Impact of graft colonization with gram-negative bacteria after lung transplantation on the development of bronchiolitis obliterans syndrome in recipients with cystic fibrosis

J. Gottlieb, F. Mattner, H. Weissbrodt, M. Dierich, T. Fuehner, M. Strueber, A. Simon, T. Welte

Department of Respiratory Medicine OE6870, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany
Department of Microbiology, Hannover Medical School, Hannover, Germany
Department of Thoracic and Cardiovascular Surgery, Hannover Medical School, Hannover, Germany

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Summary
Bronchiolitis obliterans syndrome (BOS) represents the leading cause of late mortality after lung transplantation (LTx). Cystic fibrosis (CF) patients frequently show airway colonization with gram-negative bacteria (GNB) both before and after LTx. Graft colonization with GNB and its relevance towards BOS development were investigated in a CF population after LTx.

Adult CF patients receiving LTx and surviving at least 6 months were included in this prospective observational study between 1/1/2002 and 30/6/2006 in a single center and followed until 31/3/2007. Pre- and post-LTx respiratory culture samples were compared for the presence of identical GNB. BOS-free survival was compared in colonized and non-colonized patients.

Fifty-nine adult CF patients with a median age at LTx of 25.5 (18–49) years were included and had a median follow-up of 966 (128–1889) days. Seven patients (15%) demonstrated immediate eradication of GNB in lower respiratory tract samples. A further 18 patients (34%) demonstrated transient colonization. Thirty-four recipients had further positive samples after LTx. Eighteen patients (31%) developed BOS stage 1, 508 (114–1167) days after LTx. Freedom of graft colonization with pseudomonads was independently associated with less frequent development of BOS (p = 0.006).

* Corresponding author. Tel.: +49 5115323560; fax: +49 5115323353.
E-mail address: gottlieb.jens@mh-hannover.de (J. Gottlieb).
Introduction

Lung transplantation is an accepted therapeutic option for patients with a variety of end-stage pulmonary disorders. Quality of life and short-term survival are improved with transplantation, but long-term survival is limited by the development of chronic organ dysfunction as the leading cause of late mortality. Bronchiolitis obliterans syndrome (BOS) is the clinical definition of chronic organ dysfunction and refers to a progressive obstructive ventilatory disorder. BOS affects approximately one-third of lung transplant recipients 3 years after lung transplantation, and half of all recipients after 5 years. It remains the leading cause of death after first post-operative year. Infections are potential risk factors for the development of BOS.

Lower airway tract colonization is a common phenomenon in patients with impaired pulmonary defences and airway remodeling, as in chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis (CF). Pseudomonas aeruginosa (PSA) and other non-fermenting bacteria are the dominant pathogens in CF patients presenting for lung transplantation. Four separate categories (free, never, intermittent or chronic) of chronic P. aeruginosa infection in children with cystic fibrosis (CF) have been previously defined, based on airway cultures taken over the previous year. Pseudomonad colonization remains detectable intermittently after lung transplantation in most recipients with CF after lung transplantation.

The aim of this study was to assess the impact of airway re-colonization with gram-negative bacteria in CF patients after LTx and to investigate the association to the development of BOS.

Methods

Transplant population

This cohort study was performed in a single university hospital with a high-volume lung transplant program and a large adult cystic fibrosis service. The recruitment period lasted from 1st January 2002 until 30th June 2006. Throughout this period, all adult cystic fibrosis patients (>18 years) who exhibited at least one confirmed isolate of GNB in the year prior to transplantation were included in the study. GNB was defined as isolation of at least one pseudomonad (P. aeruginosa, Burkholderia cepacia, Achromobacter or Stenotrophomonas). All patients exhibiting a post-operative survival of less than 6 months or undergoing re-do-transplantation were excluded. Additional exclusion criteria loss of follow-up and patients with less than five suitable post-operative culture samples.

All lung transplant recipients received frequent individual center-based follow-up care scheduled throughout the post-operative period with a maximum interval between assessments of 3 months. Follow-up data were recorded until 31st March 2007. At each follow-up visit, patients received comprehensive clinical examination. Quantitative assessment consisted of spirometry in conjunction with blood gas analysis, measurement of immunosuppressive drug levels and chest radiographs.

