with *P. aeruginosa*, and so one might be led to think that they were already sinus-colonised at the time of the study.

According to the Leeds criteria, CF-patients are capable of having *P. aeruginosa* sinusitis but being categorised as being free from *P. aeruginosa* (non-infected) (14). In my opinion, it would be clinically relevant to characterise CF infections both according to their sino-nasal bacteriology and according to the lower-airway colonisations/infections. This will require more focus on treating the upper airways, a general collaboration with ORLs, and that clinicians bear in mind that non-BAL lower-airway samples can be cross-

contaminated from the upper airways. Furthermore, in order to characterise CF infections and select the right CF patients for FESS, it is essential to find a combination of tests that can diagnose CF-pathogenic sinusitis with high sensitivity. Nasal lavage, as described by Mainz (69) or in paper II, is a very easy way to obtain samples with little patient discomfort. However, these samples also contain bacteria from the upper pharynx and thereby do not solely represent sinus bacteria. It is also uncertain if saline from nasal irrigations represent material from all sinuses. In unpublished data, we have found a relatively low positive predictive value when doing middle meatus cultures in early intermittently colonised patients not previously having sinus surgery, which makes us conclude that this test cannot stand by itself. However, IgA can easily be obtained and quantified by ELISA, and if this is combined with regularly obtained cultures from the middle meatus, cultures from nasal irrigations and other paraclinical measures like serum antibodies and pulmonary function, we believe it has a high diagnostic value.

It may also be clinically relevant to subdivide intermittently colonised patients based on the colonisation pattern and bacteria genotypes as previously described (21): a) patients with single or multiple events of short colonisation periods (<6 months) followed by eradication; (b) intermittently colonised patients with multiple recurrent colonisation events with the same genotype of bacteria and a low systemic immune response (77); (c) patients with a rapid development of chronic lung infections with increasing precipitating antibodies. Thus, it is most likely that intermittently colonised patients from group (b)

have an additional sinonasal infectious focus. This would help us to select patients for upper-airway treatment by FESS and/or conservative treatment.

Though we have shown that nearly all chronically infected CF patients have CF-pathogens in their sinuses, which in some cases could be eradicated (paper III, IV), we did not expect that they would have a significant decrease in positive lower-airway cultures (paper IV). In particular, four chronically infected patients had a pronounced effect of the treatment and we put forward the theory that such patients could be false-positive categorised if the lower-airway samples are cross-contaminated by the upper airways. Thus, the result of true chronically infected patients having an effect of the treatment is more uncertain. However, it is accepted that the lung damage in CF patients with chronic infections characteristically is focal (153) leading to a focal loss of alveoles and an annual decline of lung function of about 1–2% (76). In that way, true chronically infected patients may benefit from having their sinus bacteria eradicated, as it may prevent further spread of the infection by aspiration from the sinuses to new areas of the lungs.

It can be argued that in paper III and IV we gave no answers as to whether the same results could have been achieved by conservative treatment comprising nasal irrigations and endoscopical cleansing. Studies on otherwise healthy patients with CRS have shown that an ostial dimension should be >4 mm to ensure that irrigations penetrate the maxillary sinus, and that the frontal sinuses are more difficult to irrigate (154;155). By comparison, when using nebulizers the dimension requirements are thought to be smaller

(138;156). Literature addressing nasal irrigations in CF patients mainly focus on the maxillary sinuses, but one should remember that CF patients may have frontal sinuses, which contain CF-pathogens as often as the maxillary sinuses (paper III and unpublished data). To ensure permanent drainage from the sinuses adequate extensive surgery may be considered; this could comprise a modified endoscopic medial maxillectomy (127) or a Draft III (129), the latter ensuring drainage from the frontal sinuses, which are the most challenging sinuses to operate. We advocate that it is important to ensure permanent access to the sinuses, both to reduce symptoms of CRS but also to facilitate postoperative treatment preventing sinus infections and spread of bacteria to the lungs. We agree that more extensive surgery is needed in CF patients than in patients without CF, but have to await studies on FESS comparing surgical methods with postoperative clinical examinations, symptoms, adverse effects and cultures.

In paper III and in section 2.8, the possibilities of using different or additional ways to treat the upper airways are summarised but the most optimal combination is not yet defined. In addition, a synergistic effect has been suggested when using tobramycin and colistimethate sodium for inhalation, thus, this should also be considered when doing research on which drugs to use for nasal irrigations/nebulizations (157). Especially when aiming at eradicating *A. xylosoxidans* and *B. cepacia* complex, one must be aware of their antibiotic susceptibility (described in 2.4). While others have described a good effect of nebulizers such as the PARI sinus (138), the patients in our study have been able to choose between two devices for nasal irrigations (Figure 15–16). We have no conflicts of interest

and find the device in Figure 15 creates a higher pressure than the one in Figure 16, thus the saline being more likely to penetrate the sinuses.

Finally, based on our findings, I want to stress the importance of focussing on upper airway bacteriology in CF patients, especially in the outpatient routine treatment and the importance of guidelines for upper-airway treatment being established. This requires collaboration between the CF physicians, microbiologists and ORLs.



Figure 15–16: Two methods of doing postoperatively nasal irrigations. (Nicely demonstrated by my 10-year-old son Bertram).

6.1 Study strength and weaknesses

The major strength of our set-up is the establishment of a unique, effective, collaboration focusing on CF; the microbiologists and CF physicians have had a strong collaboration through many years. This project has allowed ORLs to be a part of this collaboration making it multidisciplinary. We have a very large group of CF patients, which are all seen on a monthly basis, which is very frequent compared with other CF

centres. This results in an abundance of data that can be evaluated for the benefit of the CF patients. The patients seem very committed to the research, the adherence is high, and only two patients did not wish to be enrolled in the IgA study (paper II) and only one patient turned down the offer of FESS (paper IV). The willingness to attend the postoperative controls and return the questionnaires was also high (80–99%).

A project is always strengthened by having one single committed coordinator; this improves adherence and reduces bias. To maintain and develop our high quality of treatment, I find it necessary that the ORLs are keep on seeing CF patients, evaluating their CRS symptoms, doing endonasal endoscopy and sino-nasal cultures. Furthermore, it is also important that the CF physicians on a standardized basis ask for CRS symptoms and focus on possible upper airway infections.

The main outcome of all the studies (I–IV) is based on culture results and antibody measurements. The Department of Clinical Microbiology has few, but very dedicated and experienced, laboratory technicians who are responsible for doing the bacterial and antibody CF analyses. Thus, the possibility of inter-observer errors is low.

In paper II, a weakness is that the study was not prospective; that is why our hypothesis that high IgA actually reflects sinus colonisations cannot be finally proven. IgA against alginate can, in small concentrations, be present in non-infected individuals and IgA against St-Ag can cross-react with other Gram-negative bacteria and the test is therefore not totally specific towards *P. aeruginosa* (16;158). Even if our theory is correct,

one should bear in mind that it only should be used as a supplementary test creating awareness of possible colonisations.

In paper III, a drawback might be that due to ethical considerations, only a minority of the postoperative samples were taken during general anaesthesia, which is why false negative culture results from the sinuses cannot be excluded. However, I was the only one who obtained the samples, the majority of patients had persistent opening to their sinuses, and the follow-up procedure was standardized. Except when doing FESS, the same point applies in all our studies where one can always discuss how representative the material is. There are advantages and disadvantages in concern of doing regular culture compared with molecular methods; these two methods will be compared in an upcoming paper from our study group. In short, the true positive diagnostic value is high using both methods. Though the molecular methods in a few cases did detect CF-pathogens missed by regular methods, a case was also seen where the abundance of different sinonasal bacteria resulted in a false-negative result of CF-pathogens by the molecular methods. Sinus samples could in both cases become cross-contaminated by bacteria from the anterior nasal cavity, but we find this fact clinically unimportant. What is not unimportant is that cross-contamination of lower-airway samples from the upper airways remains as a potential confounder. Hence, when the CF-pathogen bacteria were eradicated from the upper airways, there would be a smaller risk of false-positive lower-airway culture results.

Regardless of cross-contamination, CF-patients who do not show growth of CF pathogens for a longer period will get their antibiotic treatment reduced. Consequently, we could have analysed whether the use of antibiotics decreased as a consequence of the decreased positive lower-airway cultures, or if unchanged, if the higher rate of antibiotics compared with positive lower-airway cultures might have been a confounder. The reason that this analysis was not included in paper IV was that we would have had to differentiate between types of administration (oral, inhalation and intravenous) and differentiate between prophylactic, eradication and maintenance therapy. We found that this would have taken focus from our main outcome.

A general weakness concerning paper I, III and IV is that the FESS-project was step-wise initiated. As a consequence, at the beginning of the study period, we were more reluctant including patients for FESS, making FESS extensive, and encouraging patients to be thorough with the postoperative treatment. As the existing research on FESS in CF patients, including the extent and postoperative treatment, was very sparse, the FESS by itself has not been a part of the research but solely the outcome of an established treatment. As a consequence, some CF-patients with no symptoms of CRS have not been offered FESS as early as we would now recommend and some not at all. Furthermore, we have not done surgery so extensive and explored all sinuses if the symptoms were not present. On the other hand, these facts ought not to influence the results in a positive way. What may weaken the way our results can be interpreted is that we did not have a control group to the FESS group. Instead we have to use the knowledge of the natural history of

CF. Generally, a confounder could be that patients' way of being treated changed during follow-up, but in our case the treatment strategy has mainly been unchanged throughout the study period (paper IV).

We are aware that CF patients are a heterogeneous group with confounders such as different co-morbidities of CF, large age distribution, different lung infection patterns (including LTX), and a wide span in the use of medicine. Despite this, we found it most correct to include all patients; however, this should be kept in mind when interpreting our results, but we have partly dealt with the confounders by doing sub-analyses.

Finally, we now stand in a classical dilemma: the need for a randomized case-control study is in conflict with the positive results from paper IV and the positive feedback we have got from the vast majority of CF patients. This subject has been discussed with the Head of the Copenhagen Trial Unit, Centre for Clinical intervention Research. In conclusion, based on all our summarised results, our studies can be compared to a "Fase IV clinical trial"(159) and it would be unethical to randomise CF patients to FESS or no treatment. The next step is to randomise patients to either conservative treatment, minimal FESS or extensive FESS, and let the outcomes, especially postoperative IgAs and lower-airway cultures, be evaluated by someone blinded to the treatment.

7. Perspectives

The determination that the sinuses play a role in the initial colonisation and infection in CF patients opens a lot of unanswered questions and still requires an active role from ORLs.

First off all, we do not have the perfect tool to diagnose whether a CF patient has CF-lung-pathogenic bacteria in their sinuses without doing sinus surgery. A prospective study of nasal and saliva IgA against *P. aeruginosa* must be carried out, as well as prospective studies concerning cultures of nasal irrigations (69) compared with meatus media cultures (111) and perioperative findings. Research on whether biomarkers as BPI-ANCA (160) and other inflammatory markers can play a role in determining sinusitis is also advisable.

Secondly, our postoperative treatment have partly been empiric and based on knowledge from the CF-lungs. Studies involving animal experiments are recommended to determine the most suitable drug(s), dose and administration interval for nasal irrigations.

Thirdly, a prospective trial randomizing CF patients to either sinus surgery or conservative treatment with nasal antibiotic and saline irrigation is highly important.

Fourth, using molecular methods it will be interesting to determine the diversity of the bacteria in the sinuses, the bacteria-bacteria interaction, presence of biofilm, and how bacteria changes phenotype after sinus surgery. It will be of clinical interest if certain bacterial phenotypes and genotypes can be correlated with severity of the disease so aggressive treatment can be early initiated in these cases.

Finally, in the same way as CF patients, patients with primary ciliary dyskinesia (PCD) may have sinusitis initiating colonisation and infection though the mechanisms are different (161). In general, there is a lack on research on PCD, which is why CF treatment often is used on this patient group (161). I recommend that a study be done on extensive sinus surgery and adjuvant therapy in PCD patients.

