Breath volatile organic compounds of lung transplant recipients with and without chronic lung allograft dysfunction

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Abstract

Introduction. Chronic lung allograft dysfunction with its clinical correlative of bronchiolitis obliterans syndrome (BOS) remains the major limiting factor for long-term graft survival. Currently there are no established methods for the early diagnosis or prediction of BOS. To assess the feasibility of breath collection as a non-invasive tool and the potential of breath volatile organic compounds (VOC) for the early detection of BOS, we compared the breath VOC composition between transplant patients without and different stages of BOS. Methods. 75 outpatients (25 BOS stage 0, 25 BOS stage 1 + 2, 25 BOS stage 3) after bilateral lung transplantation were included. Exclusion criteria were active smoking, oxygen therapy and acute infection. Patients inhaled room air through a VOC and sterile filter and exhaled into an aluminum reservoir tube. Breath was loaded directly onto Tenax® TA adsorption tubes and was subsequently analyzed by gas-chromatography/mass-spectrometry. Results. The three groups were age and gender matched, but differed with respect to time since transplantation, the spectrum of underlying disease, and treatment regimes. Relative to patients without BOS, BOS stage 3 patients showed a larger number of different VOCs, and more pronounced differences in the level of VOCs as compared to BOS stage 1 + 2 patients. Logistic regression analysis found no differences between controls and BOS 1 + 2, but four VOCs (heptane, isopropyl-myristate, ethyl-acetate, ionone) with a significant contribution to the discrimination between controls and BOS stage 3. A combination of these four VOCs separated these groups with an area under the curve of 0.87. Conclusion. Breath sample collection using our reservoir sampler in the clinical environment was feasible. Our results suggest that breath VOCs can discriminate severe BOS. However, convincing evidence for VOCs with a potential to detect early onset BOS is lacking.

Introduction

Despite improvements in early survival after lung transplantation (LTx), chronic lung allograft dysfunction (CLAD) remains a major limiting factor for long-term graft survival [1]. CLAD is considered an overarching term for various clinical entities ranging from the classical bronchiolitis obliterans syndrome (BOS) to the restrictive allograft syndrome [2]. While restrictive allograft syndrome is characterized by interstitial lung disease leading to restrictive pulmonary function, BOS primarily involves fibro-obliterative remodeling of the bronchioles culminating in complete or partial small airway occlusion [3, 4]. Beyond the first year post-LTx, BOS is currently considered the leading cause of death [5, 6]. BOS affects 50% of LTx recipients within the first 5 years and confers a subsequent 5-year survival of 26% [7, 8]. Pathogenesis is not entirely understood, but appears multifactorial, with a wide range of known risk factors. Grant injuries due to alloimmunity and single or repetitive episodes of acute graft rejection are known to predispose to the
development of BOS [9, 10]. Both microbial airway colonization and acute respiratory infections are common in LTx recipients and may contribute to BOS [11]. Airway ischemia–reperfusion and gastroesophageal reflux are discussed as potential risk factors for BOS [12, 13]. The participation of human leukocyte antigen mismatching in BOS remains controversial [14]. All of these potential etiologies may predispose to recurrent epithelial injury, resulting in a pro-inflammatory state and dysregulated tissue repair that eventually leads to the irreversible fibrotic remodeling of the small airway epithelium [15].

BOS symptoms are unspecific and include dyspnea, cough, fatigue and decreased exertional threshold [16]. Diagnosis is one of exclusion, based primarily on spirometric changes, with supporting findings on imaging and bronchoalveolar lavage. The International Society of Heart and Lung Transplantation BOS classification relies entirely upon the decline in forced expiratory volume (FEV) from a standardized individual baseline to stage severity (BOS 1–3) [16, 17]. In response to a BOS diagnosis, macrolides, especially azithromycin have been used because their anti-inflammatory and immunomodulatory effects, were shown to improve FEV and to slow disease progression [18]. Gottlieb et al demonstrated that 30% of patients had improved pulmonary function at 6 months [19]. In macrolide non-responders additional strategies including extracorporeal photopheresis and leukotriene receptor antagonists have been suggested [20, 21]. Retransplantation is conducted as an ultima ratio with the result of lower survival rates than in the initial transplantation [22].

Improved specificity of tests used at initial diagnosis leading to early, reliable confirmation would greatly enhance clinical management. Breath volatile organic compounds (VOCs) may well be such an option. Increasing evidence suggests that the composition of exhaled VOCs correlates with malignant and inflammatory lung diseases [23–25]. VOCs are products of complex metabolic and pathophysiological processes inside the human body [26]. Volatile metabolites are transported via blood and can be detected in feces, urine, saliva, blood, skin emanations and exhaled breath [27]. The human volatolome consisting of at least 1840 VOCs was recently published [28]. Breath VOCs were also shown to be related to oxidative stress caused by pathological processes in cardiovascular and digestive tract systems [29, 30]. There is evidence that pathogen derived VOCs can be detected in breath offering a chance to non-invasively diagnose and monitor infectious lung diseases, where bacterial pathogens might also play an important role [31]. In 2001 Studer et al found no difference in the level of selected breath hydrocarbons and acetone between stable lung transplant patients and those with acute rejection [32]. There is electronic nose data available, showing that lung transplant recipients can be discriminated by their breath patterns from healthy subjects [33]. This data has been corroborated by Dolch et al who demonstrated increased levels of markers for lipid peroxidation in lung transplant patients [34]. There is, however, no breath VOC data available from patients with different degrees of BOS.

