ORIGINAL CLINICAL SCIENCE

Diagnostic performance of electronic nose technology in chronic lung allograft dysfunction

Nynke Wijbenga, MSc, a,e Rogier A.S. Hoek, MD, a,e Bas J. Mathot, MD, a,e Leonard Seghers, MD, PhD, a,e Catharina C. Moor, MD, PhD, a Joachim G.J.V. Aerts, MD, PhD, a Daniel Bos, MD, PhD, b,c Olivier C. Manintveld, MD, PhD, d,e and Merel E. Hellemons, MD, PhD a,e

From the aDepartment of Respiratory Medicine, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; bDepartment of Radiology & Nuclear Medicine, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; cDepartment of Epidemiology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; dDepartment of Cardiology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; and the eErasmus MC Transplant Institute, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands.

BACKGROUND: There is a need for reliable biomarkers for the diagnosis of chronic lung allograft dysfunction (CLAD). In this light, we investigated the diagnostic value of exhaled breath analysis using an electronic nose (eNose) for CLAD, CLAD phenotype, and CLAD stage in lung transplant recipients (LTR).

METHODS: We performed eNose measurements in LTR with and without CLAD, visiting the outpatient clinic. Through supervised machine learning, the diagnostic value of eNose for CLAD was assessed in a random training and validation set. Next, we investigated the diagnostic value of the eNose measurements combined with known risk factors for CLAD. Model performance was evaluated using ROC-analysis.

RESULTS: We included 152 LTR (median age 60 years, 49% females), of whom 38 with CLAD. eNose-based classification of patients with and without CLAD provided an AUC of 0.86 in the training set, and 0.82 in the validation set. After adding established risk factors for CLAD (age, gender, type of transplantation, time after transplantation and prior occurrence of acute cellular rejection) to a model with the eNose data, the discriminative ability of the model improved to an AUC of 0.94 (p = 0.02) in the training set and 0.94 (p = 0.04) in the validation set. Discrimination between BOS and RAS was good (AUC 0.95). Discriminative ability for other phenotypes (AUCs ranging 0.50-0.92) or CLAD stages (AUC 0.56) was limited.

CONCLUSION: Exhaled breath analysis using eNose is a promising novel biomarker for enabling diagnosis and phenotyping CLAD. eNose technology could be a valuable addition to the diagnostic armamentarium for suspected graft failure in LTR.

J Heart Lung Transplant 000;000:1−10
© 2022 The Author(s). Published by Elsevier Inc. on behalf of International Society for Heart and Lung Transplantation. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)

Abbreviations: ACR, Acute cellular rejection; BOS, Bronchiolitis obliterans syndrome; CLAD, Chronic lung allograft dysfunction; eNose, Electronic nose; FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; LTR, Lung transplant recipients; LTx, Lung transplantation; PLS-DA, Partial least squares discriminant analysis; RAS, Restrictive allograft syndrome; TLC, Total lung capacity; VOCs, Volatile organic compounds

Reprint requests: Merel E. Hellemons, Department of Respiratory Medicine, Erasmus MC Transplant Institute, University Medical Center, Rotterdam Dr. Molewaterplein 40, 2040, 3000 CA Rotterdam, The Netherlands. Telehone: 0031-107040704.
E-mail address: m.hellemons@erasusmc.nl

1053-2498/S - see front matter © 2022 The Author(s). Published by Elsevier Inc. on behalf of International Society for Heart and Lung Transplantation. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)
https://doi.org/10.1016/j.healun.2022.09.009
Lung transplantation (LTx) is a lifesaving treatment for selected patients with end stage lung disease. The number of LTx has been rising steadily over the past years along with reductions in morbidity and mortality.1,2 Survival rates after LTx have improved with a current median worldwide survival of 6.2 years.1,2 Nonetheless, further improvement of long-term survival of LTx is hampered by chronic lung allograft dysfunction (CLAD). CLAD concerns permanent loss of allograft function after exclusion of other causes.4-6 Worldwide, around 50% of the patients are diagnosed with CLAD within five years after LTx.7 CLAD is associated with high mortality and morbidity, and its exact pathophysiology is only partially understood. Several phenotypes of CLAD can be distinguished; a predominantly obstructive ventilatory pattern—known as bronchiolitis obliterans syndrome (BOS)—, a restrictive pattern—known as restrictive allograft syndrome (RAS)—, or a mixed obstructive and restrictive pattern (mixed CLAD). Although the phenotypes differ in spirometric and radiological characteristics, establishing the final diagnosis can be challenging. Yet, differentiation between BOS and RAS is crucial, since prognosis of the phenotypes considerably differs.8 Furthermore, CLAD can be divided into four stages depending on the decline in forced expiratory volume in 1 second (FEV1).5

At the moment, treatment options for established CLAD are scarce and remain an unmet medical need. Being able to detect CLAD in an early or developing stage or with greater accuracy could enable quicker, personalized interventions directed at reversing or slowing the process and could lead to better overall survival outcomes.8-11 Hence, there is a need for reliable biomarkers that can diagnose CLAD early.