8. Conclusion

As answers to the aims of this thesis (1.1):

- a) There is a very high prevalence of CF pathogen sinusitis. The bacteria persist in the sinuses for years and can be a focus for initial lung colonisation and maintain the infection.
- b) In contrast to the lungs, the sinus inflammation is dominated by non-phlogistic specific IgAs; this facilitates persistence of bacteria.
- c) There is no single way of diagnosing CF-pathogens in the sinuses without being invasive. Nasal IgA may be a surrogate-marker for *P.aeruginosa* in the lungs and may be used as a supplementary diagnostic tool for *P.aeruginosa*-sinusitis.
- d) We have treated our patients with extensive FESS and standardized postoperative follow-up, IV antibiotics, prolonged nasal irrigation with saline and antibiotics in addition to nasal steroids. Further studies are needed to find the most effective treatment.
- e) By this treatment strategy (d), quality of life was improved, bacterial sinus foci could be eradicated and the frequency of pulmonary samples positive for CF pathogens could be reduced. This indicates a reduced CF morbidity.

Altogether, the CF-upper airways should not be neglected and ORLs can give a significant contribution to CF treatment.

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10. SNOT-22 questionnaire (64)

1. Need to blow nose	0	1	2	3	4	5
2. Sneezing	0	1	2	3	4	5
3. Runny nose	0	1	2	3	4	5
4. Cough	0	1	2	3	4	5
5. Postnasal discharge (dripping at the back of your throat)	0	1	2	3	4	5
6. Thick nasal discharge (snot)	0	1	2	3	4	5
7. Ear fullness	0	1	2	3	4	5
8. Dizziness	0	1	2	3	4	5
9. Ear pain	0	1	2	3	4	5
10. Facial pain/pressure	0	1	2	3	4	5
11. Nasal blokage	0	1	2	3	4	5
12. Loss of taste and or smell	0	1	2	3	4	5
13. Difficulty falling asleep	0	1	2	3	4	5
14. Waking up at night	0	1	2	3	4	5
15. Lack of a good night 's sleep	0	1	2	3	4	5
16. Waking up tired	0	1	2	3	4	5
17. Fatigue	0	1	2	3	4	5
18. Reduced productivity	0	1	2	3	4	5
19. Reduced concentration	0	1	2	3	4	5
20. Frustrated/restless/irritable	0	1	2	3	4	5
21. Sad	0	1	2	3	4	5
22. Embarrassed	0	1	2	3	4	5

0= no problem; 1= very mild problem; 2= mild or slight problem; 3= moderate problem;
4=severe problem; 5= problem as bad as it can be.

Please mark the most important items affecting your health (maximum of 5 items)

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Paper I



Original Article

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

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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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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 Testblättchen, 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 (Mikrowell, 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 polystyrene 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 H₂SO₄. 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

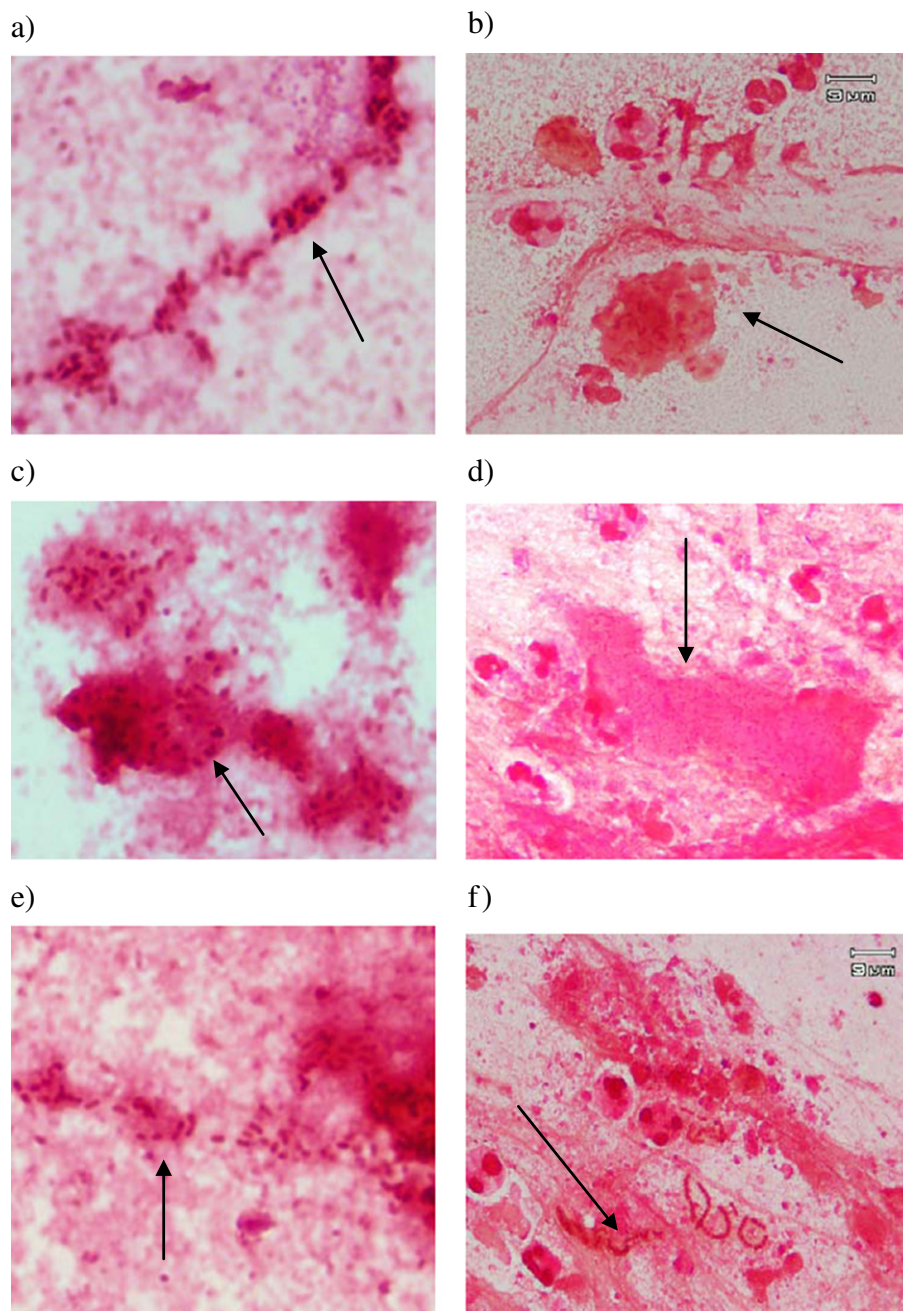


Fig. 1. a–f. Microscopic investigation of Gram-stained smears of pus from the sinuses (a, c and e) and corresponding sputum (b, d and f) obtained from three patients chronically infected with *P. aeruginosa* at the time of sinus surgery, magnification $\times 1000$. Arrows indicate biofilms.

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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Paper II



Secretory IgA as a diagnostic tool for *Pseudomonas aeruginosa* respiratory colonization

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Abstract

Background: *Pseudomonas aeruginosa* sinusitis may be the focus for intermittent lung colonization in patients with cystic fibrosis (CF). The sinusitis may induce elevated IgA levels in nasal secretion and saliva against *P. aeruginosa*.

Methods: 120 CF patients chronically infected, intermittently colonized or without *P. aeruginosa* in the lungs participated in this cross-sectional study. IgA and IgG against *P. aeruginosa* sonicate and alginate were measured in nasal secretions, saliva, and in serum by ELISA.

Results: The intermittently colonized patients had significantly higher IgA levels in nasal secretions and saliva than those without *P. aeruginosa* in the lungs, indicating that *P. aeruginosa* sinusitis may precede intermittent colonization and chronic infection of the lungs.

Conclusions: Specific IgA against *P. aeruginosa* in nasal secretions and saliva can contribute to differentiation between patients chronically infected, intermittently colonized, and without *P. aeruginosa* in the lungs. The diagnostic value of the IgA ELISA awaits a prospective study.

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Keywords: *Pseudomonas aeruginosa*; Cystic fibrosis; Colonization; IgA antibodies; Diagnosis; Sinusitis

1. Introduction

Patients with Cystic Fibrosis (CF) have increased susceptibility to infections, and *Pseudomonas aeruginosa* is the cause of most of the morbidity and mortality in these patients [1]. Lung infections with *P. aeruginosa* cause inflammation resulting in gradually declining lung function and a systemic increase of antibodies against a polyvalent *P. aeruginosa* antigen (Standard

Antigen (St-Ag)) [2] and the mucoid extracellular polysaccharide alginate [3,4]. In addition to serum, specific antibodies are present in tears and upper airways such as saliva and sputum; secretory immunoglobulin A being the dominant antibody on mucosal surfaces [4,5].

The immune system of the upper airways differs from that of the lower airways, by generating an early local response with a high plasma cell production of IgA when infected with *P. aeruginosa* [6,7]. The non-phlogistic IgA, binds *P. aeruginosa* on the mucosal surface, reducing the inflammatory response, inhibiting complement activation and the recruitment of polymorphonuclear leucocytes (PMN), and diminishing their oxidative burst by preventing phagocytosis [6,8]. *P. aeruginosa*

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probably colonizes and adapts to the paranasal sinuses acting as a reservoir, even before intermittently colonizing and chronically infecting the lungs [9]. The reduced inflammation contributes to this rather ‘silent’ adaptation, allowing *P. aeruginosa* to evolve to the chronic biofilm phenotype in the paranasal sinuses [9,10].

When CF patients become intermittently colonized in the lungs with *P. aeruginosa*, elevated levels of specific IgG against *P. aeruginosa*, measured by ELISA, can be detected 1–3 years before onset of the chronic lung infection, maybe reflecting a ‘hidden’ focus for subsequent intermittent lung colonization and chronic infection [8]. The ‘hidden focus’ may be the paranasal sinuses, which in CF patients frequently contain *P. aeruginosa* and are filled with mucus and pus [6,10]. In fact, re-infection of the lungs of transplanted CF patients often originates from the paranasal sinuses [11–14]. Chronic lung infection can be postponed by early antibiotic treatment of intermittent colonization [15]. Serological assays (IgG) have high sensitivity and specificity in distinguishing between CF patients chronically and not chronically *P. aeruginosa* infected in the lungs [16,17]. However, these methods have not been useful to distinguish between CF patients with intermittent colonization and patients without *P. aeruginosa* in the lungs [16–19], but the possible presence of *P. aeruginosa* sinusitis as a focus for subsequent lung colonization and chronic infection was not considered at the time of these publications. A diagnostic antibody assay for early detection of *P. aeruginosa* infection in the paranasal sinuses would therefore be useful [20]. As secretory IgA antibodies are detectable in CF patients with chronic *P. aeruginosa* lung infection [4], we hypothesized that *P. aeruginosa* sinusitis may cause a local rise in the specific IgA in nasal secretions and saliva, which could be used to diagnose *P. aeruginosa* sinusitis in addition to detection of *P. aeruginosa* by cultures from the sinuses. The clinical consequence could, hopefully, be successful attempts to eradicate *P. aeruginosa* from the paranasal sinuses and thereby prevent subsequent intermittent colonization and chronic infection of the lungs.

2. Material and methods

2.1. Patients

All CF patients above the age of seven, who were treated at the CF centre Copenhagen, were eligible for this cross-sectional study. The CF-diagnoses were based on characteristic clinical features, abnormal sweat electrolytes and genotypes. The CF patients are followed in the out-patient clinic every month, and the examinations are composed of sputum samples taken for microbiological examinations and regularly blood samples are analyzed for anti-bacterial antibodies [8]. In total 120 CF patients (60/60 male/female, mean age 22 years) participated in the study; saliva and nasal secretions were obtained from 73 patients, 24 patients had only saliva samples obtained and 23 patients had only nasal secretions obtained. Nasal secretions and new blood samples were obtained approximately six months later than the saliva samples. All patients had blood samples analyzed. Patients who both had sputum and nasal secretions obtained had two separate blood samples analyzed.

Twelve healthy employees from the Department of Clinical Microbiology Rigshospitalet participated as healthy controls.

2.2. Infection status

As reported previously, we defined chronic *P. aeruginosa* lung infection (CF+P (c)) as growth of this bacteria in six consecutive monthly samples taken from lower respiratory tract secretions, or a shorter period if there were two or more precipitating antibodies against *P. aeruginosa* [16,19]. Lung transplanted patients were categorized according to their infection status before transplantation.