Spirometry was performed according to ATS/ERS guidelines. Baseline FEV₁ < 80% best after transplantation according to the International Society of Heart and Lung Transplantation (ISHLT) system. Baseline FEV₁ was defined as the average of the two highest measurements obtained at least 3 weeks apart during the post-operative course.

Immunosuppression

Standard maintenance immunosuppression consisted of a triple drug regimen including a calcineurin inhibitor, prednisolone, and a cell-cycle inhibitor or an mTOR (mammalian target of rapamycin)-inhibitor. Calcineurin inhibitor (CNI)-free protocols were not applied. Target trough levels of CNIs were gradually reduced to ciclosporin levels of 100–150 ng/ml after the second post-operative year and target trough levels for tacrolimus (EMIT, Behring, Germany) of 8–12 ng/ml. Prednisolone was tapered in all recipients during the first 3 post-operative months to a dose of 0.07–0.1 mg/kg afterwards. Long-term azithromycin (250 mg orally three times/week) was the standard of care for patients with BOS in this study while augmentation of immunosuppression was avoided.

No routine induction therapy was used. Between January 2003 and May 2005 eight patients were treated with daclizumab in a randomized controlled trial compared to placebo. The perioperative antibiotic regimen in CF patients undergoing lung transplantation in our program consisted of a post-operative intravenous combination regimen (according to preoperative susceptibility testing) for 2 weeks. Nebulized colomycin (two mega units, b.i.d for 3 months) was used for patients colonized with Pseudomonas pre-transplant. Sinus surgery was performed before transplantation or thereafter if clinically indicated.

Acute rejection (AR) therapy was defined as any steroid pulse (15 mg/kg methylprednisolone on 3 consecutive days). Episodes of AR during the first 4 post-operative weeks were excluded.
Sample acquisition

Pre-transplant respiratory samples were sputum cultures, no bronchoscopy was performed before transplantation. Bronchoscopy with BAL was performed on post-operative day 0, 5 ± 1, 21 ± 2 and 100 ± 7 on all patients included in the study. Further bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy was performed based on clinical decision at any time during transplant follow-up. Regular surveillance bronchoscopies were not part of our clinical program in patients beyond the first 3 post-operative months. Samples were taken from patients using a flexible fiberoptic bronchoscope introduced via the nasal route after cleaning the nostrils. Anaesthetics were instilled via a special catheter introduced through the working channel of the bronchoscope and removed after instillation.

BAL was performed according to standard recommendations. The bronchoscope was wedged into a sub-segmental bronchus of either the lingula or the right middle lobe. BAL was performed by instilling a total of 120 ml of isotonic saline solution, warmed to 37 °C in sequential 20-ml aliquots. The first 20-ml- aliquot was discarded and was not used for quantitative cultures. The fluid fractions were aspirated with gentle low-pressure suction into a sterilised silicon container. The fluid retrieved was pooled and the total recovered volume was measured. Samples of fluid were processed immediately for cytological, viral and microbiological analyses.

Other methods of lower respiratory tract sampling (sputum samples, aspirates or bronchial washing) were performed only if BAL was not tolerated or feasible. Preoperative isolates were retrieved from sputum samples and post-operative isolates from bronchoalveolar lavage (BAL), aspirates and/or bronchial washing. Quantitative cultures with counts of colony forming units per ml in BAL fluid (BALF) were performed routinely throughout the study period. All material sent to the laboratory was cultured according to routine diagnostic procedures. Serial dilutions from each BAL sample were prepared in sterile normal saline. For BAL culture, BALF was serially diluted onto different media depending on clinical suspicion. The presence of one or more bacterial colonies after 48 h of incubation was considered significant. Quantitative cultures were expressed as colony forming units (isolated, 10^2, 10^3, 10^4, 10^5/ml). Based on the results in previous studies, BALF was considered to be not or marginally contaminated, if the specimen contained <10^2 CFU patients of oropharyngeal flora, no ciliated epithelial cells, and <1% squamous epithelial cells.