From this perspective, it is of interest to assess the role of exhaled breath analysis in the early diagnosis of CLAD. Exhaled breath contains thousands of molecules known as volatile organic compounds (VOCs). Endogenous VOCs can be associated with normal physiology, but also with pathophysiological inflammatory or oxidative activity. Thermal-desorption-gas-chromatography-mass-spectrometry has shown the potential of individual VOCs to discriminate between BOS stages 1-2 and stage 3.12 Although identification of individual VOCs is very specific, it is a very time-consuming process and hard to implement in routine clinical care. An entirely different approach is the analysis of exhaled breath by use of an electronic nose (eNose). An eNose can be used to capture the complete mixture of VOCs in exhaled air by several cross-reactive gas sensors without identifying individual components, resulting in a unique pattern, the so-called breathprint.13,14 This breathprint reflects current health status of the individual, and can be analyzed by pattern recognition using machine learning. Subsequently, real-time measurements of the breathprint with an eNose have potential as a cheap and fast point-of-care tool in clinical practice. eNose technology shows promising recent results in diagnosing and phenotyping asthma, chronic obstructive pulmonary disease, and interstitial lung diseases.15-17 Despite its potential, eNose technology has so far scarcely been explored within the field of LTx.18 In this study we aimed to assess the diagnostic accuracy of exhaled breath analysis using an eNose to detect CLAD in LTx recipients (LTR), and assess the added value of the breathprint on top of known risk factors for CLAD. Furthermore, we aimed to assess whether eNose technology could discriminate between different CLAD phenotypes and CLAD stages.

Methods

Study design and population

We performed a cross-sectional analysis of a prospective cohort study conducted at the Erasmus University Medical Center, Rotterdam, the Netherlands. Between July 2020 and September 2021, all consecutive LTR, both CLAD and non-CLAD, irrespective of transplant date (median 4 [0.1-19.3] years after LTx), visiting the outpatient clinic were asked to participate. For all patients a single measurement was included. The measurement that was included in the analysis was the first measurement, unless the treating consultant indicated that the patient was “not stable” (i.e., signs of infection or acute cellular rejection (ACR) or reduced pulmonary function). When a patient was recently transplanted the first stable measurement at least 3 months after LTx was included. When uncertainty remained about whether the patient was truly stable, we included the first consecutive measurement where the treating consultant and team were convinced a patient was stable. No subjects were excluded. This study was approved by the medical ethics committee (MEC-2019-0497). All patients provided written informed consent prior to eNose measurements.

Routine measurement and diagnosis of CLAD

Routine pulmonary function tests were performed to determine the forced expiratory volume in 1 second (FEV1) and the forced vital capacity (FVC). CLAD was diagnosed by the treating consultant and the transplant team according to the ISHLT criteria.6 CLAD is defined by a substantial and persistent decline (≥20%) in measured FEV1 from baseline. Baseline FEV1 is calculated as the mean of the two best FEV1 measurements post-LTx, with a time interval of at least 3 weeks in between. After excluding other causes of lung function decline, CLAD is confirmed when the decrease of FEV1 persists for 3 months despite clinically appropriate therapies.5,6 CLAD that presented as an obstructive ventilatory pattern (FEV1/FVC ratio <0.7) was classified as BOS. CLAD that presented as a restrictive ventilatory pattern (a ≥10% reduction in baseline total lung capacity [TLC]) along with chest computed tomography opacities was classified as RAS. In case there were insufficient TLC measurements available, a substantial and persistent decline (≥20%) in measured FVC from baseline was used as a proxy.19 If both an obstructive and restrictive pattern existed cases were classified as mixed CLAD. The remaining cases were classified as undefined CLAD. CLAD stages 0 to 4 were classified according to current FEV1 compared to baseline FEV1, with CLAD 0 as current FEV1 > 80% FEV1 baseline, CLAD 1 as current FEV1 > 65% to 80% FEV1 baseline, CLAD 2 as current FEV1 > 50% to 65% FEV1 baseline, CLAD 3 as current FEV1 > 35% to 50% FEV1 baseline, and CLAD 4 as current FEV1 ≤ 35% FEV1 baseline.6