Intermittent lung colonization was defined as growth of *P. aeruginosa* for less than 6 months, but normal levels of precipitating antibodies (0–1) against *P. aeruginosa*. If the monthly samples had never contained *P. aeruginosa*, patients were classified as without *P. aeruginosa*.

Based on these criteria the patients were divided into four groups: 1: CF patients without *P. aeruginosa* in the lungs (CF-P) 2: intermittently colonized with *P. aeruginosa* (CF+P(i)) in the lungs, 3: chronically infected in the lungs with *P. aeruginosa* (CF+P(c)) or 4: colonized/infected in the lungs with other CF pathogenic Gram-negative bacteria (*Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* or *Burkholderia cepacia* complex)(CF+GNB).

2.3. Collection of serum, saliva, and nasal secretions

The blood samples and secretions were obtained simultaneously. Mixed saliva was collected by using four sterile 6 mm diameter paper discs (Antibiotica Testblættchen, Struers, Copenhagen, Denmark) which were placed on the oral mucosa for 30 s as reported previously [5].

Nasal secretions were obtained by using four sterile 6 mm paper discs as mentioned above (Fig. 1B). With forceps, the discs were gently stroked against the mucosa for five seconds, each absorbing nasal secretions. Depending on the patients’ anatomy and co-operation the anterior part of the medial meatus could be reached, being the anatomical target for the discs (Fig. 1B). No decongestion was used. The patients decided if the samples were taken from the left, right or both sides. The saliva/nasal secretion-containing paper discs were stored at room temperature until analyzed.

2.4. IgA and IgG against *P. aeruginosa*

Serum and eluates of saliva and nasal secretions from the paper discs were examined for IgA and IgG antibodies against *P. aeruginosa* alginate and *P. aeruginosa* sonicate ((St-Ag) (serogroups 1–17)) using enzyme-linked immunosorbent assays (ELISA) as reported previously [4,5]. Saliva- and nasal secretion impregnated paper-discs (mean: 25 µl/disc as reported previously [5]) were incubated on a shaker for one hour at 35 °C in 175 µl dilution buffer to elute IgG and IgA antibodies (1:8 dilution). Serum was diluted 1:100 for the St-Ag ELISAs and 1:4000 for the alginate ELISAs. The volume of diluted serum samples for the ELISA was 100 µl, and 50 µl for the saliva and nasal

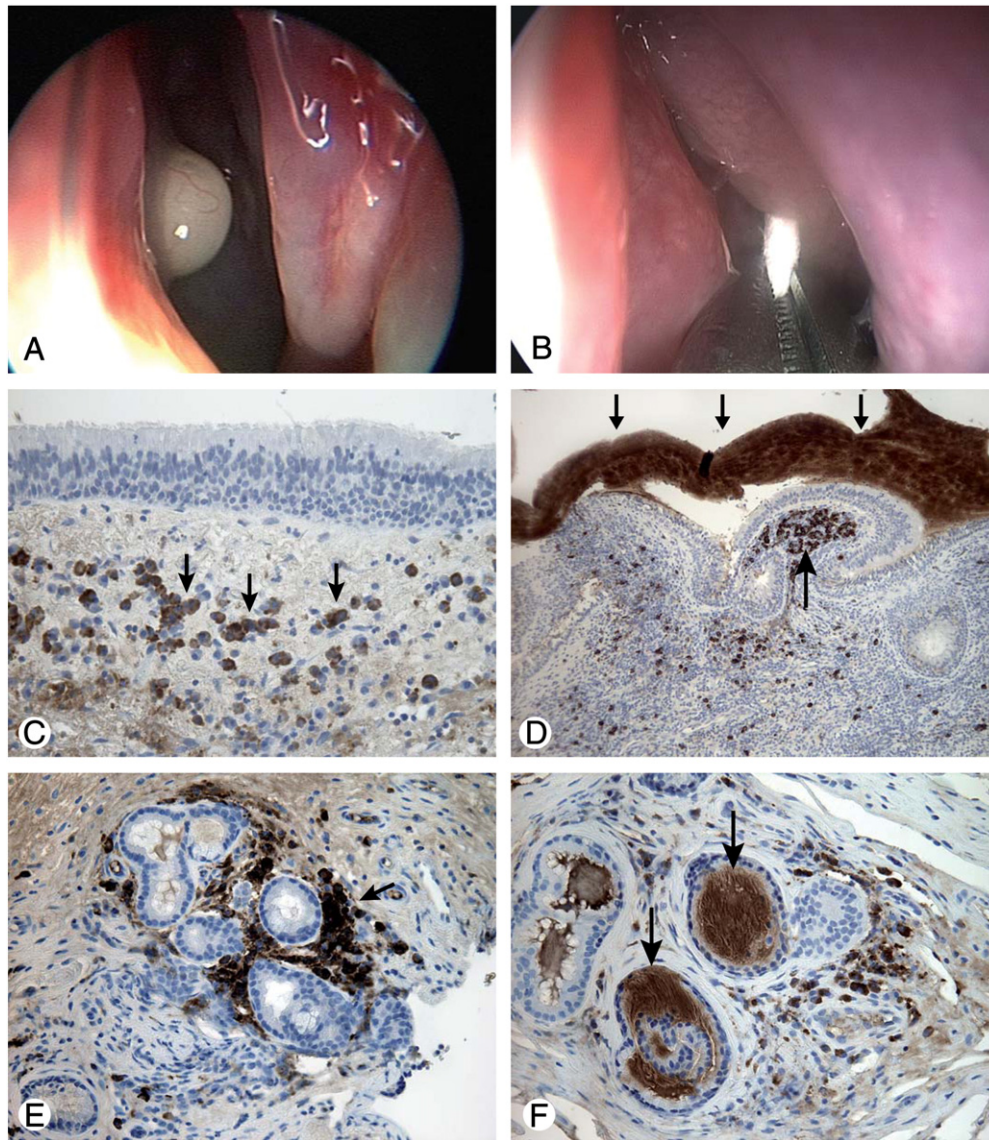


Fig. 1. A–F. A: A structure we thought was an abscess, but it turned out to be a sterile sinus-associated-lymphoid-tissue with plasma cells and IgA. B: Blotting paper within the right middle meatus absorbing nasal secretions. C: 09-33224 IgA x 40. Immunoreaction visualizing IgA immunoreactive (brown) plasma cells (arrows) in the lamina propria close to the pseudostratified respiratory surface epithelium. D: 09-32541 IgA x 10. An accumulation of IgA containing plasma cells (brown, large arrow) in the lamina propria close to the mucosal surface and also a strong IgA immunoreaction in the luminal mucosal layer (brown, smaller arrows). E and F: 01-586 IgA x 25 IgA immunoreactive plasma cells (brown) concentrated around secretory acini of the glands of the sinus tissue and also positive immunoreaction in secretions in the excretory ducts (arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

secretion ELISAs. Phosphate-buffered saline (PBS pH 7.2)+0.1% Tween-20 (Sigma) (washing buffer)+NaCl 15 g/l (= dilution buffer) was used and the plates were washed three times.

2.4.1. Antibodies against alginate (IgA-alginate, IgG-alginate)

Ninety-six-well microtiter plates (Mikrowell, BiotechLine A/S, Denmark) were coated with alginate (10 µg/ml) purified from a mucoid CF *P. aeruginosa* strain (6680NH) as previously reported [7]. The plates were coated overnight at 35 °C and blocked for one hour at 35 °C in dilution buffer. Diluted serum, saliva and nasal secretions (see above) were added and allowed to react for one hour at 35 °C. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA (P0216) and anti-human IgG

(P0214) (Dako A/S, Glostrup, Denmark) both diluted 1:10,000 were added and left to react for one hour at 35 °C.

2.4.2. Antibodies against *P. aeruginosa* standard antigen (IgA-St-Ag, IgG-St-Ag)

A sonicated cell extract of *P. aeruginosa* serogroups 1–17 was used as standard antigen (St-Ag, protein concentration 16 mg/ml) [4,5] and coated onto irrigated 96-well polystyrene plates (Maxisorb, BiotechLine A/S, Denmark) at a dilution of 1:2000. The plates were incubated for one hour at room temperature and blocked overnight with dilution buffer at 4 °C. Serum was diluted 1:100, saliva and nasal secretion 1:8 and allowed to react for one hour at room temperature. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA (P0216) and anti-human

IgG (P0214) (Dako A/S, Glostrup, Denmark)) diluted 1:20,000 were added and left to react for one hour at room temperature.

For all ELISAs TMB Plus was added (KemEnTec Diagnostics). The reactions were stopped after one hour at room temperature by adding 1 M H₂SO₄. 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).

2.5. Immunohistochemistry

The presence and localization of IgA in mucus and plasma cells in the mucosal tissue were visualized by means of immunohistochemistry. Resected nasal polyps and mucosa from the maxillary sinus from two CF+P(c) and two CF+P(i) (Fig. 1A) were fixed in 4% buffered paraformaldehyde and embedded in paraffin and 4 µm sections were cut on a microtome. The sections were incubated for 10 min in 2% bovine serum albumine followed by 18 h at 4 °C after addition of a polyclonal rabbit anti-human IgA (IMGEX, IMG-80368) diluted 1:100. For visualization of the immunoreaction the sections were incubated for 1 h with biotinylated goat anti-rabbit immunoglobulin (BA-1000, Vector) diluted 1:200, followed by StreptABComplex/horseradish peroxidase (Vectastain, PK-4000), and finally visualized by means of 3,3-diaminobenzidine for 15 min. The sections were counterstained with hematoxylin (Fig. 1C–F).

2.6. Sinus and nasal bacterial colonization/infection

We randomly chose 20 of our (CF-P) patients (13/7 male/female, mean age 16 years) and cultured their nasal cavity to determine if they were also free from *P. aeruginosa* (and other CF pathogens) in the nose and sinuses. Wearing a headlight and using a nasal speculum, the nasal cavities were examined by one ENT surgeon (one of the authors, K.A.). In each side of the nose a thin cotton swab was wiped within the middle meatus. Furthermore, a nasal irrigation was performed using a plastic Neti Pot (Yogaprocess A/S) containing 100 ml of sterile saline. The saline was poured through one nostril, passed through the nasal cavity and out of the other nostril where 10 ml were collected. Finally, a sputum sample or an endolaryngeal suction was taken [21]. This provided four different samples which were cultured the same day.

2.7. Ethics

The study was approved by the local ethics committee (H-A-2008-141). All patients gave informed consent. In patients <18 years a consent was also obtained from their parents. Obtaining the saliva samples did not involve any discomfort for the patients. Obtaining nasal secretions could result in a little discomfort; therefore in a couple of cases fewer samples were taken. The serum and saliva samples were part of the routine, thus no extra blood samples were taken. The antibody results did not result in any change of the treatment modality for the involved patients.

2.8. Statistics

SAS 9.1.3 was used for analyzing data, making receiver operating characteristic (ROC) curves and Spearman rank coefficient test. The St-Ag and alginate data were unpaired, continuous and positively skewed distributed why Log₁₀ transformations were made. The transformed data had an approximately normal distribution justifying an unpaired two-sample *t*-test for the means and a one way analysis of variance. The level of significance was set to ≤0.05 (two-tailed).

3. Results

The results of the antibody measurements are shown in Table 1A and B.

CF+P(c) had the highest IgA and IgG antibody levels against St-Ag and alginate in serum, nasal secretions (Table 1) and saliva (Table 2) compared with CF+P(i) and with CF-P (all *p*-values<0.0001 except IgA against St-Ag which was not significantly different between CF+P(c) and CF+P(i)).

Generally, the nasal/serum (Table 1) and the saliva/serum (Table 2) IgA ratios were high in CF+P(c) in accordance with a local mucosal production of IgA, in contrast to the low IgG ratios being in accordance with a high systemic production and subsequent transudation of IgG to the mucosa secretions.

CF+P(i) had higher IgA and IgG antibody levels against St-Ag and alginate than CF-P (all *p* values<0.0001) (Tables 1 & 2). Nasal IgA against alginate was also higher in CF+P(i) compared to CF-P (Fig. 2G).

Table 1
Mean nasal and serum antibodies against *P. aeruginosa* (PA).