Before using breath VOC analysis in longitudinal studies within the follow up of lung transplant patients, we first compared breath VOCs of patients with and without BOS to evaluate if there are detectable differences in the VOC patterns that are related to the severity of BOS. We were also interested to see if breath VOC measurements are commonly accepted in the clinical environment and by the patients and how the analysis is affected by differences in the specific therapeutic regimens between groups. In our single center setting at the Hanover Medical School lung transplantation aftercare ambulance we collected breath samples of 75 bilateral lung transplanted patients.

### Methods

#### Subjects

In total 78 patients were recruited according to BOS stage (none, early and advanced stages) from the Hannover Medical School outpatient clinic between July and December 2016. All had undergone either bilateral lung or combined heart–lung transplantation. Patients were divided in three different groups (25 BOS stage 0 (controls), 25 BOS stage 1 + 2, and 25 BOS stage 3). The 25 controls included 12 cases with BOS 0p, which is defined as a decline in FEV1 in 1 s (FEV1) of 10%–19% and/or a decline in forced expiratory flow 25–75 > 25% compared to post-operative personal best [35]. Three patients, one out of each study group, were excluded due to insufficient breath sampling or instrument failure during analysis. Patient’s demographics are shown in table 1. The patients were recruited during their regular follow up visit and provided their written informed consent prior to the measurement. Key exclusion criteria were active smoking, acute infection, oxygen therapy and infection with multi- or pan-resistant bacteria. The study was approved by the Ethical Committees of the Hannover Medical School.

#### Study design

Before breath VOC sampling, patients underwent their regular examination and diagnostic procedures such as blood gas analysis, pulmonary function test, and physical examination. Potential patients were selected based on their current clinical condition and BOS stage. The majority of VOC samplings were performed before noon in the lung function laboratory. After a short introduction to the method, patients were ask to breath normally through the mouthpiece for a total of 8 min.
Collection of breath samples
Room air was inhaled through an A2 VOC filter (Dräger, Lübeck, Germany), a y-valve (Hans-Rudolph, Shawnee, USA) and standard lung function sterile filter (to protect these immune suppressed patients against infections). They exhaled through the sterile filter and the y-valve into an aluminum reservoir tube with a flow restrictor mounted at the end of the tube (figure 1). Patients exhaled against minimal resistance sufficient to close the nasal velum and were asked to maintain a certain flow rate indicated on a flow meter. For some patients with BOS stage 3 the resistor had to be removed. Following a pre-collection period of 3 min (to clean the breath from acutely accumulated room air contaminants), the breath from the reservoir was loaded simultaneously into two separate Tenax® TA adsorption tubes (Perkin Elmer, Waltham, USA). Using a small suction pump and a

Table 1. Patient demographics.

| BOS 0 | BOS 1 + 2 | BOS 3 | p |
|-------|-----------|-------|---|
| n     | 25        | 25    | 25 | *** |
| Time since TX years | 3.1 (3.5) | 4.3 (6.2) | 8.3 (6.6) |
| Age years | 51.0 (24.0) | 52.0 (21.0) | 56.0 (15.0) |
| Gender | f/m       | f/m   | f/m |
| Weight kg | 69.0 (17.5) | 68.5 (19.0) | 65.0 (17.0) |
| BMI kg m$^{-2}$ | 24.0 (4.1) | 22.5 (6.3) | 23.0 (5.7) |
| WBC count 10$^9$ μl$^{-1}$ | 6.0 (2.3) | 7.0 (2.7) | 6.8 (4.2) |
| CRP mg l$^{-1}$ | 1.3 (2.1) | 1.5 (1.7) | 2.9 (4.9) |
| FEV$\text{1}$ l | 2.6 (0.7) | 1.8 (0.9) | 1.1 (0.6) |
| FEV$_1$ (%) best. after TX | 95.9 (5.2) | 67.7 (10.9) | 43.5 (12.2) |
| Major TX cause: | | | *** |
| Fibrosis n | 8 | 6 | 6 |
| Emphysema n | 5 | 8 | 13 |
| Cystic Fibrosis n | 7 | 3 | 3 |
| Donor gender f/m | 15 l / 10 m | 14 l / 10 m | 13 l / 12 m |
| Donor age years | 44.0 (21.0) | 49.0 (14.8) | 45.0 (16.0) |
| Donor smoking status y/n | 7/15 | 8/13 | 13/9 |
| Tacrolimus | 21 | 16 | 16 |
| Ciclosporin | 4 | 8 | 9 |
| Everolimus | 0 | 1 | 0 |
| Target level§ | 0/18/7 | 5/17/3 | 2/16/7 |
| Mycophenolat Mofetil | 18 | 23 | 24 |
| Virostatic agents | 10 | 15 | 11 |
| Azithromycin | 8 | 22 | 24 |
| LTRA | 4 | 3 | 12 |
| Photopheresis | 0 | 5 | 15 |

TX: transplantation, BMI: body mass index, WBC: blood leukocytes, FEV$_1$: forced expiratory volume in 1 s, CRP: C-reactive protein, LTRA: leukotriene receptor antagonist, § = blood level of medication below/within/above target level. Median (Interquartile range), ***p < 0.001.
calibrated resistor, adjusting flow to 500 ml min$^{-1}$, 2.5 l of breath passed through the tubes over the 5 min collection period. As a control, 2.5 l of room air were collected close to the sampling device. The sterile filter and mouthpiece were discarded after collection, the valve was disinfected and rinsed prior to the next use and the reservoir tube was first disinfected with ethanol and then rinsed thoroughly with warm tap water and cold desalted water.