Patient demographics, type of LTx, time after LTx, occurrence of any prior ACR episodes, medication use and CLAD phenotype and stage at time of the eNose measurement were collected from electronic medical records. ACR was defined as: (1) clinical suspicion for ACR with confirmatory trans-bronchial biopsy (classified
as ≥A2 or ≥B1), (2) clinical suspicion for ACR with suspicious transbronchial biopsy (classified as A1), after exclusion of other causes (such as viral infection) and with clinical response to antirejection treatment (3) clinical suspicion for ACR after exclusion of other causes and with clinical response to antirejection treatment.

**eNose - Exhaled breath measurements**

Exhaled breath was analyzed real-time using a cloud-connected eNose; SpiroNose (Breathomix, Leiden, the Netherlands). The SpiroNose consists of seven different types of cross-reactive metal-oxide semiconductor sensors. Each sensor is present on the sensor arrays in a duplex configuration, both inside and outside of the SpiroNose. With this configuration, the SpiroNose is able to measure the VOCs in both exhaled breath and ambient air. Detailed description of the methods and set up was published previously. In short, a SpiroNose measurement consists of 5 tidal breaths, followed by an inspiratory capacity maneuver to total lung capacity, a five second breath hold, and slow expiration to residual volume. All measurements were performed in duplicate. Sensor responses were sent directly to the online analysis platform, BreathBase. The BreathBase platform includes the secured online database of Breathomix and is developed conform the requirements of standards ISO 27001 (information security) and NEN 7510 (information security in healthcare). eNose sensor responses were processed and corrected for ambient air as previously described. Of each sensor, the peak value was determined and normalized to the most stable sensor. To minimize inter-array differences, sensor-to-sensor ratios were used. Ratios between the sensor peaks and breath hold point were calculated. The normalized sensor peaks and the ratios between the sensor peaks and breath hold points were both used for data analysis.

**Standard treatment protocols post LTx**

Induction therapy consists of basiliximab (Simulect; Novartis Pharma, Basel, Switzerland). Maintenance immunosuppression consists of a calcineurin inhibitor (CNI)-based immunosuppressive regimen (trough levels 7-10) combined with prednisolone (0.05-0.15 mg/kg) and mycophenolate mofetil (CellCept; Roche, Basel, Switzerland). Tacrolimus (Prograft; Astellas Pharma, Staines, UK) is the most commonly used CNI in our center. Upon significant decline of renal function patients receive a quadruple immunosuppressive scheme adding everolimus (Certican; Novartis Pharma, Basel, Switzerland) to enable CNI dose reduction (tacrolimus trough levels 3-4; everolimus trough levels 3-4). All patients receive Pneumocystis Jiroveci prophylaxis (co-trimoxazole or inhaled pentamidine) and cytomegalovirus prophylaxis (valganciclovir) if applicable. Inhaled amphotericin B is used as fungal prophylaxis in the first months and azithromycin is used as valganciclovir (valganciclovir) if applicable. Inhaled amphotericin B is used as fungal prophylaxis in the first months and azithromycin is used as fungal prophylaxis in the first months and azithromycin is used as standard CLAD prophylaxis in all patients unless patients experience side-effects.

**Statistical analyses**

Descriptive statistics were used to analyze baseline data. Between-group comparisons were conducted using independent samples t-tests and Pearson’s Chi-squared tests as appropriate.

We investigated the diagnostic value of eNose-based exhaled breath analysis using the following strategy.

First, we achieved a dimensionality reduction of the eNose sensor data by applying a partial least squares discriminant analysis (PLS-DA). PLS-DA is a supervised machine learning approach. In short, it is a modeling technique for data reduction, creating simplified new explanatory variables, known as latent variables, while retaining as much information as possible from the complete dataset. These variables are subsequently used for supervised classification and discrimination problems, and can be visualized using a scatter plot where the axes present these latent variables.

Second, to perform the actual analyses, we randomly divided the dataset into a training set and a (internal) validation set by using a ratio of 2:1. The training set was used to train the model by using a 10-fold internal cross-validation procedure and the predictive potential of the fitted model was assessed in the validation set.