	Nasal IgA	Serum IgA	Nasal IgG	Serum IgG	Nasal IgA	Nasal IgG
					Serum IgA	Serum IgG
Non-CF control group, n=9	0.17	0.05	0.09	0.12	3.29	0.92
CF-P, n=32	0.63 (0.20)	0.51 (0.08)	0.28 (0.05)	0.96 (0.09)	1.74 (1.83)	0.24 (0.40)
	0.28 ‡ (0.20)	0.13 (0.08)	0.09 ‡ (0.05)	0.12 * (0.09)	2.57 ‡ (1.83)	0.84 * (0.40)
	0.77 ‡ (0.28)	0.73 * (0.57)	0.19 ‡ (0.13)	1.01 ‡ (0.45)	1.99 (2.28)	0.19 □ (0.13)
CF+P(i), n=24	1.03 (0.68)	0.14 (0.14)	0.50 (0.50)	0.30 (0.26)	27.92 (83.42)	2.80 (3.64)
	1.33 (0.35)	1.21 (0.87)	0.64 (0.59)	1.68 (0.64)	1.99 (1.87)	0.38 (0.27)
CF+P(c), n=25	1.70 □ (0.60)	0.45 ‡ (0.31)	0.63 * (0.50)	0.85 ‡ (0.71)	4.84 * (2.79)	1.16 (1.41)
	1.46 (0.47)	1.65 * (0.68)	0.96 □ (0.41)	2.52 ‡ (0.77)	1.06 * (0.58)	0.38 (0.16)

The standard deviations are shown in brackets. The ratios were first calculated for each individual and following the mean values were calculated. The white marked numbers refer to alginate; the grey marked numbers refer to standard antigen.

* :*P*<0.05, □:*P*<0.01 and ‡:*P*<0.0001 and when the CF+P(i) are compared with CF-P or when the CF+P(i) are compared with CF+P(c). None of the values from the control group differed significantly from the CF-P group.

Table 2
Mean sputum and serum antibodies against *P. aeruginosa*.

	Saliva IgA	Serum IgA	Saliva IgG	Serum IgG	Saliva IgA	Saliva IgG
					Serum IgA	Serum IgG
Non-CF control group, n=12	0.19	0.05	0.10	0.12	4.00	1.07
CF-P, n=19	0.48 (0.22)	0.52 (0.06)	0.07 (0.03)	0.98 (0.17)	1.21 (2.49)	0.12 (0.29)
CF+P(i), n=47	0.23 (0.21)	0.09 (0.49)	0.07 (0.03)	0.17 (0.86)	2.88 * (0.75)	0.58 (0.06)
CF+P(c), n=23	0.26 * (0.39)	0.54 * (0.04)	0.07 (0.06)	1.51 (0.34)	0.80 (5.87)	0.07 (0.27)
	0.38 (0.33)	0.08 (0.73)	0.08 (0.02)	0.26 (0.72)	5.62 (0.72)	0.51 (0.02)
	0.45 (0.48)	0.92 (0.14)	0.07 (0.22)	1.68 (0.66)	0.77 (3.99)	0.05 (0.18)
	0.82 ‡ (0.71 ‡)	0.20 ‡ (1.23 ‡)	0.21 ‡ (0.25 ‡)	0.74 ‡ (2.59 *)	5.06 (0.67)	0.35 * (0.09 □)
	(0.33)	(0.56)	(0.21)	(0.37)	(0.44)	(0.07)

The standard deviations are shown in brackets. The ratios were first calculated for each individual and following the mean values were calculated. The white marked numbers refer to alginate; the grey marked numbers refer to standard antigen.

* :P<0.05, □:P<0.01 and ‡:P<0.0001 and when the CF+P(i) are compared with CF-P or when the CF+P(i) are compared with CF+P(c). None of the values from the control group differed significantly from the CF-P group.

CF+P(i) also had significantly higher ratios between alginate IgA in nasal secretion/serum (Table 1) and in saliva/serum (Table 2) compared with CF-P.

ROC-curves were used for finding the best cut-off values between CF+P(i) and CF-P:

Nasal IgA: A cut-off value of 1.09 for nasal IgA-St-Ag gave 76% sensitivity and 87% specificity. A cut-off value of 0.54 for nasal IgA-alginate gave 75% sensitivity and 94% specificity. When these cut-off values of the two nasal IgA tests (IgA-alginate and IgA-St-Ag) were combined, CF+P(i) could be discriminated from CF-P with 96%, sensitivity, 81% specificity, 79% predictive

value of a positive test (pvpos), and 96% predictive value of a negative test (pvneg) .

Saliva IgA: A cut-off value of 0.27 for saliva IgA-St-Ag gave 64% sensitivity of and 74% specificity. A cut-off value of 0.24 for saliva IgA-alginate gave 55% sensitivity and 74% specificity. When the cut-off values of the two saliva IgA tests were combined, CF+P(i) could be discriminated from CF-P with 72%, sensitivity, 74%, specificity, 87% pvpos, and 52% pvneg .

Nine sputum samples and 17 nasal secretions were obtained from CF-P. In general, the antibody levels were higher in CF-P than in CF-GNB but lower than in CF+P(c).

There was a significant correlation between IgA-alginate in nasal secretions and saliva ($\rho=0.60$) and between IgA-St-Ag in nasal secretions and saliva ($\rho=0.51$) from the seventy-five CF patients where these values were available (P-values<0.0001).

In eighteen of 20 CF-P patients (90%), sputum samples with *S. aureus*, *H. influenzae*, *S. pneumoniae* or *Aspergillus* spp. were cultured. There was agreement in 12/20 (60%) of the nasal irrigation cultures and in 9/20 (45%) of the nasal swabs from the middle meatus cultures when compared with the sputum bacteriology. In one CF-P patient, *S. maltophilia* were found in sputum and nasal irrigation but these bacteria were not found by culture from the middle meatus swabs.

By immunohistochemistry examinations we found excessive amounts of IgA producing plasma cells in the sino-nasal tissue. They were mostly found in the connective tissue of the lamina propria (Fig. 1C), with higher concentration in focal areas close to the mucosal surface (Fig. 1D). Accumulations of the IgA producing cells were also seen around the secretory acini (Fig. 1E). IgA was also detected in the layer of mucus on the luminal mucosal surface (Fig. 1D) and in the secretions in the excretory ducts from the submucosal glands (Fig. 1F).

4. Discussion

We have previously reported that CF+P(c), in contrast to healthy persons, had high levels of IgA against *P. aeruginosa* alginate and St-Ag in sputum, saliva and tears and that the IgA contained a secretory component (sIgA) [4]. The high ratio of IgA in saliva, tears, and sputum versus serum also showed a local IgA production in contrast to the low ratio of IgG in the secretions versus serum in accordance with the systemic production of this immunoglobulin class [4]. The specific IgA response to *P. aeruginosa* in these secretions reflects the common mucosal immune response to offending microbes. Since our previous report (4;), *P. aeruginosa* sinusitis has attracted increasing attention as a focus for subsequent lung colonization and chronic infection [6,9,11–14,20,22]. Thus, we have expanded the study [4] in order to evaluate whether sIgA against *P. aeruginosa* can contribute to the diagnosis of *P. aeruginosa* sinusitis before onset of the chronic lung infection. In another article, more details are given about the bacteriological and clinical results from functional endoscopic sinus surgery on a large number of CF patients with sinusitis [6].

In the present study we also investigate IgA against *P. aeruginosa* alginate and St-Ag in saliva and serum, but in contrast to our earlier study [4] we now include nasal secretions and a greater number of CF patients, now subdivided according

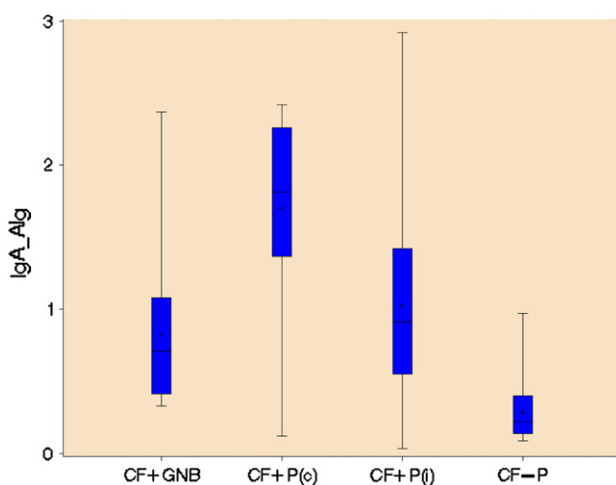


Fig. 2. A Box-and-whisker plot of the IgA values (OD) against alginate in nasal secretions distributed on four infection categories, showing the 2½, 25, 50, 75 and 97½% percentiles. CF+P(c), CF+P(i) and CF-P all differed significantly (P<0.0001) from each other.

to the lung infection status. Significant differences of IgA levels to the *P. aeruginosa* antigens were seen between both CF+P(c), CF+P(i) and CF-P. Accordingly, we also found an abundance of local IgA producing plasma cells in the nasal mucosa of CF+P(c) CF+P(i). Many plasma cells have previously been found in sinonasal tissue from CF patients [23]. Importantly, our results allow a differentiation between CF+P(i) and CF-P. Nevertheless, the test is limited by the possibility of cross-reaction with other pathogenic Gram-negative bacteria (e.g. *S. maltophilia*, *A. xylosoxidans* or *B. cepacia complex*) [15]. We conclude that the sensitivity, specificity, pvpos, and pvneg of each of the results of the IgA ELISAs or combined ELISA results are high enough to be evaluated as a diagnostic tool for *P. aeruginosa* sinusitis in a prospective study in our CF centre.

IgA concentration is in general elevated as a result of the local mucosal antibody response, whereas IgG is elevated as a consequence of the systemic inflammatory response [4,7]. Our results indicate an early, local production of IgA in the sinuses when these are infected with *P. aeruginosa*. This assumption is fortified, as we did not find *P. aeruginosa* in the nasal irrigation or middle meatus cultures from CF-P, which indicates that the sinuses are also free from *P. aeruginosa* [22,24]. This result can be compared with our previous findings of pathogenic bacteria in the majority of the sinuses in CF+P(i) and CF+P(c) [6].

Our results are therefore in accordance with our previous findings of high IgA levels in saliva, tears and sputum [4] and additionally show that nasal secretions contain even higher levels. This is in agreement with the increasing evidence of *P. aeruginosa* sinusitis being a focus for subsequently intermittent lung colonization and infection [6,9,11–14,22]. When *P. aeruginosa* settles in the paranasal sinuses, a local increase of mucosal IgA response is initiated. However, the inflammatory response is reduced compared with that of chronic lung infection since the secretory IgA is non-phlogistic in contrast to IgG which forms immune complexes with *P. aeruginosa* antigens and activate complement and attract PMNs in the respiratory zone of the lungs [1,6,10]. The result may be that IgA keeps *P. aeruginosa* at a distance from the cells of mucosal membranes [25] and thereby diminishing the clinical symptoms of sinusitis [10]. The same mechanism may be working in the conductive airways of CF+P(c), where most *P. aeruginosa* are located inside sputum together with the highly active PMNs [1,26,27], probably also due to IgA in mucosa and mucus in the conductive airways [4]. Keeping the bacteria at a distance from the mucosal membrane cells may be a common strategy in the respiratory and intestinal tracts [25] by means of the mucus layer and the anti-bacterial molecules such as IgA which prevent inflammation. *P. aeruginosa* sinusitis in CF may therefore resemble secretory otitis media with effusion where bacterial biofilms and IgA have also been found [28–30].

There are no standardized guidelines for detection of upper airway *P. aeruginosa* colonization or how or when to eradicate CF-pathogenic bacteria from the CF sinuses by sinus surgery and/or with antibiotic treatment [24,31]. The potential of surgical and/or conservative antibiotic treatment for eradication of *P. aeruginosa* sinusitis requires further prospective evaluation; for now an early

stage attempt of eradicating *P. aeruginosa* may be considered when detected in the sinus [6,9,11,12,22,32].