All microorganisms isolated were identified by standard laboratory methods. The presence or absence of a mucoid phenotype on Pseudomonas isolation agar plates was recorded for each isolate. Antibigrams of all pseudomonads pre- and post-transplantation were compared for every isolate to identify identical GNB. Occasionally pulsed-field gel-electrophoresis was used as described previously to genotype GNB in cases demonstrating equivocal results. Identical GNB—colonization of the graft was defined by any growth of the same pre-transplant gram-negative bacterial pathogen in a respiratory sample. Colonization of the graft was defined by repeated detection on at least two occasions by pulmonary pathogens regardless of the presence of signs of infection. No cut-off value for quantitative BAL cultures was used. Loss of colonization was defined of three or more consecutive GNB-negative materials more than 14 days apart.

Ethics

Approval from the local Ethics Committee was obtained to conduct the investigation. Patients gave informed consent prior to inclusion into the study.

Statistical analysis

Data are reported as means (±standard deviation), time dependent variables are expressed as median (minimum and maximum). All reported p-values are two-sided, unless otherwise indicated. For all analyses, p-values <0.05 were considered statistically significant.

Univariate analysis: Categorical variables were analyzed by the Chi-squared test or Fisher’s exact test. Medians were analyzed with the Mann—Whitney test. Means were analyzed with Student’s t-test. Univariate analysis of risk factors for BOS was performed using the log rank test. Multivariate analysis included Cox stepwise forward regression analysis (for endpoint BOS-free survival). All variables with a p-value <0.10 were included and variables with a p-value of >0.10 were excluded in multivariate analysis.

Results

Patient characteristics are shown in Table 1. Group A (n = 25) consisted of seven of the 59 recipients remaining GNB-free from the outset along with 18 patients who were transiently colonized with identical GNB but subsequently cleared GNB in the following 224 (77—1173) days after LTx. Group B were those patients in whom intermittent or chronic GNB in respiratory samples were cultured (n = 34) (Fig. 1). No patient became GNB-negative after developing BOS during follow-up. Nine patients (group A, n = 5; group B, n = 4) demonstrated transient de-novo colonization with a second gram-negative pathogens after LTx.

One thousand four hundred and thirty-two respiratory samples in total and 532 BALF (37%) were collected after

<table>
<thead>
<tr>
<th>Table 1 Patient demographics (n = 59).</th>
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<tbody>
<tr>
<td>Age, years</td>
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<tr>
<td>Gender, female</td>
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<tr>
<td>Follow-up, days</td>
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<tr>
<td>Best FEV1, % predicted</td>
</tr>
<tr>
<td>Double lung transplantation</td>
</tr>
<tr>
<td>Liver—lung transplantation</td>
</tr>
<tr>
<td>Operation time, min</td>
</tr>
<tr>
<td>Cardiopulmonary bypass use</td>
</tr>
<tr>
<td>Minimal invasive surgery</td>
</tr>
<tr>
<td>ICU length of stay &lt; 96 h</td>
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<td>Pre-transplant mechanical ventilation</td>
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transplantation in 59 patients, averaging 24 samples (range 6–56) and 9 ± 5 BALF/patient. The majority of all samples (52%) contained no identical GNB. In samples with an identical GNB, 8% grew less than 10^2 CFU/ml, 15% 10^3, 11% 10^4 and 5% grew 10^5 colony forming units (CFU)/ml. The spectrum of isolated pre-transplant gram-negative pathogens is displayed in Fig. 2. Nine transplant recipients exhibited polymicrobial pre-transplant colonization, all of which contained *Pseudomonas* spp. (+ methicillin-sensitive *Staphylococcus aureus* n = 2, + methicillin-resistant *S. aureus* n = 2, + *Achromobacter* = 2, + *Pandorea* n = 1, + *Escherichia coli*, + *Stenotrophomonas* n = 1).