VOC analysis
Loaded Tenax® TA adsorption tubes were immediately closed with Swagelok Caps (Swagelok®, Solon, USA) containing polytetrafluorethylene fittings to allow transportation and storage. The tubes were transferred to the Fraunhofer ITEM gas-chromatography–mass-spectrometry (GC–MS) laboratory and stored for a maximum of three days prior to analysis by thermodesorption–GC–MS (TD–GC–MS). Details for the analysis parameters and settings are provided in the online supplement (table S1 is available online at stacks.iop.org/JBR/12/036023/mmedia). VOCs were identified using TurboMass Software 5.4 (Perkin Elmer, Shelton, USA). The different sample constituents were identified by their unique retention time, reference compounds and by library search using the National Institute of Standards and Technology Mass Spectral Search Program Version 2.2 (NIST, Boulder, USA). The Tenax® TA tubes were re-conditioned (250 °C, 5 min) after the analysis. 157 VOCs were quantified using peak height of specific target ions which most commonly matched with m/z signals of highest intensity in the respective VOC mass spectrum.

Data preprocessing and statistics
VOCs were first checked with respect to the quality of correlation between the double determinations of the two simultaneously collected tubes. We also checked for drift over the study period, the relationship to the respective room air sample, and for the distribution characteristics using QQ-plots and histograms [36]. Table 2 lists the 123 VOCs that were included into the final analysis. We excluded those VOCs with a clear indication for a drift over time, a clear relationship to room air, and VOCs known to be related to TENAX decomposition. VOCs that were normally distributed without ($n = 5$) and after log-transformation ($n = 23$) underwent parametric testing, otherwise non-parametric tests were used. The false discovery rate (expected proportion of erroneous rejections among all rejections of null hypothesis) was checked by correcting raw p-values with the p.adjust function in R (using the Benjamini and Hochberg option). Random forest analysis was performed using the R package rattle. First, all VOCs were included into the analysis, BOS stage was set as target and the analysis was performed using different data splits. VOC levels were log-transformed prior to the analysis. The dataset was split into training/validation/test sets in the ratio 50/25/25%, 60/20/20%, 70/15/15%, 80/10/10% and 90/10/0% and 1000 trees were calculated (the analysis showed that generally 300 trees would have been sufficient). VOCs were ranked according to their importance to discriminate between states. Random forest analyses were performed including all three BOS stages ($n = 75$) as well as including only controls and BOS 3 ($n = 50$) and controls and BOS 1 + 2 ($n = 50$). Using arbitrary cut-off values, the top most important VOCs were selected to reduce the dataset. Two lists were generated including the 52 VOCs that contributed most to the discrimination between controls versus BOS stage 3 and including 43 VOCs for the respective discrimination between controls versus BOS stage 1 + 2. These VOCs were tested by logistic regression analysis and receiver operating characteristic (ROC) analysis.

Results
Patients
The three groups of patients were matched with respect to age and gender. As displayed in table 1 the groups differed with respect to time since transplantation, the distribution of underlying disease, and treatment regimes. While pulmonary fibrosis patients were nearly equally distributed over the three groups, patients with cystic fibrosis were more often found in the BOS 0 group, whereas emphysema was more prevalent in the BOS 3 group. Azithromycin was more frequently used in BOS patients, leukotriene receptor antagonists were mainly prescribed in BOS 3 stages and photophoresis treatment was also predominantly performed in more severe BOS stages. Some patients, especially with severe BOS stage 3 had to interrupt the sampling procedure due to discomfort and the feeling of dyspnea, but most patients tolerated the sampling well.