A prospective study is planned to examine if the IgA response in nasal secretion or saliva is helpful for early diagnosis of *P. aeruginosa* sinusitis. It would also be relevant to investigate whether IgA in nasal secretions, collected through a gentle nasal lavage, is an easier way to collect IgA or if the method is complicated by dilution problems. Nasal lavage is already used as a supplementary diagnostic tool for CF infections [22], and as a research tool for rhinitis [33].

5. Conclusions

Both CF+P(c) and CF+P(i) have significantly higher IgA antibody levels in nasal secretions and saliva against *P. aeruginosa* alginate and St-Ag compared to CF-P. In CF+P(i) the elevated IgA probably reflect *P. aeruginosa* sinusitis where the bacteria adapt to the environment of CF airways CF patients; sinusitis may then be the focus for subsequent intermittent *P. aeruginosa* colonization and chronic infection of the lungs. The diagnostic value of IgA in saliva and nasal secretions for sinusitis will be studied prospectively.

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Paper III

The effect of sinus surgery with intensive follow-up on pathogenic sinus bacteria in patients with cystic fibrosis

AQ:1 Kasper Aanaes, M.D.,¹ Christian von Buchwald, D.M.Sc.,¹ Thomas Hjuler, Ph.D., M.D.,¹ Marianne Skov, Ph.D., M.D.,³ Mikkel Alanin, M.D.,¹ and Helle Krogh Johansen, D.M.Sc.²

ABSTRACT

Background: Most patients with cystic fibrosis (CF) have chronic rhinosinusitis; their sinuses are often colonized with bacteria that can initiate and maintain deleterious pulmonary infections. Theoretically, eradication of the sinus bacteria should reduce the frequency of lung infections and thereby reduce pulmonary morbidity. This article addressed whether bacteria in CF sinuses are eligible for eradication by sinus surgery and postoperative treatment.

Methods: A prospective study including 58 CF patients, who had extensive sinus surgery and growth of *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, and/or *Burkholderia multivorans* in their sinuses, was initiated. All patients followed a systematic postoperative treatment program of nasal irrigations with saline and colistimethate sodium and systematic endoscopic cleansing. All patients had follow-up examinations including sinus cultures; each side of the nose was cultured separately.

Results: At the 6-month follow-up visit, 49 patients were cultured; 66 of 98 maxillary-ethmoidal complexes (67%) showed no growth of pathogenic bacteria. Some patients were not free from CF pathogenic bacteria at all cultures; however, 20 (41%) patients had no bilateral regrowth ($p < 0.01$) and 4 patients had no unilateral regrowth at any time during 6 months of follow-up. Both patients intermittently lung colonized as well as chronically infected and in lung transplanted could show no regrowth of CF pathogenic bacteria in the sinuses. The patient with the longest follow-up had no bacterial growth for 3 years.

AQ:2 **Conclusion:** Extensive sinus surgery combined with intensive follow-up can eradicate pathogenic bacteria from CF sinuses.

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AQ:3 **R**hinosinusitis is present in patients with cystic fibrosis (CF) due to defective CFTR channels in the sinonasal mucosa.^{1,2} This gene defect causes viscous mucus, making CF patients susceptible to paranasal sinus and lung infections with CF pathogenic Gram-negative bacteria, *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, and *Burkholderia multivorans* causing the vast majority of lung morbidity and mortality. CF sinuses are often colonized with concordant bacteria, as are the lungs.³ The bacteria adapt to the CF environment in the sinuses and successively develop into different phenotypes capable of infecting the lungs.⁴ As a result of impaired mucociliary clearance, blockage of the sinuses, biofilm formation, low antibiotic bioavailability, and secretory immunoglobulin A immune response, eradication of sinus bacteria is difficult.⁵⁻⁷

There is no consensus on whether sinus surgery has an impact on subsequent lung infections.⁸⁻¹⁴ Theoretically, bacterial eradication by sinus surgery can reduce the migration of bacteria from the upper to the lower airways, reducing morbidity. Nevertheless, whether pathogenic bacteria in CF sinuses are eligible for eradication has, to our knowledge, never been addressed in nonlung transplanted (LTX) CF patients. We performed a prospective, observational, nonrandomized study, investigating peri- and postoperative sinus cultures in a cohort of CF patients undergoing extensive functional endoscopic sinus surgery (FESS), combined with intensive postoperative local and systemic medical treatment; we focused on whether bacteria that are pathogenic in CF lungs are potentially eradicated from the sinuses.

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MATERIALS AND METHODS

Patients

CF patients from the CF Center Copenhagen undergoing FESS during 2009-2011 were eligible for the study. Patients were selected for FESS based on the following criteria: (1) intermittent lung colonization with declining lung function, despite intensive antibiotic-chemotherapy, and/or increasing antibodies against CF pathogenic Gram-negative bacteria; (2) patients who had undergone lung transplantation within 1 year; (3) patients with severe symptoms of rhinosinusitis in accordance with the European position paper on rhinosinusitis and nasal polyps.¹

FESS Procedures

The purpose of surgery was creating ventilation and drainage of the sinuses, making them accessible for postoperative cleaning and medical irrigations. Preoperatively, patients were evaluated for symptoms¹ and had a clinical examination and a CT scan of the sinuses. We applied classic image-guided FESS comprising uncinectomies, anterior ethmoidectomies, and medial antrostomies, leaving significantly enlarged maxillary ostia comprising more than one-half of the medial maxillary wall as recommended.¹⁵⁻¹⁹ Accessibly swollen and inflamed sinus mucosa was removed. The frontal and sphenoid sinuses were entered in the majority of cases. Finalizing the surgical procedure, the sinuses were irrigated with sterile saline and colistimethate sodium.

Perioperative Samples

To optimize culture results, multiple samples for culture were prioritized during surgery, including nasal secretions, pus, mucosa, polyps, and bone. Samples taken from same anatomic location at the same side were cultured together. An average of 4.8 (3-8) different anatomic sampling locations was taken from each included patient. Samples taken for culture were collected with sharp instruments or by suction tubes, and the anatomic localizations were noted. The material obtained was immediately cultured.

Postoperative Regimen and Samples

All patients followed a postoperative regimen: (1) 2 weeks of broad-spectrum i.v. antibiotics against the expected bacteria; (2) min-

Table 1 Number of unilateral sinus cultures with CF pathogenic growth according to time after FESS

Lung Status at Surgery	Perioperative	1 mo	3 mo	6 mo	12 mo
LTX	24 of 24 (100%)	8 of 24 (33%)	9 of 20 (45%)	11 of 22 (50%)	9 of 20 (45%)
Chronically infected	25 of 26 (96%)	8 of 24 (33%)	12 of 24 (50%)	13 of 26 (50%)	8 of 18 (44%)
Intermittently colonized*	55 of 66 (83%)	5 of 60 (8%)	11 of 60 (18%)	8 of 50 (16%)	12 of 48 (25%)

Fifty-eight patients (116 sinus complexes) with CF underwent FESS and follow-up during 2009–2012; each patient had separate sinus cultures taken from the left and right side. The postoperative frequencies all differed significantly from the perioperative ones ($p < 0.01$). *Intermittently colonized is defined as growth of CF pathogenic bacteria (*Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, and/or *Burkholderia multivorans*) in 1 to <50% of the monthly samples taken from the lower airways with in the last year.

CF = cystic fibrosis; FESS = functional endoscopic sinus surgery; LTX = nonlung transplanted.

Table 2 Number of patients with no growth and unilateral and bilateral CF pathogenic growth in cultures taken from the sinuses according to time after FESS among 58 patients with CF undergoing FESS and follow-up

	Perioperative	1 mo	3 mo	6 mo	12 mo
No. of patients with no growth	0 (0%)	40 (69%)	29 (50%)	25 (47%)	23 (50%)
No. of patients with unilateral growth	12 (21%)	7 (12%)	14 (24%)	16 (30%)	11 (24%)
No. of patients with bilateral growth	46 (79%)	7 (12%)	9 (16%)	8 (15%)	9 (20%)
Did not show for follow-up	—	4 (7%)	6 (10%)	4 (8%)	3 (7%)

CF = cystic fibrosis; FESS = functional endoscopic sinus surgery.

imum 6 months of topical nasal steroids (mometasone furoate); (3) minimum of 6 months of daily nasal irrigations with saline and antibiotics (colistimethate sodium when susceptible bacteria were cultured); and (4) visits to the ear, nose, and throat outpatient clinic, 1 week and 1, 3 and 12 months postoperatively, where crusts and secretions were endoscopically cleansed from the nose and sinuses. At each follow-up the patients were bilaterally cultured using endoscopic guidance. No local anesthetics were used. In the majority of patients, a 2- or 3-mm curved suction tube was used to enter and collect secretions from the maxillary sinuses, ethmoids, and/or frontal recesses. The left- and right-side samples taken for culture were treated separately. A cotton swab was wiped within each middle meatus in cases where the maxillary sinuses were not accessible as a consequence of mucosal swelling. The samples were immediately cultured. In some cases the samples were taken while the patient was under general anesthesia for other reasons. The strategy of antibiotic treatment was postoperatively unchanged and performed according to guidelines and susceptibility testing.²⁰

Microbiological Samples

Sinus samples were cultured aerobically and anaerobically at 37°C on standard agar media for 5–7 days as previously described.²¹

Ethics

The study was approved by the local ethics committee (H-A-2008-141). All patients gave informed consent. In patients <18 years of age, informed consent was obtained from their parents.

Statistics

A McNemar's test was used in SAS 9.1.3 to compare the postoperative frequencies of growth with the perioperative frequencies.

RESULTS

A total of 81 consecutive patients had FESS during 2009–2011. All had bacteria in their sinuses; 60 patients had growth of *P. aeruginosa*, *A. xylosoxidans*, and/or *B. multivorans* in their perioperative sinus samples. One patient was lost to follow-up and one patient only had pathogenic bacteria in the sphenoid sinuses and was excluded, because the sphenoid sinuses do not drain into the middle meatus. Consequently, 58 patients were included in our prospective study

(Tables 1 and 2). They had the following lung infection status at time of surgery: 12 LTX; 13 chronically lung infected; and 33 intermittently lung colonized with *P. aeruginosa*, *A. xylosoxidans*, and/or *B. multivorans*.²² In one patient, the bacteria found in the sinuses (*A. xylosoxidans*) were different from the pulmonary bacteria (*P. aeruginosa*); in the remaining patients the sinus bacteria were in accordance with the pulmonary bacteria the year before surgery or before LTX being as follows: 42 with *P. aeruginosa*, 13 with *A. xylosoxidans*, and 2 with *B. multivorans*. The majority of patients fulfilled criterion 1 or 2 for surgery, but 45 patients also fulfilled criterion 3. Except for one patient who only had pathogenic bacteria in one of her frontal sinuses, the 57 remaining patients had growth of pathogenic bacteria in their maxillary sinuses; in 12 patients the growth was only unilateral.

All patients attended at least two postoperative follow-up visits. The adherence to participation in the follow-up visits was high; at every postoperative follow-up, >90% of the patients attended (Tables 1 and 2). In addition, 14 patients were additionally cultured outside the planned schedule (9–36 months postoperatively).

Peri- and Postoperative Culture Results

Overall, 430 postoperative sinus samples were obtained and cultured; 123 of these had growth of CF pathogenic bacteria (*P. aeruginosa*, *A. xylosoxidans*, and/or *B. multivorans*). The results of the sinus cultures are shown in Tables 1 and 2 and described in the following paragraphs.

Initially, the 12 LTX patients (6 men and 6 women aged 21–45 years [mean, 33 years]) had bilateral CF pathogenic bacterial growth in their sinuses, in accordance with their lung bacteriology before transplantation. Five of these patients (eight sinuses) already had regrowth of CF pathogenic bacteria at the first postoperative culture after 1 month. At the 6-month follow-up, five patients only had unilateral regrowth, and three patients showed no bilateral regrowth. This was a significant decrease when compared with the perioperative results ($p < 0.01$). One of these patients had the longest follow-up period of 36 months where no CF pathogenic regrowth was seen.

The 13 patients with chronic lung infections (10 men and 3 women aged 19–42 years [mean, 29 years]) initially consisted of 24 left and right sinus sides with pathogenic bacteria present at surgery. At the 6-month follow-up, 13 sinus complexes showed no regrowth of CF pathogenic bacteria, where 6 of these had no regrowth at any time during the 6–12 months of follow-up ($p < 0.01$).