Eighteen recipients developed BOS after a mean of 508 (114–1167) days. The likelihood of remaining BOS-free after 1 year, 2 and 3 years was found to be 82, 78 and 63%, respectively, in the total cohort. The onset of BOS in patients harbouring an identical GNB was 671 (114–1054) days vs. 362 (191–1167) days in recipients without or transient colonization (*p* = 0.63). Twenty-three out of 59 patients (34%) required at least one treatment for acute rejection during the first 12 months. Recipients developing BOS had 1 ± 1 steroid pulses in the first post-operative year vs. 0.5 ± 0.8 in patients without BOS (*p* = 0.06).

Seven patients died 480 (128–855) days after LTx. The cause of death in three patients was sepsis, with a further two developing respiratory failure/BOS. One died following unrelated trauma and the remaining patient after re-do-transplantation.

Univariate analysis demonstrated significantly lower risk for the development of BOS in the recipients without early or late detection of identical GNB after LTx (Table 2). BOS-free survival was significantly better in patients without or with transient colonization (Fig. 3). In addition to acute rejection episodes during the first post-operative year, the loss of colonization (early or late) proved to be a significant covariate for BOS-free survival in multivariate Cox regression analysis (Table 3).

### Discussion

The present study provides further insight on the natural course of pre-transplant colonization early and late after LTx. The flow-chart of study design is shown in Fig. 1. Table 2 displays the univariate analysis comparing CF-patients losing GNB—airway colonization (group A, n = 25) and CF-patients further colonized (group B, n = 34).

### Table 2 Univariate analysis comparing CF-patients losing GNB—airway colonization (group A, n = 25) and CF-patients further colonized (group B, n = 34).

<table>
<thead>
<tr>
<th>Colonization &amp;</th>
<th>Further colonized</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>loss (n = 25)</td>
<td>(n = 34)</td>
<td></td>
</tr>
<tr>
<td>Daclizumab (%)</td>
<td>4 (16)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>Tacrolimus (%)</td>
<td>6 (24)</td>
<td>9 (27)</td>
</tr>
<tr>
<td>Everolimus (%)</td>
<td>3 (12)</td>
<td>3 (9)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> aeruginosa (%)</td>
<td>17 (68)</td>
<td>30 (88)</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em> (%)</td>
<td>2 (8)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Airway Stents (%)</td>
<td>3 (12)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>Rejection treatments during first post-operative year (%)</td>
<td>9 (36)</td>
<td>14 (41)</td>
</tr>
<tr>
<td>BOS (%)</td>
<td>4 (16)</td>
<td>14 (41)</td>
</tr>
<tr>
<td>Death (%)</td>
<td>1 (4)</td>
<td>6 (18)</td>
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</table>
lung transplantation in CF patients. Eighty-eight percent of all CF recipients harbouring GNB pre-transplant were colonized with an identical GNB after LTx. In identically transplant recipients with an identical pre-transplant pseudomonad, subsequent clearance of colonization occurred in one-third. Eighty-two percent of these transiently colonized recipients cleared GNB from the allograft during the first 2 post-operative years, and 68% during the first post-operative year. In this study, a link between persistent identical gram-negative airway colonization and BOS after lung transplantation was demonstrated. This effect seems to be independent from other BOS risk factors.

It was shown by our group that transplant recipients with CF harboured different pseudomonas strains. Most of the patients remained colonized with the same strain as was cultured before lung transplantation. The frequency of re-colonization was comparable in this study, to data from the literature. Our genotype data support the hypothesis that a reservoir of \textit{P. aeruginosa} exists in patients with cystic fibrosis who undergo lung transplantation, presumably located in the upper respiratory tract or trachea. The phenomenon of transient colonization is common in the CF population. It has been previously demonstrated that recipients harbouring pan-resistant GNB and especially \textit{B. cepacia} have worse survival following lung transplantation. This was explained by an increased number of early post-operative infections but GNB may have additional late negative sequelae such as BOS.