Breath and room air VOCs
The collected breath samples and the respective room air samples showed the typical spectrum of VOCs. Acetone and isoprene were predominantly found in breath samples, the median level being 24 and 14 fold higher compared to the respective matched room air samples. The VOCs with the highest levels in room air were ethanol, propan-2-ol, propanol-1, and butanone. These are all related to cleaning and disinfection solutions and were expected at high levels as the breath collection was performed in a hospital environment. As we were using an A2 carbon inhalation filter the level of these compounds in breath was reduced to 4%, 11%, 7% and 10% of the room air sample, respectively. Butanone is used in Germany as denaturant for ethanol therefore we saw the expected significant...
| VOC            | RT  | Validated | BOSA - C1 | BOSy/n1 | BOSH/β/BS3 | + 2    | Gender | Donor Gender | Pseudomonas spp. | Tacrolimus | Cyclosporin | Virostat | Azathioprine | Tacrolimus - Receptor Ant. | Photoreresist | control vs | control vs |
|----------------|-----|-----------|-----------|---------|-----------|--------|--------|--------------|-----------------|------------|-------------|---------|---------------|----------------------|--------------|-----------|-----------|
| Acetaldehyde   | 1.2 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Ethanol        | 1.5 | x         |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Pentane        | 1.6 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 2-Propanol     | 1.8 | x         | **        |         | ~         | *      |        |              |                 |            |             |        |               |                      |              | x         | x         |
| Isoprene       | 1.8 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Acetone        | 1.9 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Acetonitrile   | 2.0 | x         |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Dimethylsulfide| 2.1 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| tert-Butanol   | 2.1 | x         |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 1,3-Dichloro-2-propanol | 2.2 |           | **        |         | **        |         |        |              |                 |            |             |        |               |                      |              |           |           |
| 1,3-Cyclopentadiene | 2.3 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Dichloromethane| 2.4 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 1-Hexene       | 2.6 | x         | ~         |         | ~         |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Methylpentane  | 2.6 | x         | ~         |         | ~         |        |        |              |                 |            |             |        |               |                      |              | x         | x         |
| Hexane         | 2.8 | x         |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Tetramethylethylene | 3.0 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Butanal        | 3.3 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 2-Methylfuran  | 3.3 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Butanon        | 3.4 | x         | **        |         | ~         |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Ethyl acetate  | 3.7 | x         | ~         |         | ~         |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 2,4-Hexadiene  | 3.8 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 4-Methyl-1,3-pentadiene | 3.9 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 1,4-Cyclohexadiene | 4.0 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 1,3-Cyclohexadiene | 4.2 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Cyclohexane    | 4.4 | x         | ~         |         | ~         |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 2,2,4-Trimethylpentane | 4.6 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 1-Butanol      | 4.7 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Benzene        | 4.8 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 1-Heptene      | 4.8 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Heptane        | 4.9 | x         | ~         |         | ~         | *      |        |              |                 |            |             |        |               |                      |              |           |           |
| Propionic acid | 5.3 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              | x         |           |
| 2-Pentanone    | 5.5 | x         | ~         |         | ~         |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 2-Ethyl furane | 5.6 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| 2,5-Dimethyl furane | 5.8 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Pentanal       | 5.8 | x         |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Methyl cyclohexane | 5.8 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 1-Methylthiopropane | 6.0 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Cycloheptadiene | 6.2 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 2-Methyl thiopropanol | 6.4 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Methylbutanole | 6.5 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              | x         |           |
| 1,2-Propanol   | 7.0 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| unidentified Diene | 7.0 |           |           |         |           |        |        |              |                 |            |             |        |               |                      |              |           |           |
| Pyridine       | 7.4 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              | x         |           |
| Octane         | 7.5 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              | x         |           |
| Toluene        | 7.5 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 2 Ethyl-5-Methylfuran | 8.3 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 2-Methylbutenol | 8.5 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Hexanal        | 8.6 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              | x         |           |
| Tetrachlorethene | 8.7 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              | x         |           |
| Thujone        | 8.7 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Butylacetate   | 8.8 | x         |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| unidentified Terpene | 9.3 |           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| 1-Noren        | 10.2|           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Ethylbenzene   | 10.2|           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
| Nonane         | 10.3|           |           |         |           | ~      |        |              |                 |            |             |        |               |                      |              |           |           |
Table 2. (Continued.)

| VOC1          | RT2²      | Validax³     | BOSSa – C³   | BOSSb/BOS³ | BOSSb/BOS³ + ² | Gender³   | Donor/Grade³ | Tacro/dimm/Cyclosporin³ | Viro³¹² | Anti-tumor³¹³ | Leukotrien – Receptor³¹⁴ | Photophoresis³¹⁵ | controlsvs: BOS³¹⁷ | controlsvs: BOS³¹⁷ |
|---------------|-----------|--------------|--------------|------------|----------------|-----------|---------------|------------------------|---------|---------------|------------------------|-----------------|---------------------|---------------------|
| 1,3-Butandiol | 10.7      | x            | x            |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Styrene       | 11.2      | x            | ~            |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| a-Pinene      | 11.8      | x            | ~            |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Pentamethylheptane | 12.0     | x            | ~            |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Cytetrasiloxan | 12.3      | x            |               |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Terpen        | 12.6      | x            | ~            |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2,2,4,6,6,-   | 12.7      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Pentamethylheptane |          |              |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Decane        | 13.0      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| b-Pinene      | 13.2      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Myrcen        | 13.2      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2,6-Dimethyl-2,6-octadiene | 13.5 | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2-Pentyl furane | 13.5   | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Phenole       | 13.7      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Trimethylbenzene/ Mesitylene | 13.9 | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 3-Carene      | 14.0      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 1-p-Menthene  | 14.2      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2-Ethyl hexanole | 14.4    | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| p-Cymole      | 14.6      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| D-Limonene    | 14.7      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 1,4-Dichloro benzene | 14.8 | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Eucalyptol    | 14.8      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Benzyl alkohol | 15.5     | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 3,7-Dimethyl-3-octanol | 15.5  | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Methylphenole | 15.5      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| b-Linalol     | 15.9      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Cyclopentasiloxane | 16.0    | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| unidentified  | 17.0      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Triethylphosphate | 17.6   | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Cyclododecane | 17.7      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Dodecane      | 17.9      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Benzoic acid  | 18.2      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Menthon       | 18.2      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 3,7-Dimethyl-1-octanol | 18.4  | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Menthol       | 18.4      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2,4-Dichlorphenole | 18.7    | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 2-PropylHeptanol | 19.3     | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Citronellol   | 19.4      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Naphthalene   | 19.4      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Cyclohexasiloxane | 19.7  | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Nonanacid     | 19.9      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Linalyl-Acetate | 19.9    | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Tridecane     | 20.1      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 1-Decanol     | 20.2      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| 1-Phenoxy-2-propanol | 20.5  | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Heptamethylene | 20.6      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Octylether    | 21.0      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Undecanal     | 21.1      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Decanoic acid methyl ester | 21.2 | x |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Butyloctanol  | 21.3      | ~            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Menthyl acetate | 21.5     | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Decanoic acid | 22.0      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
| Tetradecane   | 22.2      | x            |              |            |                |           |               |                        |         |               |                        |                  |                     |                     |
correlations between these two VOCs in room air samples.