All 33 patients with intermittent lung colonization (18 male and 15 female patients aged 6–43 years [mean, 17 years]), except for 1, had concordant bacteriology in their sinuses and lungs at surgery; the single exception was a girl intermittently lung colonized with *P. aeruginosa* but harbored *A. xylosoxidans* in several of her sinuses. In addition, two patients who were intermittently lung colonized with *P. aeruginosa* also harbored *A. xylosoxidans* in their sinuses together with *P. aeruginosa*. At the 1-month follow-up, only four patients had one or more sinus samples that showed regrowth. Of the patients who were followed for 6 months, 52 of 60 sinus complexes had no CF pathogenic growth ($p < 0.01$); 37 sinus complexes showed no pathogenic growth at any time during the 6 months ($p < 0.01$). Among the intermittently colonized patients, the longest follow-up without any regrowth was 28 months.

At the 6-month follow-up, 49 patients were cultured; 66 of 98 maxillary–ethmoidal complexes (67%) showed no growth of pathogenic bacteria. Some patients were not free from CF pathogenic bacteria at all cultures; however, 20 (41%) patients had no bilateral regrowth ($p < 0.01$) and 4 patients had no unilateral regrowth at any time during 6 months of follow-up.

Sampling Accuracy

Postoperative samples were obtained for culture eight times during a second general anesthesia because of revision surgery or other procedures; in these cases the quality of the sampling could be compared with the one during surgery. Eleven of 16 sinus complexes had no regrowth of pathogenic Gram-negative bacteria according to this thorough sampling. Six months after FESS, 84% of the maxillary sinuses were accessible and cultured. In the remaining 14%, samples were collected from the middle meatus and anterior ethmoidal cells. We did not compare our results with sinus cultures from nonoperated CF patients because such samples would have required general anesthesia.

DISCUSSION

We present the largest prospective cohort study, to date, of CF patients undergoing FESS. We showed that extensive FESS with postoperative treatment eradicated the CF pathogenic bacteria from the paranasal sinuses in many CF patients, in several patients for >1 year. Bacteria were most successfully eradicated in patients with intermittent lung colonization. In some cases, the bacteria were only unilaterally eradicated, giving weight to the proposition that the left and right sinuses should be regarded as two separate compartments.⁴ Our results show that sinus bacteria can be eradicated independent of lung infection status. Otolaryngologists have an important role in CF management in focusing on the upper part of the airways; we suggest that this include regular endoscopic guided sinuscopy, and obtaining of representative material for culture in operated as well as in nonoperated patients.

No standardized guidelines comprising criteria for FESS or postoperative treatment exist for patients with CF.^{1,23} We offer FESS as a supplementary diagnostic tool and a method to attempt eradication of bacterial sinus infections. This methodology has been introduced as a consequence of the general recognition of bacterial sinusitis as a focus for intermittent colonization of the lungs.^{3–5} The present study shows that extensive FESS with postoperative treatment can eradicate CF sinus bacteria; nevertheless, we stress that intensive postoperative treatment is mandatory to obtain this achievement. It is evident that saline nasal irrigations alleviate symptoms of chronic rhinosinusitis and is broadly used postoperatively together with endoscopic removal of nasal crusts as we did in this study.^{1,11,19,24,25} Other studies have reported use of nasal irrigations with antibiotics or suggest it to have efficacy in chronic rhinosinusitis treatment.^{8,24–26} Colistimethate sodium is broadly used as inhaled therapy in CF patients with *P. aeruginosa* or *A. xylosoxidans* lung infections. Thus, we used this drug for nasal irrigations when the bacteria were susceptible. Nasal irrita-

tion was the only side effect reported. Because this treatment strategy has a low level of evidence, the dosage, administration intervals, and drug(s) of choice should be considered when making postoperative guidelines for CF patients. Overall, there is no evidence for using topical steroids in patients with CF and nasal polyposis.²⁷ However, we recommend topical steroids as a part of the postoperative treatment, because they may reduce inflammation and the size of polyps and have limited side effects.²⁷ Adding baby shampoo to the saline,^{28,29} using nebulizers for irrigations, and use of mucolytics^{30–32} are all modalities that have been suggested by others as postoperative treatments.

The impact of FESS on CF lung bacteriology has been previously addressed.^{8–14} Apart from our study, Holzmann *et al.*⁸ and Lewiston *et al.*,¹¹ who included only LTX patients, are the only groups conducting prospective studies aimed at the implementation of FESS for eradication of bacteria, thus making the surgery and postoperative care more extensive. Consequently, these two studies showed the most promising results. As in a study by Holzmann *et al.*,⁸ we explored the majority of sinuses during FESS, including those with minimal opacification, because CF sinuses may harbor CF pathogenic bacteria independent of the CT findings and the clinical examination.⁶

We found a striking concordance between sinus and lung bacteria, which is in agreement with studies showing coherence between bacteria genotypes and phenotypes in the sinuses and lungs in CF patients.^{3,4} Nevertheless, only one other study,⁸ focused solely on LTX patients, has addressed the possibility of eradicating CF sinus bacteria. They found that 54% of the LTX patients had three or fewer positive sinus cultures during follow-up after FESS, which strongly correlated with the absence of tracheobronchitis. This finding is in accordance with our results among CF LTX patients. By showing that bacteria can potentially be eradicated from the paranasal sinuses, we show that extensive FESS with postoperative treatment may reduce lung morbidity in patients intermittently lung colonized, by preventing bacteria from the sinuses infecting the lungs. Whether sinus bacteria can be used as a surrogate marker for the positive effect of FESS still needs to be elucidated. Covariates as pulmonary function test, quality of life, antibody levels, and frequency of lung colonizations should be taken into account.

Our study is strengthened by having a prospective design, a large adherent cohort, a minimum of dropouts, and the same FESS surgeon performing the follow-up visits, as well as having a standardized program for a thorough sampling for culture during surgery. However, there were two limitations in our study. Our study was observational, because we found it unethical to include a control group who would not receive any treatment. Second, although some of the postoperative samples were obtained during cleansing under general anesthesia, and some of these did not show any bacterial regrowth, the majority of samples were obtained for culture in our outpatient clinic. Although being thorough, the value of a negative culture sample obtained by suction tube versus a biopsy during general anesthesia will always be questionable; it is uncertain whether cultures of secretions will reveal presence of bacteria, especially when patients are doing nasal irrigations with antibiotics or if the bacteria only were present embedded in submucosal abscesses or biofilm. Thus, when stating that we eradicated the bacteria from the paranasal sinuses, we at least reduced the quantity of colony-forming units, so the bacteria could not be redetected by sinus cultures for longer periods.

CONCLUSION

Our study shows that extensive FESS combined with postoperative treatment in several cases can eradicate pathogenic bacteria from the sinuses, especially in CF patients with intermittent lung colonization. This supports the hypothesis that FESS reduces the frequency of bacterial lung colonization.

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Paper IV

Title page:

Clinical Effects of Sinus Surgery and Adjuvant Therapy in Cystic Fibrosis Patients — Can Chronic Lung Infections be postponed?

Short title: Sinus surgery can reduce lower airway pathogens

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Keywords:

Cystic fibrosis; paranasal sinus surgery; lower airway cultures; *Pseudomonas aeruginosa*; quality of
life; pulmonary function

ABSTRACT

Background: In patients with cystic fibrosis (CF), the paranasal sinuses are often colonised by CF pathogens (*Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex). The sinuses may be a bacterial reservoir for pulmonary infections.

Objectives: In this Prospective, non-randomised, uncontrolled, intervention cohort study, the aim was to assess the clinical effect of extensive endoscopic sinus surgery followed by two weeks' intravenous antibiotics, 6 months' antibiotic nasal irrigations, and 12 months' topical steroids.

Methods: 106 CF patients underwent extensive sinus surgery. Their data were pre- and postoperatively compared regarding: frequency of lower airway cultures positive for CF pathogens, spirometry, BMI, levels of specific serum IgG antibodies, symptoms of chronic rhinosinusitis, disease-specific health-related quality of life, and surgical complications.

Results: One year after sinus surgery, the prevalence of intermittently colonised patients had decreased by 38%, while the prevalence of non-colonised patients had increased by 150% ($p < 0.01$). The frequency of pulmonary samples with CF pathogens was reduced (38% before vs. 30% after; $p < 0.01$). Specific IgG against *P. aeruginosa* decreased after six months ($p < 0.05$). No effects were seen on BMI or lung function, which remained stable. Additionally, the self reported symptoms of chronic rhinosinusitis and quality of life improved ($p < 0.05$). No irreversible surgical complications were seen.

Conclusions: Combined extensive sinus surgery and postoperative systemic and topical antibiotic treatment significantly reduced the frequency of pulmonary samples positive for CF pathogens in the first year after sinus surgery. Moreover, we substantiate that sinus surgery relieves symptoms of chronic rhinosinusitis in CF patients and improves quality of life.

INTRODUCTION

A marked association exists between upper and lower airway cultures in patients with cystic fibrosis (CF)[1-3] due to the paranasal sinuses often being colonised by CF-lung-pathogenic Gram-negative bacteria [2;3]. The environment in the sinuses and the lower airways is similar, [1;4;5] albeit the sinus colonisations are dominated by the non-inflammatory IgA antibodies and lack of polymorphonuclear leukocytes [3;6]. The sinuses can therefore be an evolutionary ‘nest’ in early airway colonisations, where the bacteria diversify, evolving antibiotic resistance and other phenotypes associated with adaptation to the CF airways in general. Subsequently the bacteria migrate and intermittently colonise the lungs and may ultimately cause chronic lung infections [2-4;7]. Likewise, the sinuses in lung transplanted (LTX) CF patients are regarded as a bacterial reservoir, causing lung allograft infections and rejections [8].

CF pathogens can potentially be eradicated with extensive functional endoscopic sinus surgery (FESS) and postoperative antibiotic treatment [9;10]. It has been hypothesized that FESS can reduce the frequency of lung colonisations and may postpone chronic lung infections [2;3]. Nevertheless, large-scale prospective studies on the effects of FESS on lung colonisation and infection in CF are lacking [1], and data on surgical therapy for CF patients with chronic rhinosinusitis (CRS) are primarily level III evidence [1;11]. Thus, the aim of this prospective cohort study was to assess various clinical effects of extensive FESS followed by topical steroids and intravenous and nasal antibiotics.

MATERIAL AND METHODS

Study population

Patients were recruited among the 300 CF patients treated at the CF Centre in Copenhagen. The diagnosis of CF was based on clinical characteristics, abnormal sweat electrolytes, and genotype. Patients were recruited among the 300 CF patients treated at the CF Centre in Copenhagen. The diagnosis of CF was based on clinical characteristics, abnormal sweat electrolytes, and genotype. CF patients followed a routine protocol with monthly medical examinations including lung function tests and lower airway samples taken for microbiological culture. Additional lower airway samples were taken whenever patients were hospitalised or when clinical and/or paraclinical parameters indicated a risk of lung colonisation or infection. Approximately every third month, blood samples were taken for analyses including specific antibodies against relevant Gram-negative bacteria [12]. LTX patients followed a different outpatient setting with fewer routine samples taken. All CF pathogens were treated with antibiotics regardless of clinical symptoms according to the Copenhagen CF centre's treatment protocols [13].

Grading of pulmonary infection

Modified Leeds criteria[14] were employed:

1. Non-infected: no growth of CF pathogens (*Pseudomonas aeruginosa*, *Achromobacter xylosoxidans* or *Burkholderia cepacia complex*) over 12 months **(CF-)**.
2. Intermittently colonised: growth in >0% and ≤ 50% of samples **(CF+(i))**.
3. Chronically infected: growth in >50% of a patient's monthly lower airway samples **(CF+(c))**.

Design

A prospective, non-randomized, uncontrolled, intervention study was conducted over 3½ years including CF patients aged more than six years with FESS from July 2007 to January 2012. Those included had approximately one year of follow-up with postoperative treatment, lower airway samples cultured, spirometry, body weight, serum samples, and questionnaires (Figure 1). These data were compared with preoperative data from the same patients extracted from our CF database. In patients having revision surgery, only the outcome of the primary extensive FESS was included in the calculations, except for four cases where no CF pathogens were detected in the lungs the postoperative year; in these cases revision surgery performed more than a year later was included instead.