Third, we investigated the discriminative ability of the eNose sensors to distinguish in LTR with CLAD from LTR without CLAD. Furthermore, we assessed the diagnostic value of the eNose measurements combined with available known risk factors of CLAD (age, gender, type of LTx, time after LTx and occurrence of any prior ACR episodes) by adding these risk factors to the diagnostic model from the eNose results. Additionally, a multivariate logistic regression model with only clinical parameters was compared to a multivariate logistic regression model with eNose parameters added to the known predictors of CLAD. The final model ultimately consisted of the first two PLS-DA components, age, gender, type of LTx, time after LTx, and occurrence of any prior ACR episodes.

Discrimination between different CLAD phenotypes and stages was analyzed without dividing the dataset into a training and validation set. To assess discrimination between CLAD phenotypes, we first tested BOS versus RAS, and subsequently tested all four CLAD phenotypes. Additionally, the discrimination between CLAD stage 1 versus CLAD stage 2 or 3 versus non-CLAD was tested.

The performance of the obtained models were evaluated by computing the area under the receiver operating characteristic curve (AUC) and the associated model sensitivity, specificity, and accuracy. Furthermore, the 95% confidence interval (CI) was calculated. Additionally, the cross-validation accuracy and Cohen’s kappa were calculated. The Cohen’s kappa indicates how much better the model is over a random classifier. A value >0.75 is considered as excellent, 0.40 to 0.75 as fair to good, and <0.40 as poor agreement beyond chance. Differences in performance between the models were estimated by performing a pairwise comparison of the obtained AUCs using a bootstrap method.

To check whether the primary disease in the native lung of unilateral transplanted patients influenced the discrimination between CLAD and non-CLAD using the eNose, a sensitivity analysis was performed by excluding unilateral transplanted patients. The influence of the exclusion of unilateral transplanted patients was estimated by performing a pairwise comparison of the obtained AUCs using a bootstrap method.

The data were analyzed using RStudio (version 2021.09.1 +372) with R software (version 4.1.2) and supported with the following packages: mixOmics, tableone, pROC, ggplot2, caret.

**Results**

**Baseline characteristics**

A total of 152 out of the 160 approached LTR participated in the study and were included at outpatient clinic visits. Of
these patients 131 (86%) underwent a bilateral transplantation, 49% of the patients were female and the median age was 60 [range 18-77] years. Median time between LTx and study inclusion was 4.0 [range 0.3-19.3] years. A diagnosis of CLAD was established in 38 patients; of these patients 20 (53%) were diagnosed with BOS, 5 (13%) with RAS, 5 (13%) with mixed CLAD, and 8 (21%) with undefined CLAD. There were no significant differences in age (p = 0.20) and gender (p = 0.85) between LTR with and without CLAD. Median time after LTx was 2.4 [range 0.3-13.2] years for LTR without CLAD and 10.3 [range 2.5-19.3] years for LTR with CLAD (p < 0.001). For the analysis, we have used the first available measurement of 114 (75%) patients, the second measurement from 16 (11%) patients, and a consecutive measurement of 22 (14%) patients. Baseline characteristics are shown in Table 1.

The training set consisted of 78 LTR without CLAD and 26 LTR with CLAD. The validation set consisted of 36 LTR without and 12 LTR with CLAD. Training and validation set were very similar, the only small difference was found in CLAD phenotype (p = 0.002, Supplementary Table S1).

### Classification of CLAD versus non-CLAD

For the training set the estimated classification accuracy of the PLS-DA model achieved with cross-validation was 83% (±9.6% SD) with a kappa of 0.47 (±0.31 SD). The results of the PLS-DA model for the training set, without incorporation of the known predictors for CLAD, are shown in Figure 1, along with the corresponding ROC curve. In the training set, the AUC was 0.86 (0.76-0.96) with a sensitivity of 88%, a specificity of 76%, and an accuracy of 79%. In the validation set, the AUC reached 0.82 (0.66-0.98) with a sensitivity of 83%, a specificity of 83%, and an accuracy of 83%.

Multivariate logistic regression with only known available predictors of CLAD and supplemented with the eNose parameters was performed (Table 2). eNose parameters are significantly associated with CLAD on top of the known predictors of CLAD.