Smoking
Due to the fact that active smoking patients were excluded from this study, the level of typical smoking related VOCs were much lower compared to our previous study with chronic obstructive pulmonary disease (COPD) patients [37]. Using a different GC–MS device and a slightly different analysis protocol in this study, the levels are not directly comparable, but the relationship of smoking related breath VOCs to the respective levels in room air samples differed drastically between our studies. Breath benzene, toluene and xylene in lung transplant patients were all below room air levels, while in active smokers of our COPD study breath levels exceeded room air levels by 3–6 fold. No difference in these aromatic hydrocarbons were seen between patients that received a transplant lung from a smoker and those that received a non-smoker lung.

Gender
Nine VOCs were different between male and female patients. Most of these differences were found in the control group, but to a lesser extent in patients with BOS. Testing the gender difference just in BOS 0 patients showed a significant difference for 17 VOCs and a trend \((p < 0.08)\) for additional eight VOCs. No VOCs were found that showed at least a trend to a gender difference in all three groups. Nine VOCs showed significant differences for the gender of the lung donor. Interestingly for some VOCs there was an overlap with respect to recipient gender differences (table 2).

Correlation with clinical data
A significant correlation \((p < 0.05)\) between age and dimethylsulfoxide, toluene and phenol was found when all subjects were included into the analysis. The correlation within the three groups were also significant or showed a respective trend. For nonane, octane and isoprene we observed significant correlations \((p < 0.01)\) with body mass index, that were also significant or showed a respective trend in the three groups separately. No robust correlations of breath VOCs were found for the number of blood leukocytes, the level of C-reactive protein, and lung function parameters.

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Table 2. (Continued.)

| VOC1        | RT2  | Validated3 | BOS A – C4 | BOSy/n5 | BOS0/BOS1 + 26 | Gender8 | DonorGender9 | Pseudomonaspos 10 | Tacrolimus/Cyclosporin11 | Virostat12 | Azithromycin13 | Leukotrien – ReceptorAnt14 | Photophoresis15 | controls vs: BOS1 + 216 | controls vs: BOS217 |
|-------------|------|------------|------------|---------|----------------|---------|---------------|----------------------|--------------------------|-------------|----------------|---------------------------|----------------|---------------------------|------------------|
| Indole      | 22.2 | *          | ~          | ~       | **             | ~       | x             | x                    |                          |             |                |                           |                |                           |                   |
| Gerany LAcetate | 22.4 |            |            |         | *              | ~       | x             | x                    |                          |             |                |                           |                |                           |                   |
| Phthalic acid anhydrid | 23.1 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Cycloheptasiloxane | 23.1 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Pentadecane | 24.1 | x          |            | ~       |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Ionone      | 25.3 | *          | ~          | ~       |                | *       | x             |                      |                          |             |                |                           |                |                           |                   |
| Buthylhydroxytoluene | 25.6 | x          |            |         |                | ~       | ~             | x                    |                          |             |                |                           |                |                           |                   |
| Hexadecane  | 26.0 |            |            | ~       |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| unidentified | 26.9 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Dietyltoluamide | 27.5 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Heptadecane | 27.7 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Cyclopentadienionph | 28.8 |            |            |         |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Isopropylmyristate | 30.2 |            | ~          | ~       |                | *       | x             |                      |                          |             |                |                           |                |                           |                   |
| Diisobutyl phthalate | 32.1 | x          |            |         |                | ~       | x             |                      |                          |             |                |                           |                |                           |                   |
| Homosolate  | 32.4 |            |            | ~       |                |         |               |                      |                          |             |                |                           |                |                           |                   |
| Dibutyl phthalate | 34.1 | x          | ~          | ~       |                | ~       | x             |                      |                          |             |                |                           |                |                           |                   |