During the study period, the protocol was supplemented by additional clinical outcome measures: the sinonasal outcome test (SNOT-22) was included from May 2009, the Cystic Fibrosis Questionnaire-Revised (CFQ-R) questionnaire from November 2009, and bronchoalveolar lavage (BAL) during FESS from December 2009.

Inclusion criteria

The patients were selected for FESS based on the following criteria:

- 1: Search for an infectious focus: (CF+(i)) patients with increasing frequency of positive lower airway cultures or repeatedly declining lung function (> 10%), despite intensive antibiotic chemotherapy. Patients with an unknown infectious focus and increasing antibodies against *P. aeruginosa*, *A. xylosoxidans* or *B. cepacia complex* were given the highest priority.
- 2: Patients who had recently been LTX.
- 3: Patients with severe symptoms of chronic rhinosinusitis (CRS) according to the European Position Paper on Rhinosinusitis [1].

Perioperative BAL and functional endoscopic sinus surgery (FESS)

A BAL was performed under general anaesthetic. The subsequent FESS was to ventilate and drain the paranasal sinuses and to make these accessible for postoperative instrumental cleansing and irrigation with saline and topical antibiotics. Each patient was evaluated for symptoms of chronic rhinosinusitis[1] followed by a clinical examination. The extension of surgery (e.g., exploration of the frontal or sphenoid sinuses) was undertaken based on the preoperative CT scan and perioperative findings. As a standard, we applied FESS with an uncinectomy, an anterior ethmoidectomy and a medial antrostomy, leaving a significantly enlarged maxillary ostium comprising more than half the medial maxillary wall as recommended [1]. Visible intramucosal abscess-like structures (especially found in the maxillary sinuses) were resected along with other inflamed mucosal tissue when accessible. Following the surgical procedure, the opened and now accessible sinuses were irrigated with saline and colistimethate sodium.

Bacteriology

To optimise sinus culture results, multiple perioperative samples were obtained including nasal secretions, pus, mucosal tissue, polyps, and bone. Samples were collected with sharp instruments or by suction. Sinus and lower airway samples were cultured aerobically and anaerobically at 37 °C on standard agar media for 5–7 days as previously described [15].

Postoperative treatment

Postoperative adjuvant therapy included: two weeks of IV antibiotics [13], at least 6 months of twice daily nasal irrigation with saline and antibiotics (starting Day 1 with colistimethate sodium but could be adjusted according to susceptibility), and 12 months of topical nasal steroids (mometasone furoate). As a standard each patient had four postoperative visits to the ENT outpatient clinic where crusts and secretions were endoscopically cleansed (Figure 1).

Lower airway cultures

The percentage of the lower airway samples taken monthly and positive for CF pathogens (*P. aeruginosa*, *A. xylosoxidans* or *B. cepacia complex*) 365–0 days before FESS was compared with cultures taken 1–365 days after FESS. Additionally, the grade of pulmonary infection (CF-, CF+(i), CF+(c)) at surgery was compared with the grade one year postoperatively [14].

In patients who underwent LTX the year before FESS, only the days after the LTX were evaluated. If more than one CF pathogen was cultured, only the most frequently cultured bacterium was evaluated. We did not differentiate between how the lower airway samples were obtained (expectoration, endolaryngeal suction or BAL).

Spirometry

Spirometry was performed at every clinical visit. The average of all measurements expressed as percent of predicted values [16;17] and trend slopes of FVC and FEV₁ were calculated 6 and 12 months pre-and postoperatively in 104 patients (two LTX patients had no postoperative values).

Body mass index

Body mass index standard deviation scores (BMI z-scores) [18] were calculated at every clinical visit. The average percentages and z-BMI trend slopes were calculated 6 and 12 months pre-and postoperatively.

Serum antibodies

Elevated levels of specific anti-Pseudomonas IgG antibodies measured by ELISA is a risk factor for developing chronic *P. aeruginosa* infection [19]. Averages of anti-Pseudomonas IgG values measured 6 and 12 months pre-and postoperatively were compared in patients with no chronic infection.

SNOT-22

The SNOT-22 questionnaire focuses on sinonasal conditions [20]. It contains 22 items graded from 0 (no problem) to 5 (problem as bad as it can be). Some items are specific while others are more general, for example cough and fatigue. We initiated a back and forth translation from English to Danish according to accepted international criteria.

Health-related quality of life (HRQOL)

The Cystic Fibrosis Questionnaire-Revised (CFQ-R) measures disease-specific HRQOL [21]. The questionnaires and domains can be viewed at: http://www.psy.miami.edu/cfq_QLab. Two questionnaires were used: one for patients aged more than 13 years (maximum score of 201), and one for those aged 6–13 years (maximum score of 177). As we were interested in the changes over time and not the actual score, the scores of the two questionnaires were analysed together.

Ethics

In consultation with the local ethics committee, it was determined that only approval of the additionally BAL was needed. The study was approved by the local ethics committee (H-A-2008-141). All patients gave informed consent, for patients <18 years of age, consent was also obtained from their parents.

Statistics

SAS 9.1.3 was used for statistical analyses. The data were continuous and in all cases the differences between the pre- and postoperative data had an approximately normal distribution. The data fulfilled the criteria for normality and equal variance. A two-sample paired t-test or an ANOVA test was used to compare the pre- and postoperative data.

When comparing the change in lung infection status, the nominal data were analysed by McNemar's test (two categories were merged).

RESULTS

Study population characteristics

Demographic data on the 106 included patients are shown in Table 1 and their criteria for FESS appear in Figure 2; in the study period, 21 patients had surgery twice and 4 had surgery three times; 4 patients had revision surgery during the first postoperative year because of a satisfactory but transient effect of the primary surgery.

The average time between LTX and FESS was 3.5 years ($\frac{1}{2}$ –11 years). None of the patients had LTX during the year after FESS.

Upper airway culture results

All but one patient had bacterial growth from at least one sinus in their perioperatively obtained samples, but only 8 patients showed fungus isolates (*Aspergillus*, *Candida species*, and unidentified yeast). In 71 patients (67%) the same type of CF pathogen was perioperatively cultured from the sinuses and in the lungs the previous year. In 35 patients (33%) there was no correlation between lung and sinus bacteriology; these also included one (CF+(i)) patient with *A. xylosoxidans* in the sinuses but *P. aeruginosa* in the lungs, one (CF+(c)) patient without CF pathogens in the sinuses, and 4 (CF-) patients (2 LTX) where *P. aeruginosa* was found only in the sinuses.

Lower airway culture results

The one-year prevalence of CF+(i) patients decreased by 38% (CI: 24%–51%) after FESS (58% at surgery compared with 36% one year postoperatively; $p < 0.01$). The one-year prevalence of (CF-)

patients increased by 150% (CI: 71%–310%) after FESS (15% at surgery compared with 38% one year postoperatively; $p<0.01$) (Table 2).

The frequency of positive pre- and postoperative lower airway cultures in grades of pulmonary grades is illustrated in Figure 3 and shows a significant decrease in occurrence of CF pathogens in the postoperative samples. In subgroup analyses, the significant reduction of positive lower respiratory cultures was seen in the (CF+(i)) patients with simultaneous growth of CF pathogens in their sinuses and lungs (27% vs. 16%; $p<0.01$), and in (CF+(c)) patients (87% vs. 76%; $p<0.05$); the latter mainly due to four successful cases shown in Table 3. There was a high correlation between the number of positive lower airway cultures and the number of days from a positive culture to the next negative culture ($\rho=0.93$; $p=0.01$).

Spirometry results

A negligible, but nevertheless, significant decreased was seen in FVC and FEV₁ the year after FESS (Table 4).

Body mass index results

No significant changes were seen in the BMI z-scores (Table 4).

Specific serum antibody levels

Specific anti-Pseudomonas IgG antibodies decreased significantly during the first 6 postoperative months; however, the decrease was non-significant within 12 months after FESS (Table 4).

SNOT-22 results

A decrease was seen in sinonasal symptoms 3 and 12 months after FESS ($p<0.01$) (Table 5). As expected, cough, waking up tired, and fatigue were rated high, but nasal blockage, facial pressure and sense of smell/taste were also highly rated and improved after 3 months ($p<0.01$). After 12 months an improvement could still be detected in nasal blockage and facial pressure ($p<0.01$).

Quality of life results

An increase in HRQOL was seen 6 months postoperatively ($p < 0.05$). However, at 12 months postoperatively no significant differences in HRQOL were observed (Table 5).

Surgical complications

No perioperative major blood loss was observed. One patient was hospitalized three days after surgery because of epistaxis, one patient experienced a month of paresthesia of the skin below the eye and discomfort when looking to the side (the orbit was not entered during surgery), one patient had a watery eye requiring intervention, and one patient had an accidental penetration of the orbital lamina (with unaffected vision). Other than one patient who experienced decreased sense of smell (surgically unexplained), no persistent sequelae were noted.

DISCUSSION

In this prospective cohort study, we present data from a large cohort of CF patients undergoing extensive FESS and adjuvant therapy. A significant reduction in the frequency of lower airway cultures with CF-pathogens accomplished after FESS is shown for the first time. Subsequently, the proportion of intermittently colonised (CF+(i)) patients decreased as a consequence of the increased number of non-infected (CF-) patients. Specific IgG against *P. aeruginosa* decreased and quality of life including sinonasal symptoms was improved. Only a small decrease in lung function was seen after a year, which is in agreement with an annual average decline of 1–2% in CF patients [22].

Other studies have addressed the effect of sinus surgery on CF lungs using varying parameters and showing inconsistent results [10;23-32]. Comparable to our study, one prospective study performed extensive sinus surgery intending to eradicate sinus bacteria in a group of 82 LTX patients [10], showing that *P. aeruginosa* and *B. cepacia complex* could be eradicated from the sinuses, resulting

in reduced lung allograft infections. *Shatz* [31] found decreased antibiotic use, a lower hospitalisation rate, and an increase in FEV₁ six months after FESS among 15 non-LTX patients. *Lewiston et al.* [27] postoperatively installed Tobramycin in the sinuses and reported a lower rate of *P.aeruginosa* in the lungs of 11 LTX patients. Other studies differ in several ways: as they were retrospective, most did less extensive surgery compared with our procedures, none focused on the grading of pulmonary infection and only few included intensive follow-up with use of nasally applied antibiotics. These studies found no postoperative reduction in lung colonisation; however, some found a slight improvement of lung function, lower hospitalisation rates, and reduced use of antibiotics [23;24;26;28;29;32;33].

In the present study, (CF+(i)) patients simultaneously sinus and pulmonary colonised experienced the greatest benefit from our treatment protocol; it is likely that the treatment protected the lungs in this group of ‘risk’ patients from subsequent recurrent lung colonisation and chronic infection. The challenge is to detect these specific patients and to estimate when they may benefit from FESS. Our results support previously published studies showing that LTX patients benefit from FESS [27;34], and further indicate that patients at the early stage of chronic lung infection with well-preserved lung function and low levels of specific antibodies, such as the patients in Table 3, may benefit from FESS. We put forward the possibility that these patients may be false positively categorized as (CF+(c)) since lower airway samples can be cross-contaminated by secretions from the upper airways; in these cases the antibody response has shown to be helpful in distinguishing (CF+(i)) patients from (CF+(c)) [12;19].

The SNOT-22 questionnaire is suitable as a disease-specific instrument in sinonasal research and is widely used in non CF patients [20]. We found that the questionnaire is a good tool for evaluating CRS symptoms; however, the risk of CF patients underreporting symptoms of CRS should be taken

into account [35]. The majority of patients reported decreased nasal discharge and decreased nasal blockage after FESS despite very low preoperative scoring of these two items.

The CFQ-R has undergone validity testing [36] and is a widely used HRQOL measure for CF. CRS affects quality of life [37] but the CFQ-R contains only one question on CRS. Nevertheless, in our population, CFQ-R improved six months after FESS.

All our results, both positive and negative, highlight the need for guidelines for when to offer CF patients sinus surgery and/or nasal care.