Subsequently, the logistic regression model for CLAD was trained using the two PLS-DA components obtained from the breathprints and known predictors for CLAD (age, gender, type of LTx, time after LTx and occurrence of any prior ACR episodes). For the training set, the estimated

| Table 1  | Baseline Characteristics |
|----------|--------------------------|
|          | non-CLAD (n = 114) | CLAD (n = 38) | p-values |
| Age      | (years [range]) | 60 [18-75] | 63 [32-77] | 0.20* |
| Gender   |             | Male 58 (51%) | 18 (47%) | 0.85* |
|          |             | Female 56 (49%) | 20 (53%) |  |
| Time after LTx | (years [range]) | 2.4 [0.3-13.2] | 10.3 [2.5-19.3] | <0.001* |
| Type of transplantation | Unilateral | 14 (12%) | 7 (18%) | 0.50* |
|          | Bilateral   | 100 (88%) | 31 (82%) |  |
| Underlying diagnosis | COPD/A1AT/OB | 42 (37%) | 22 (58%) | 0.29* |
|          | CF          | 14 (12%) | 7 (18%) |  |
|          | PAH         | 2 (2%) | 0 (0%) |  |
|          | IPF         | 25 (22%) | 4 (11%) |  |
|          | non-IPF PF  | 26 (23%) | 5 (13%) |  |
|          | Other       | 5 (4%) | 0 (0%) |  |
| Medication regime | Tacrolimus | 111 (97%) | 38 (100%) | 0.31* |
|          | Everolimus  | 22 (19%) | 7 (18%) | 0.91* |
|          | Cyclosporin | 3 (3%) | 0 (0%) | 0.31* |
|          | Mycofenolat Mofetil | 100 (88%) | 32 (84%) | 0.58* |
|          | Prednisone  | 114 (100%) | 38 (100%) |  |
|          | Azithromycin | 83 (73%) | 26 (68%) | 0.60* |
|          | Montelukast | 4 (4%) | 11 (20%) | <0.001* |
| Time till onset CLAD | (years [range]) | 5.7 [0.23-18.8] |
| CLAD stage | 1 | 21 (55%) |
|           | 2          | 13 (34%) |
|           | 3          | 4 (11%) |
|           | 4          | 0 (0%) |
| CLAD phenotype | BOS | 20 (53%) |
|          | RAS        | 5 (13%) |
|          | Mixed      | 5 (13%) |
|          | Undefined  | 8 (21%) |

*Abbreviations: A1AT, alpha-1 antitrypsin; BOS, bronchiolitis obliterans syndrome; CF, Cystic fibrosis; COPD, Chronic obstructive pulmonary disease; IPF, Idiopathic pulmonary fibrosis; LTx, lung transplantation; OB, Obliterative bronchiolitis; PAH, Pulmonary arterial hypertension; PF, Pulmonary fibrosis; RAS, Restrictive allograft syndrome.

*Independent samples t-test.

*Pearson’s Chi-squared test.
classification accuracy of the complete model achieved with cross-validation was 85% (±9% SD) with a kappa of 0.55 (±0.29 SD). The results for the training set are shown in Figure 2. In the training set, the AUC was 0.94 (95% CI 0.87-1.00) with a sensitivity of 96%, specificity of 85%, and an accuracy of 88%. In the validation set, the AUC reached 0.94 (95% CI 0.87-1.00) with a sensitivity of 100%, a specificity of 78%, and an accuracy of 83%.

Bootstrapped comparison of the AUCs showed that the logistic regression model—with the additional parameters—outperformed the PLS-DA model with breathprint only in both the training ($p = 0.02$) and the validation set ($p = 0.04$).

**CLAD phenotype**

Out of the 38 LTR with CLAD, 20 were diagnosed with BOS, 5 LTR with RAS, 5 LTR with mixed CLAD, and 8 LTR with undefined CLAD phenotype. The results of the PLS-DA model are shown in Figure 3. The SpiroNose accurately discriminated between BOS and RAS with an AUC of 0.95 (95% CI 0.87-1.00), a sensitivity of 100%, specificity of 90%, and an accuracy of 92%.

Likewise, as shown in Figure 4, the SpiroNose could adequately discriminate between the BOS and undefined phenotypes. However, the SpiroNose could not adequately discriminate between the other phenotypes (Table 3).

**CLAD stages**

The results of the PLS-DA model are shown in Figure 5. The eNose could reliably distinguish between non-CLAD LTR (CLAD 0) and CLAD stages 1 and 2-3. However, for discrimination between CLAD 1 and CLAD 2-3, the AUC was only 0.56 (95% CI 0.38-0.75) with a sensitivity 100%, specificity of 18%, and an accuracy of 54%.

**Influence of unilateral lung transplantation**

In a sensitivity analysis, unilateral LTR were excluded from the dataset. This resulted in a dataset of 131 bilateral LTR of which 31 LTR were diagnosed with CLAD.