Pseudomonads (e.g. \textit{P. aeruginosa}, \textit{Achromobacter}, \textit{Stenotrophomonas maltophilia} and \textit{B. cepacia}) are opportunistic pathogens. Colonization of the lower respiratory tract occurs only in those with impaired host defences. In cystic fibrosis, there is a unique host—bacterial interaction with an inflammatory response to the chronic bacterial infection. Alginate produced by pseudomonas bacteria leads to a characteristic biofilm within the bronchioles. The process of phagocytosis around the biofilm leads to liberation of proteolytic enzymes such as elastase and oxygen radicals which gradually destroy the tissue of the lungs in patients with cystic fibrosis. It has been shown that colonization triggers expression of diverse cytokines by structural airway cells, inducing neutrophil recruitment and thereby perpetuating a cycle of airway inflammation and destruction. Structural abnormalities seem to be more important in the clearance of gram-negative colonies than altered host defences. In LTx recipients aside from immunosuppression, airway homeostasis is abnormal. Airway ischemia and disruption of lymphatic vessels, impaired cough reflex, and disturbed mucociliary clearance may predispose to bacterial colonization in the pulmonary allograft.

Loss of colonization after LTx may be due to gradual reduction of immunosuppression but this appears unlikely as most of our patients experienced de-colonization during the period of maximal immunosuppression (first 2 post-operative years). In our experience breakdown of mucosal barriers due to post-anastomotic airway ischemia (resulting in necrotic bronchitis) is an important obstacle, with healing occurring usually during the first 6 post-operative months.

![Figure 3](image-url) BOS-free survival of CF patients losing GNB colonization (group A) and with further gram-negative colonization (group B), \(p = 0.02\), log rank test.

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**Table 3** Univariate analysis (log rank test) comparing patients developing BOS with patients without BOS and multivariate Cox proportional hazards model for BOS-free survival.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Univariate analysis (BOS (n = 18) vs No BOS (n = 41))</th>
<th>Multivariate Cox regression model</th>
<th>Adjusted Hazard ratio</th>
<th>95% Confidence interval</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daclizumab induction (%)</td>
<td>4 (22) vs 3 (7)</td>
<td>p = 0.50</td>
<td></td>
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<tr>
<td>Tacrolimus (%)</td>
<td>6 (33) vs 9 (22)</td>
<td>p = 0.13</td>
<td></td>
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<tr>
<td>Everolimus (%)</td>
<td>0 (0) vs 6 (15)</td>
<td>p = 0.30</td>
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<tr>
<td>\textit{Pseudomonas aeruginosa} (%)</td>
<td>14 (78) vs 32 (78)</td>
<td>p = 0.86</td>
<td></td>
<td></td>
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<tr>
<td>\textit{Burkholderia cepacia} (%)</td>
<td>1 (6) vs 4 (10)</td>
<td>p = 0.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airway Stents (%)</td>
<td>2 (11) vs 5 (12)</td>
<td>p = 0.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rejection treatments/1st year (%)</td>
<td>11 (61) vs 12 (29)</td>
<td>p = 0.001</td>
<td>8.01</td>
<td>2.68–24.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Colonization lost (%)</td>
<td>4 (22) vs 21 (51)</td>
<td>p = 0.02</td>
<td>0.18</td>
<td>0.05–0.62</td>
<td>0.006</td>
</tr>
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</table>

Cut-off values used if factor was present.
Lower airway tract colonization may trigger neutrophil-mediated airway inflammation. It is accepted that accumulation of GNB, particularly *P. aeruginosa*, leads to expression of inflammatory cytokines (interleukin-1β, -6, and -8 and tumor necrosis factor-alfa by airway epithelial cells). These cytokines attract neutrophils and lead to a vicious cycle of airway inflammation and destruction.\(^{20,21,25,26}\) It has been recently demonstrated that colonized BAL samples contain significantly more total cells, along with neutrophilia and raised IL8-concentrations. Patients within this group displayed a significant reduction in FEV1 compared to matched non-colonized. BAL samples. Airway colonization could therefore potentially increase airway inflammation, leading to airway obstruction in lung transplant patients. Aside from infection, pronounced neutrophilia is a frequent finding in recipients displaying BOS and even precedes the onset of BOS.\(^{28}\)

Two recent studies have documented an association between the pseudomonads and the development of BOS.\(^{29,30}\) In our study 3-year BOS-free survival of pseudomonal colonized CF patients was comparable to these studies (53% vs. 62 and 50%). In the Belgian study, 26 transplanted patients with CF colonized with different pseudomonads (including *P. aeruginosa*, *Acinetobacter*, *Stenotrophomonas* and *B. cepacia*) were included (total \(n = 92\)).\(^{10}\) As in our study, BOS risk was significantly increased in the 16 colonized vs. 10 non-colonized CF patients. In a control of 66 non-CF recipients of whom 23 were de-novo colonized BOS-free survival was not different compared to non-colonized subjects.