Notes: 1: VOC name, identification by NIST database (compound with highest probability). 2 RT: retention time (min). 3: Validated: VOC identification confirmed by standard compound (x). 4: BOS A–C: Comparison between all BOS stages (ANOVA). 5: BOS y/n: Comparison between controls and BOS. 6: Comparison between BOS 0 and BOS 3. 7: Comparison between BOS 0 and BOS 1&2. 8: VOCs differing between genders. 9: VOCs differing between lung donor genders. 10: Comparison between Pseudomonas pos. and neg patients. 11-15: Comparison between treatment groups. 16,17: included into multivariate analysis (x). * * \(p < 0.01\), ** \(p < 0.05\), ∼ p < 0.08 (trend).
Breath VOC composition and BOS stage
Table 2 lists the 13 VOCs that were significantly different between BOS stages. Additional seven VOCs demonstrated a trend ($p < 0.08$). Five of these 20 VOCs remained significant when the 25 controls (BOS 0) were compared with the 50 BOS patients (column BOS y/n, table 2). Eleven VOCs were significantly different between the 25 controls and the 25 patients with severe BOS 3 (plus seven VOCs with $p < 0.08$, column BOS 0 versus BOS 3). When comparing the 25 controls with the 25 BOS 1 + 2 patients, just six VOCs showed a significance level below $p < 0.05$ (plus three VOCs with $p < 0.08$, column BOS 0 versus BOS 1 + 2).

Looking at the distribution of $p$-values for each of these analyses, there is a certain enrichment in lower $p$-values visible for the analysis of variance and for the comparison of controls with BOS 3 (figure S1, online supplement). Adjustment of $p$-values shows, that a false discovery rate of 0.2 would be required to keep three VOCs statistically significant in the analysis of variance (indicating that 20% of these three VOCs are likely to be false positive) and two for the comparison of BOS 0 with BOS 3. The VOCs iodone and iso-propylmyristat were among these in both tests. Using the same assumptions no significant results would remain for the comparison of BOS 0 versus all BOS and for BOS 0 versus BOS 1 + 2.

Potential confounding factors
To test the influence of potential confounding factors, we performed the analysis of variance also in the respective subgroups. In the 51 patients without a diagnosis for *Pseudomonas* colonization, in the 65 patients under treatment with Mycophenolat-Mofetil, in the 53 patients receiving Tacrolimus, and in the 54 patients treated with azithromycin very similar differences between groups were found, as indicated by the correlation between $p$-values derived from the total and subgroup analysis. The agreement was less pronounced for the comparison of the subgroups treated with leucotriene receptor antagonist (n = 56), virostatic drugs (n = 36), and in those treated with cyclosporine (n = 22); the latter two most likely due to lower case numbers.

Colonization
Some of the patients in this study were colonized by different pathogens and treated accordingly. The number of cases for most pathogens were too low to be tested for VOC differences. Patients with a positive diagnosis for *Pseudomonas aeruginosa* (n = 24) showed a different level for 11 VOCs compared to those without (table 2).

Therapy
Detailed information on VOCs related to the treatment of patients is provided in table 2. The patients treated with tacrolimus (n = 53) and those treated with cyclosporine as immune-suppressant therapy differed in 15 VOCs. However, none of these correlated significantly with the respective blood levels of these medications. For 2,2,4-trimethylpentane this difference was seen in all three groups of patients. The 54 patients treated with azithromycin showed a different level in nine VOCs compared to the untreated patients. The most pronounced difference was seen for indole, a VOC known to derive from bacteria. The 39 patients under virostatic treatment differed from the untreated patients in the level of two VOCs. The 19 patients treated with a leukotriene receptor antagonist differed in the level of 12 VOCs from those not under such treatment. The 20 patients treated by photopheresis differed in the level of 10 VOCs from those not receiving this treatment. Nearly all subjects were treated with Co-trimoxazole. For two VOCs (dimethyl-sulfoxide, methylthiopropan) we observed significantly ($p < 0.001$, analysis of variance) lower levels in subjects treated with a high dose (2 × 960 mg per week).

Multivariate analysis
We first reduced the number of VOCs by random forest analysis. We selected those VOCs with the highest levels of importance scores for the discrimination between all three groups, between controls and BOS 3 and between controls and BOS 1 + 2. From this data we generated 2 lists of VOCs. The first list included VOCs that best discriminated between controls and the severe BOS stage 3 patients to test if there are any BOS related VOC patterns at all. The second list included VOCs that best discriminated between controls and BOS stage 1 + 2 patients to test if there are VOC patterns that could potentially enable the early diagnosis of BOS. Most of the VOCs that were significant or showed a trend for a difference between groups in the univariate analysis (table 2) were also among the VOCs that received the highest ‘importance scores’ in these 2 lists.

We then performed a logistic regression and ROC analysis. For the comparison between controls and BOS stages 1 + 2 patients, no significant results were found, indicating that the detection of early BOS stages is not possible with the VOCs in our respective list. For the comparison between control and BOS stage 3 we found four VOCs that showed a significant contribution for the discrimination between groups and/or a large area under the curve (AUC) in the ROC analysis. The levels of heptane and isopropyl-myristat were lower and the levels of ethyl-acetate and iodone were higher in BOS stage 3 patients (figure 2).

Then we transformed the individual levels of these VOCs into counts based on their cut-off values from their ROC curves. The count 0 was given for BOS stage 3 positive high levels of iodone and ethyl-acetate and low levels of heptane and isopropyl-myristat. A count of 1 was given for BOS stage 3 negative low levels of...
ionone and ethyl-acetate and high levels of heptane and isopropyl-myristat. The table in figure 2 shows that 16/17 patients with a total count of 0 or 1 were BOS stage 3 patients and that 10/11 patients with a maximal total count of 4 were control patients. The AUC for the total score was 0.87. Adding the nine BOS stage 2 patients to the BOS stage 3 patient group still correctly identified 17/18 patients with a total count of 0 or 1 and 10/12 patients with a total count of 4. The AUC decreased to 0.79.