---

**Table 2 Multivariate Logistic Regression Models**

| Clinical parameters | $\beta$ | SE  | $p$-value |
|---------------------|---------|-----|------------|
| Age                 | 0.02    | 0.03| 0.43       |
| Gender (Female)     | -0.20   | 0.63| 0.75       |
| Type of LTx (Bilateral) | 0.27 | 1.12| 0.81       |
| Time after LTx      | 0.38    | 0.09| <0.001     |
| ACR event (ACR)     | 2.03    | 0.72| 0.0048     |

| Clinical parameters | $\beta$ | SE  | $p$-value |
|---------------------|---------|-----|------------|
| Age                 | 0.02    | 0.035| 0.50      |
| Gender (Female)     | -0.23   | 0.77 | 0.76      |
| Type of LTx (Bilateral) | 0.94 | 1.56| 0.55      |
| Time after LTx      | 0.35    | 0.10| <0.001    |
| ACR event (ACR)     | 1.75    | 0.84 | 0.037    |
| eNose latent variable 1 | -0.80 | 0.25| 0.001     |
| eNose latent variable 2 | 0.73  | 0.37| 0.047     |

*Abbreviations: ACR, Acute cellular rejection; LTx, Lung transplantation; SE, Standard error.*

*p-Values were calculated using Wald test statistics.*
Subsequently, the model was trained on the new dataset and the AUCs were obtained.

In the training set the AUC was 0.94 (95% CI 0.89-0.99) with a sensitivity of 78%, specificity of 97%, and an accuracy of 92%. In the validation set the AUC reached 0.86 (95% CI 0.74-0.98) with a sensitivity of 100%, specificity of 66%, and an accuracy of 73%. Bootstrapped comparison of the AUCs showed no significant differences before and after exclusion of unilateral LTR in both the training (p = 0.84) and validation (p = 0.27) sets.

**Figure 2** ROC curves of the logistic regression model.

**Figure 3** Discrimination between the BOS and RAS phenotypes of CLAD using the SpiroNose and the corresponding ROC curve. BOS: Bronchiolitis obliterans syndrome; RAS: Restrictive allograft syndrome.
Discussion

In this study, we found that exhaled breath analysis using an eNose was able to confirm CLAD in LTx with 79% to 83% accuracy. The addition of known risk factors for CLAD to the breathprint further improved the diagnostic accuracy. Additionally, the eNose could discriminate between BOS and RAS CLAD phenotypes with 92% accuracy.

As for the technical model, eNose technology shows a good discriminative signal between LTR with and without CLAD, as indicated by the cross-validated machine learning model showing an AUC of at least 0.82 for the PLS-DA model (validation set). When known risk factors for CLAD development were incorporated into the model the discriminative power of the model further increased. In literature underlying diagnosis pre-LTx, induction treatment, time after LTx, ACR, gastroesophageal reflux disease (GERD), bacterial colonization, infections, and the presence of donor-specific antibodies (DSAs) were described as consistently associated with the development of CLAD and its main phenotypes.26,27 In our data set induction treatment, GERD, bacterial colonization, infections, and presence of DSAs were missing and could therefore not be added into the model. A larger validation set should reveal whether the breathprint alone is sufficient as predictor for CLAD or that a larger model with incorporation of other known risk factors would be preferred.

The eNose could accurately discriminate between the BOS and RAS phenotypes. However, discrimination between the other CLAD phenotypes, especially the mixed

Table 3  Receiver Operating Curve results for discrimination between CLAD phenotypes using the SpiroNose.

|                  | AUC (95% CI) | Sensitivity | Specificity | Accuracy |
|------------------|--------------|-------------|-------------|----------|
| BOS vs Mixed     | 0.54 (0.14-0.94) | 40 %        | 100%        | 88%      |
| RAS vs Mixed     | 0.60 (0.12-1.00) | 60%         | 100%        | 80%      |
| BOS vs Undefined | 0.92 (0.82-1.00) | 100%        | 85%         | 89%      |
| RAS vs Undefined | 0.50 (0.16-0.84) | 38%         | 100%        | 62%      |
| Mixed vs Undefined | 0.63 (0.17-1.00) | 100%        | 60%         | 85%      |