Although we did not address the need for revision surgery, our prospective study gives weight to the conclusions of the recently published European position paper on rhinosinusitis and fulfils the requirements for new studies [1]: “Future prospective studies are needed to further elucidate the role of medical and surgical therapy in CF patients with chronic rhinosinusitis (CRS), but the data on surgical therapy support the safety and efficacy of endoscopic sinus surgery”.

The strengths of our study are the large cohort, its prospective design taking multiple parameters into consideration, the extensive surgery done to eradicate CF pathogens, and, in particular, the unchanged CF treatment protocol throughout the intervention [13]. Longer follow-up times and larger cohorts with the possibility of randomising patients into different treatment groups are needed to further elucidate the role of medical and surgical therapy among CF patients. With this study design, the effect of FESS on pulmonary infections could not be sufficiently evaluated in (CF-) patients who had CF pathogens only in the sinuses and not in the lungs.

Our study is limited by the lack of a control group and the tardy initiation of the questionnaires.

Further, we were initially reluctant to explore all sinuses. Nevertheless, for each FESS procedure,

we aimed for extensive surgery with exploration of all sinuses with mega-antrostomies, now also recommended in the recently published guidelines[1]. Apart from nasal irrigations with saline in CF sinuses, there is low level evidence to support our postoperative adjuvant therapy [1]. The use of topical steroids, additional dornase-alfa[1], and choice of antibiotic drug(s) may be considered in the future.

In conclusion, extensive sinus surgery combined with postoperative antibiotic treatment significantly reduced the frequency of pulmonary samples positive for CF pathogens during the first postoperative year. Our results support FESS with adjuvant therapy, especially in intermittently colonised CF patients; however, guidelines are warranted for criteria for FESS based on clinical and paraclinical parameters.

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	Non-infected	Intermittently colonized	Chronically infected	Total
Male/female	8 / 8	29 / 32	12 / 17	49 / 57
Median age in years at FESS (range)	18 (6–44)	15 (6–50)	30 (11–46)	19 (6–50)
dF508 homozygous/ heterozygous/ other mutations	13 / 3 / 0	41 / 16 / 4	17 / 12 / 0	71 / 31 / 4

Table 1. Demographic data on the included 106 CF patients.

	Non-infected	Intermittently colonized	Chronically infected	Total
%-pathogenic sinus bacteria	25 %	72 %	95 %	72 %
Mean no. of lower airway cultures one year prior/ one year after FESS	10.4 (2–15) 10.4 (2–15)	13.5 (8–22) 11.2 (3–24)	14.1 (2–35) 12.3 (1–42)	13.2 11.4
Mean no. of lung function tests and z-BMI one year before / one year after FESS	9 (0–15) 8 (1–13)	13 (1–30) 11 (1–20)	10 (1–18) 10 (0–18)	12 10
Mean FEV ₁ 12/6 months before and 6/12 months after FESS (%)	82/80 79/79	85/86 86/84	72/72 70/68	81/81 81/79*
Mean FEV ₁ slope (%) 12/6 months before and 6/12 months after FESS	-4.33/-1.48 12.71/-0.89	-0.67/1.44 2.38/-1.41	0.57/5.63 -8.88/-1.33	-0.85/1.15 0.27/-1.33
Mean FVC 12/6 months before and 6/12 months after FESS (%)	93/91 91/91	95/95 96/94	85/85 84/83	92/92 92/91*
Mean FVC slope (%) 12/6 months before and 6/12 months after FESS	-4.05/-2.90 10.80/1.74	-1.16/0.22 -0.47/-1.04	0.62/6.37 -6.77/0.33	-1.11/1.27 -0.71/-0.52
Mean z-BMI 12/6 months before and 6/12 months after FESS	-0.67/-0.64 -0.65/-0.72	-0.05/-0.01 -0.04/-0.17	-0.45/-0.62 -0.46/-0.49	-0.25/-0.27 -0.25/-0.34
Mean IgG 12/6 months before and 6/12 months after FESS (No. of patients)	2.1/1.8 2.0/2.0 (10)	3.4/3.7 2.7/2.7 (54)		3.1/3.6 2.6*/2.6 (64)

Table 4. Pre and post-FESS obtained clinical data from the 106 included CF patients. *: P<0.05

	Pre-operatively	Three months	Six months	Twelve months
Patients who completed SNOT-22, (response rate), [mean time after FESS]	86	79 (92%) [103 days]		64 (80%) [367 days]
Mean SNOT-22	20	11*		14*
Patients who completed CFQ-R, (response rate), [mean time after FESS]	67		53 (79%) [168 days]	42 (69%) [369 days]
Mean (CFQ-R)				
6–13 years:	143		145	144
More than 13 years:	165		170*	163
Total:	156		162*	157

Table 5. Results of the questionnaires before and after FESS. *: P<0.05

Table 2. The distribution of 106 CF patients in different groups according to grading of pulmonary infection at FESS and the year **after** FESS; a significant increase of (CF-) patients and a decrease of (CF+(i)) is observed. CF-: non-colonised; CF+(i): intermittently colonised; CF+(c): chronically infected.

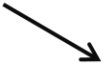
Lung infection status at FESS		Lung infection status a year after FESS	
CF- / 16 patients		CF- / 14 patients	
		CF+(i) / 2 patients	
		CF+(c) / 0 patients	
CF+(i) / 61 patients		CF- / 22 patients	
		CF+(i) / 35 patients	
		CF+(c) / 4 patients	
CF+(c) / 29 patients		CF- / 4 patients	
		CF+(i) / 1 patient	
		CF+(c) / 24 patients	

Table 3. An overview of four chronically infected CF patients showing a long postoperative period without re-growth of *P. aeruginosa* (PA) or *A. xylosoxidans* (AX) in the lower airway samples.

CF patient	Lung bacteriology	Sinus bacteriology	Precipitating antibodies against PA or AX prior to FESS	Mean PA IgG six months prior to FESS	Mean expected FEV ₁ six months prior FESS	Lower airway samples with PA or AX one year prior to FESS	First post FESS bacterial re-growth from the lower airways
11-year-old girl	PA	PA	0	1.15	97 %	62 %	523 days
16-year-old boy	PA	PA	2	2.64	94 %	60 %	593 days
22-year-old man. LTX	AX	AX	4	-	57 %	77 %	Still no re-growth after 982 days
31-year-old woman. LTX	PA	PA	4	5.75	60 %	75 %	508 days

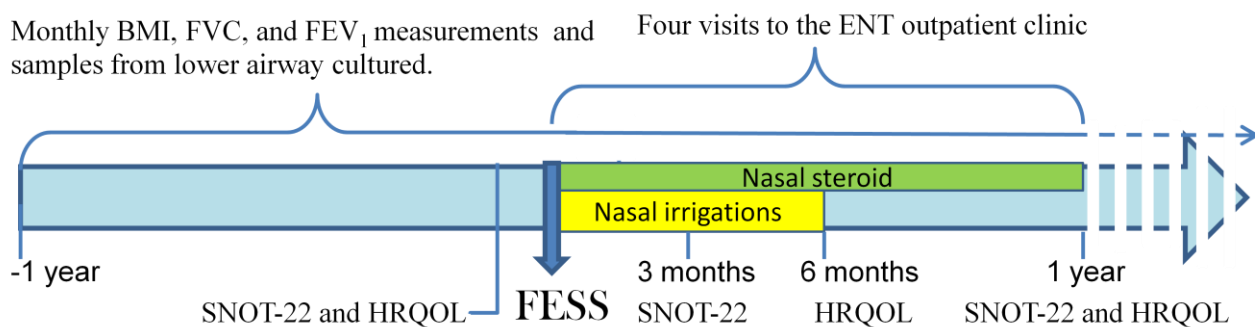


Figure 1. Time-line showing the interventions in the study.

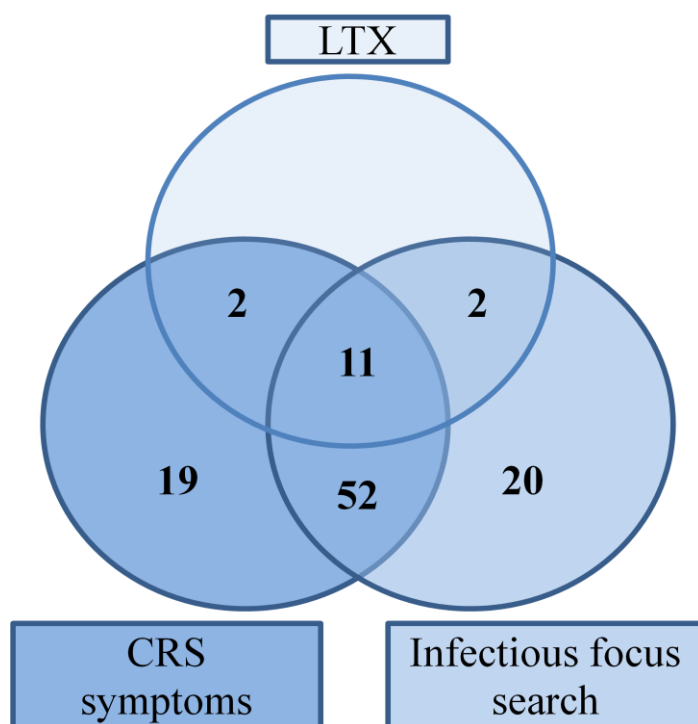


Figure 2. The criteria fulfilled for FESS in 106 CF patients undergoing surgery. (CRS: chronic rhinosinusitis; LTX: lung transplantation).

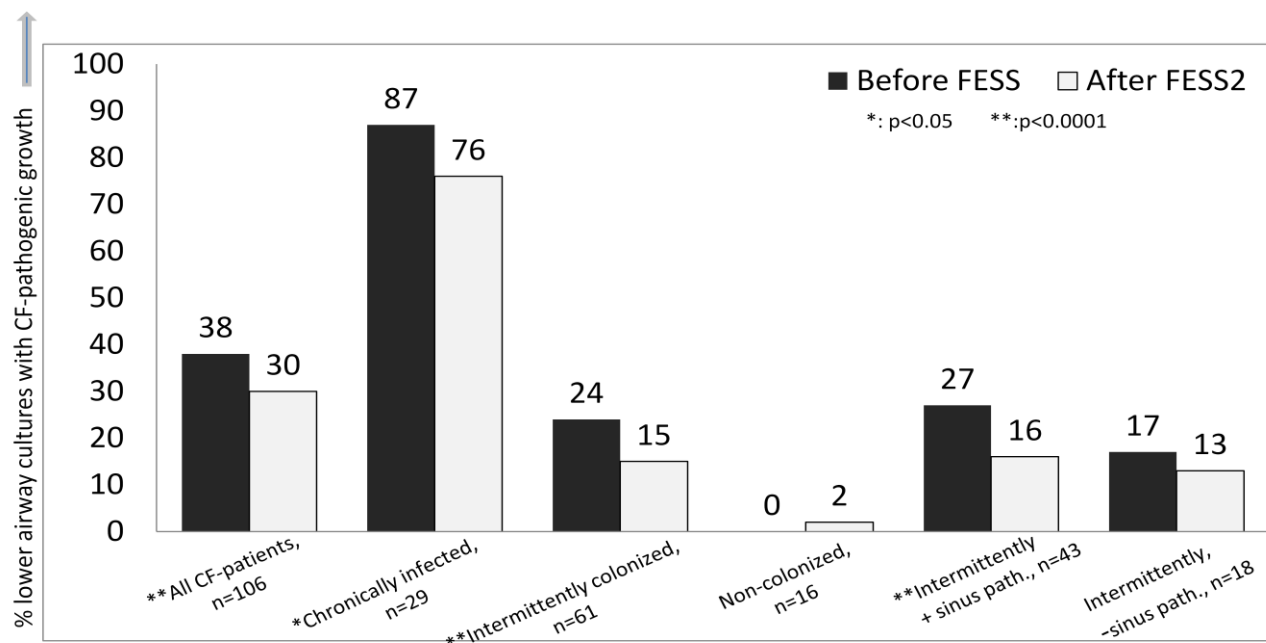


Figure 3. Frequencies of lower airway cultures with growth of CF-pathogenic Gram-negative bacteria a year before and after FESS, distributed on lung infection status and on the perioperatively bacterial findings in the sinuses.