To check these results we performed a linear discriminant analysis. Including all three groups and the VOCs from the above mentioned lists showed a separation between groups with 77.3% accuracy (without cross validation). Including only controls and BOS stage 3 patients showed 82% accuracy (figure S2 online supplement). Ionone, heptane, and ethyl-acetate were among VOCs that were included into the model.

### Discussion

In this cross-sectional investigation we showed that breath sample collection using our reservoir sampler in the clinical environment was feasible. We found evidence for a breath VOC pattern able to discriminate between patients without BOS and patients with severe BOS stage 3. While endogenous or bacterial origins are likely, these specific VOCs are also lifestyle related and therefore it remains to be determined, if they actually play a role in the patho-mechanism of BOS. Among the 123 breath VOCs included into our analysis we found no VOC pattern that was sufficiently able to discriminate between control patients and early stages of BOS.

### Study design

The pathogenesis of BOS is not fully understood. The multifactorial processes beginning before symptom onset and before lung function decline [38]. There are currently no non-invasive methods available that could be used to frequently screen patients after transplantation in an effort to detect and to treat these processes as early as possible. Breath analysis is very simple to perform and breath VOCs are thought to reflect metabolic processes within the body [23]. Therefore, it was the aim of this study to evaluate, if BOS related pathogenic processes could be detected by this novel methodology. Ideally, this would have involved a prospective longitudinal study starting out with patients immediately following lung transplantation. Such a design would have required a study period of several years. Therefore, we decided to use a cross-sectional approach and searched for differences in the breath VOC pattern between transplant patients without BOS and 2 groups with mild to severe stages of BOS. Only if differences of breath VOC patterns...
between BOS stages can be detected, it would justify a long-term longitudinal follow up study.

Data quality and methodological issues

The breath and room air VOC composition and levels suggest that the breath sampling and analysis were adequately performed. Our data is comparable to a previous study in COPD patients [37] in which we used the same kind of breath sampling equipment. The lower benzene and toluene concentrations in these non-smoking transplant patients relative to room air are in line with the results of this study. We loaded the breath samples to Tenax® TA adsorption tubes. This material has been used in other breath studies and appears to be especially suited for the analysis samples with a relatively high level of humidity. Nevertheless, the focus on Tenax® TA limits the spectrum of detectable VOCs, and we cannot exclude that due to this, we missed clinically relevant VOCs, which have a lower affinity to Tenax® TA. Our sampler allows a direct loading of two Tenax® tubes simultaneously. The results of the separate analysis of these tubes show a high level of agreement, which we use as quality criteria. Each subject was required to breathe into the sampler for a period of 8 min, which is much longer compared to studies using several breaths to fill a e.g. Tedlar bag. This additional effort, however, leads to much cleaner samples and avoids all potential contaminants and drawbacks known from using bags [39]. While our sampler uses a restrictor that helps to close the nasal velum and avoids potential contamination from the upper airways, we have not enriched our samples with peripheral lung air by excluding the first part of the exhaled breath. According to our knowledge, there is currently no strong evidence that excluding the dead space air has a major advantage. Using such an approach would require increasing the collection time, which was not possible especially for BOS stage 3 patients, or would have reduced the amount of air loaded onto the adsorption tubes. The latter would have limited the potential increase in sensitivity that is supposed to be an advantage of methods excluding the dead space air.

Patient selection and potential confounding factors

The patients were consecutively recruited from the outpatient clinic of Hannover Medical School with the aim to include 25 patients of each group into the study. The patients were well matched with respect to demographics, but the composition of diseases leading to the need of lung transplantation differed and due to the different severities of BOS there were naturally pronounced differences in the treatment regimens between groups. To test, if the breath VOC composition was biased by differences in treatment, we compared the results of the univariate analysis performed with all patients with the results of the analysis performed with the respective largest treatment subgroup. The \( p \)-values for the differences between BOS stages in these subgroups correlated with the \( p \)-values in all patients suggesting that no major bias due to these factors exists. This was also true for the four VOCs that showed a significant contribution to the discrimination between controls and BOS stage 3, although these were influenced by some of these factors (table 2).

We also compared if patient subgroups differed in the breath VOC composition from other subgroups irrespective of BOS stage. Due to low subject numbers these results have to be interpreted with care. However, we included this data into table 2 to allow the comparison with results from other trials, which might enable a more solid interpretation when combined. Interestingly, we found that the level of indole was reduced in patients treated with Azithromycin. Indole is known to be derived from bacteria [40], suggesting that treatment with this macrolide decreases bacterial numbers within the lung or digestive tract.

Data analysis approach

After deleting room air related VOCs and known Tenax decomposition products from our dataset, we first performed a univariate analysis. As expected, it showed the largest number and most pronounced differences between control and BOS stage 3 patients. Nevertheless, considering the multiplicity of these comparisons, all these results have to be interpreted with care. In summary, there appears to be no single VOC marker able to discriminate sufficiently between groups among those that were included into our analysis.