Abbreviations: AUC, area under receiver operating curve; CI, confidence interval; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome.
and undefined phenotypes was insufficiently accurate. Whereas this could be related to the overall small numbers in the subgroups it may also give insight into the underlying characteristics of the various phenotypes. There are some putative mechanisms described for RAS including inflammation, innate immune response, and humoral immune response, which may result in irreversible tissue remodeling and fibrosis. Putative mechanisms described for BOS include different injurious processes, such as allo- and autoimmune responses, external stimuli (such as gastroesophageal reflux, bacterial or viral infections, pollutant exposure), and airway ischemia, ultimately leading to remodeling of the small airways while other compartments of the lung remain relatively intact. Nonetheless, the mechanisms of BOS and RAS often overlap. The inability to discriminate undefined and mixed CLAD patients may be explained by the fact that these phenotypes express characteristics of both RAS and BOS. Although highly speculative, the patients with mixed phenotype seem to cluster more towards the BOS phenotype, and patients with undefined phenotype cluster more towards the RAS phenotype. This does however not align with data as published by Levy et al. on prognosis of these phenotypes, as they found that patient with RAS or mixed phenotype had a worse allograft survival than BOS, while patients with an undefined phenotype did not differ in allograft survival from patients with BOS. Unsupervised analyses of breathprint in a larger and multicenter CLAD dataset may yield useful insights into how patients will naturally cluster together as opposed to how they are grouped according to the current classification. This might confirm or challenge the current CLAD classification.

To date, there are limited treatment options for CLAD with no treatment able to reverse the onset of CLAD. Addition of azithromycin has shown to delay CLAD-onset and improve long-term survival, and is yet regarded standard therapy before allowing a diagnosis of CLAD. Therapeutic options for CLAD (BOS) include total lymphoid irradiation (TLI), extracorporeal photopheresis (ECP), or rescue treatment with alemtuzumab. All of these have not been studied extensively or in controlled studies, but may have some effect in slowing down progression of CLAD. Also, a beneficial effect of ATG as a second line therapy is suggested, with a response rate of 63%. Some beneficial effects with add-on montelukast have also been suggested in two small studies, but its effect remains unclear. As CLAD is histologically often accompanied by interstitial fibrosis, novel antifibrotic agents are currently under study in various clinical trials, both in BOS and RAS phenotypes of CLAD, with disappointing initial results for BOS. More results are expected later in 2022. Finally, if all treatment options fail re-transplantation can be a last resort to treat advanced CLAD. Nevertheless, a minority of patients qualifies for re-transplantation and outcomes are inferior to primary transplantation.

Despite the limited treatment options of established CLAD, timely diagnosis is highly desirable. Earlier diagnosis could potentially enable more successful treatment. Regarding future perspectives, if patients that are at risk of developing CLAD could be identified before pulmonary function actually deteriorates, early treatment such as increment of the net state of immunosuppression, might be initiated with the possible potential for better outcomes. Our study shows that the eNose might be a suitable point of care test to rapidly and noninvasively establish reliable diagnosis of CLAD. Future studies will be directed at external validation of the current results as well as the potential of a more timely diagnosis or even predicting development of CLAD before actual decline in pulmonary function becomes apparent.
This is the first time that exhaled breath using eNose technology was demonstrated to be a very promising novel biomarker for CLAD. Despite the potential of eNose technology, it has barely been explored in the field of lung transplantation.\(^4\) The ability of eNose technology to discriminate between different pulmonary diagnoses have been evaluated by numerous clinical studies for a wide spectrum of lung diseases.\(^18\) Accordingly, evidence is accumulating that eNose may be a valuable diagnostic tool for clinical and inflammatory phenotyping of for example asthma, COPD, or interstitial lung diseases.\(^15\)−\(^17\) Real-time measurements of the breathprint by an eNose is a low-cost and fast point-of-care tool that may provide useful as addition to the current repertoire in clinical follow-up after LTx. Further study into the application and validation is warranted given these promising first results. Using eNose technology, only the patterns of VOCs in exhaled breath are captured by cross-reactive gas sensors. Subsequently, these patterns are analyzed and classified using machine learning algorithms. In order to identify individual VOCs, other techniques—such as gas chromatography—mass spectrometry (GC-MS)—are needed, which are very complex and time-consuming, but could theoretically be combined with the current eNose. Despite this inherent characteristics and inability to characterize individual VOCs, main advantages are that this technique is cheap, easy to execute, fast and can be made available in real-time at the outpatient clinic, thus offers opportunities for widespread implementation.