In the second study, 54 out of 66 involved LTx recipients with CF (total cohort \(n = 155\)) had persistent colonization with pseudomonads (cepacia and pseudomonas).\(^{29}\) In contrast to our results and that from the Leuven group, 3-year BOS-free survival (nearly 80%) was significantly better in this study in persistent colonized transplant recipients (mainly CF patients) while it was significantly lower in 20 de-novo colonized non-CF patients (50%). Whether persistent pseudomonal colonization (representing spread of organisms from an upper airway reservoir) and de-novo colonization of the allograft (may represent a true infection of damaged airways) after LTx are both risk factors for BOS remains elusive.

Limitations of our study include the potential risk of contaminating respiratory samples with material from the upper respiratory tract. Several actions were taken to try to eliminate this confounder. These included nasal cleaning preceding every bronchoscopy and on occasions when any BAL fluid was considered to be contaminated according to our criteria, it was excluded from further analysis. Furthermore all first BAL aspirate samples were discarded prior to quantitative culturing to ensure only material retrieved from the alveolar compartment was analyzed. Finally quantitative cultures were consistently used during the study duration. Another limitation of this study was the lack of a strict bronchoscopic surveillance protocol beyond the first 3 post-operative months. Furthermore, in 80% of the recipients pseudomonas species were isolated. Data on other pseudomonads were limited. In 12 patients with non-pseudomonas GNB, the BOS-free survival was not significant \((p = 0.45)\) when comparing negative \((n = 8)\) and further positive \((n = 4)\) patients following transplantation, but number were small. De-novo colonization with non-identical pseudomonads after LTx was unlikely to influence our results because it was rare, transient and not different between groups.

Overall, BOS shows multifactorial predisposition with alloimmune and non-alloimmune risk factors, with colonization being only one of them. Similar prevalence of BOS in CF patients was observed than in other diseases.\(^{31}\) Reflux disease and primary graft dysfunction are two other recently recognised risk factors for BOS. Reflux is more common in the CF population as well, while primary graft dysfunction may be less frequent due to avoidance of cardiopulmonary bypass and extended donors in our CF cohort. CMV and other virus infections were not different between the two groups as well as reflux disease. We believe it is required to study larger patient populations prospectively using regular, standardised assessments in follow-up of lung transplant recipients with CF and non-CF to answer the influence of various risk factors for BOS.

Current data suggest it may be important to protect lung allografts from harvesting GNB. Modification of the perioperative management of lung transplant recipients in this regard may be crucial. New antimicrobials against GNB are unlikely to be available within the next years. Non-antimicrobial strategies may therefore gain importance in the eradication of GNB from the respiratory tract of LTx recipients with cystic fibrosis. Sinus surgery combined with antimicrobial lavage may reduce the risk of post-operative infection in lung transplant recipients with cystic fibrosis. Some transplantation groups therefore advocate surgical drainage of the sinuses immediately before or after transplantation.\(^{32}\) Topical administration of nebulized antimicrobial agents via a face mask or nasal plugs to target the sinus tract is another potential adjunctive measure. Further potential eradication strategies include bacterial immunization, individual tailoring to minimize immunosuppression and topical application of bacteriophages.

In conclusion our data demonstrate a link between persistent pseudomonal airway colonization and BOS after LTx. Re-Colonization with pseudomonads after LTx is frequent but further clearance of such colonies reoccurs later in a significant proportion of recipients with CF. Strategies to eradicate GNB early after LTx or reduction of bacterial load may be crucial in the future to prevent BOS.

**Conflict of interest statement**

None of the authors declare that they have a conflict of interest in relation to this work.

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