We also choose different multivariate approaches to analyze the data. First, we used random forest analysis to reduce the number of VOCs to be tested by regression and discriminant analysis. The cut-offs for the respective importance scores derived from the random forest analysis were chosen arbitrarily to obtain a list of approximately 50 VOCs that contribute to the discrimination between controls and mild to moderate and severe BOS stages. In line with the univariate analysis the logistic regression analysis found no VOCs that showed a significant contribution to the discrimination between controls and BOS stages 1 + 2. However, four VOCs showed a significant contribution to the discrimination between control and BOS stage 3 patients. Transformed to a binary count and as a combined VOC pattern, these four VOCs were able to discriminate between these groups even better, as indicated by the large AUC in the ROC analysis. We also used linear discriminant analysis based on the same reduced dataset that was used for the logistic regression. Interestingly, three of the VOCs that were included into our summary count, were also included into the model suggested by the discriminant analysis.

We are aware that there are other statistical strategies to analyze such a dataset. Depending on the VOCs
that are initially entered into the analysis or depending on the setting of specific parameters of the respective statistical tests, the outcome, e.g. the VOCs included into discrimination models, is likely to differ. We found a number of VOCs that showed evidence for differences between groups in all our tests. Therefore we consider these VOCs as potential candidates that contribute to the discrimination between controls and BOS stage 3 patients, but we cannot exclude that there are others.

### Table 3. VOC origin.

| VOC                  | Pubchem                  | mVOC                  | hmdb ontology                  |
|----------------------|--------------------------|-----------------------|---------------------------------|
|                      |                          |                       | Origin | Biofunction | Cellular locations |
| Ethyl acetate        | [43]                     | Alcohol beverages     | Endogenous | Anti-arthritis | Cytoplasm          |
|                      |                          | Crops                 | Food | Anti-inflammatory | Extracellular      |
|                      |                          | Fruits                | Anti-mycotic                   |
|                      |                          | Food additive         | Anti-nociceptive               |
|                      |                          | Adhesives             |                                 |
|                      |                          | Paint                 |                                 |
|                      |                          | Toner                 |                                 |
| Heptane [28, 44]     | Solvent                  | Endogenous            | Food |                          | Membrane           |
|                      |                          | Paint                 |                                 |
|                      |                          | Adhesives             |                                 |
|                      |                          | Automotive care       |                                 |
| Indole [45–47]       | Bacteria                 | Feces                 | Epidermis |                          |                   |
|                      | Perfumes                 | e.g. E. coli          | Fibroblasts |                          |                   |
|                      | Human feces              | Saliva                | Intestine |                          |                   |
|                      |                          | Urine                 | Neuron |                          |                   |
| Ionone               | Fragrances               | Food                  | Extracellular |                          | Membrane          |
|                      | Oils                     | Cell signaling        |                                 |
|                      | Foods                    | Energy storage        |                                 |
|                      | Cleaning products        | Energy source         |                                 |
|                      |                          | Membrane integrity/   |                                 |
|                      |                          | stability             |                                 |
|                      |                          | Nutrient              |                                 |
|                      |                          | Anti-proliferative    |                                 |
| Isopropyl myristat   | Food additive            | Endogenous            | Extracellular |                          | Membrane          |
|                      |                          | Food                  |                                 |
|                      |                          | Flavoring agent       |                                 |
|                      |                          | Cleaning products     |                                 |

Pubchem: https://pubchem.ncbi.nlm.nih.gov/, mVOC: http://bioinformatics.charite.de/mvoc/, hmdb: http://hmdb.ca/.

**Origin of VOCs**

The available knowledge about the pathology of BOS does not provide any solid suggestions for potential VOCs that are mechanistically related to the disease process. Therefore, we did not have a specific hypothesis for BOS related changes of breath VOCs. As inflammatory processes could play a role in the BOS pathology [41], changes of markers for oxidative stress like ethane, pentane, or hydroxyl-nonenal could have been potentially expected. Ethane, which has been shown to increase...
following outpatient surgery [42] could not be detected with our system due to its low molecular weight. Pentane was not among the VOCs that showed differences between our patient groups. To assess the origin of the VOCs that discriminated between control and BOS stage 3 patients in our study we search different databases (table 3). Indole was added to this search as it showed differences between subgroups.

For these five VOCs table 3 illustrates both a potential endogenous as well as exogenous origin. It also demonstrates that VOCs derived from food flavoring, lifestyle products and the environment will pose a serious hurdle for the proper interpretation of breath VOC data. Based on our data we like to emphasize that environmental VOCs, e.g. those detected in high concentrations in hospital air, cannot be ignored. Despite the use of A2 inhalation filters these will always be detectable in breath, potentially also due to pre-sampling exposures, and will therefore pose a problem for data derived from using unspecific detectors and electronic noses.

**Conclusion**

The use of our breath sample collection system in the clinical environment was feasible. Only minor adaptations, unlike to have substantial effect on the outcome of the analysis, had to be made for seriously impaired patients. Our results suggest, that the discrimination between control and severe BOS stage 3 patients is possible. However, among the VOCs included into our analysis we found no convincing evidence for VOCs that have a clinically relevant prognostic potential for the early detection of CLAD.

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