A potential limitation in this study is the relatively small validation set. The size of the validation set has an influence on the random uncertainty of the observed performance of the model, as to avoid bias the validation set needs to be representative for the patient population.\(^30\) Nonetheless, results from training and validation set are highly similar in all models tested, indicating good model fit. Also, eNose studies generally require small numbers to assess discrimination abilities.\(^24\) The diagnostic accuracy found in this cohort is very high and reproducible in the validation sets and thus unlikely to be based on chance findings. Additionally, the study was underpowered for discrimination between the 4 phenotypes of CLAD. Analyses of the breathprint in a larger and multicenter CLAD dataset should reveal the accuracy of eNose technology to distinguish between the four phenotypes of CLAD. A strength is the large response rate and the well-defined cohort of patients.

In the present study we for the first time demonstrate that it is possible to discriminate between LTR with and without CLAD, including RAS and BOS phenotypes, with the use of eNose technology. Hence, exhaled breath analysis using eNose technology is a very promising novel biomarker for CLAD enabling timely diagnosis and phenotyping. Furthermore, eNose technology could be a valuable addition to the diagnostic armamentarium for suspected graft failure after LTx.

**Disclosure statement**

JA: reports personal fees and non-financial support from MSD; personal fees from BMS, Boehringer Ingelheim, Amaphera, Eli Lilly, Takeda, Bayer, Roche, Astra Zeneca outside the submitted work. In addition, JA has a patent on allogenic tumor cell lysate licensed to Amaphera, a patent combination immunotherapy in cancer pending, and a patent biomarker for immunotherapy pending. OM: reports personal fees from Astra Zeneca, Boehringer Ingelheim, Novartis outside the submitted work. The remaining authors have no conflicts of interest to disclose.

**Acknowledgments**

We would like to thank the Erasmus MC Thorax Foundation for supporting our research.

**Author contributions**

Research idea and design, MH, OM, DB, and NW; patient inclusion NW; data analysis and interpretation, NW, MH, OM, and DB; drafting and/or critically reviewing of the manuscript, NW, MH, OM, RH, BM, LS, CM, DB, and JA; advise on study design RH, BM, LS, CM, and JA. All authors have read and agreed to the published version of the manuscript.

**Supplementary materials**

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.healun.2020.07.009.

**References**

1. Opelz G, Dohler B, Ruhnstroth A, et al. The collaborative transplant study registry. Transplant Rev (Orlando) 2013;27:43-5.
2. van der Mark SC, Hoek RAS, Heltemons ME. Developments in lung transplantation over the past decade. Eur Respir Rev 2020;29:190132. https://doi.org/10.1183/16000617.0132-2019.
3. Gauthier JM, Hachem RR, Kreisel D. Update on chronic lung allograft dysfunction. Curr Transplant Rep 2016;3:185-91.
4. Bos S, Vos R, Van Raemdonck DE, Verleden GM. Survival in adult lung transplantation: where are we in 2020? Curr Opin Organ Transplant 2020;25.
5. Tissot A, Danger R, Claustre J, Magnan A, Brouard S. Early identification of chronic lung allograft dysfunction: the need of biomarkers. Front Immunol 2019;10:1681.
6. Verleden GM, Glansville AR, Leased ED, et al. Chronic lung allograft dysfunction: Definition, diagnostic criteria, and approaches to treatment - A consensus report from the Pulmonary Council of the ISHLT doi: 10.1016/j.healun.2019.03.009 J Heart Lung Transplant 2019;38:493-503. https://doi.org/10.1016/j.healun.2019.03.009.
7. Chambers DC, Zuckermann A, Cherikh WS, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: 37th adult lung transplantation report — 2020; focus on deceased donor characteristics doi: 10.1016/j.healun.2020.07.009 J Heart Lung Transplant 2020;39:1016-27. http://10.1016/j.healun.2020.07.009.
8. Levy L, Huzszi E, Renaud-Picard B, et al. Risk assessment of chronic lung allograft dysfunction phenotypes: validation and proposed refinement of the 2019 International Society for Heart and Lung Transplantation classification system. J Heart Lung Transplant 2020;39:761-70. https://doi.org/10.1016/j.healun.2020.04.012.
9. Meyer KC, Raghur G, Verleden GM, et al. An international ISHLT/ATS/ERS clinical practice guideline: diagnosis and management of...
bronchiolitis obliterans syndrome. Eur Respir J 2014;44:1479. https://doi.org/10.1183/09031936.00107514.

10. Gottlieb J, Zamora MR, Hodges T, et al. ALN-RSV01 for prevention of bronchiolitis obliterans syndrome after respiratory syncytial virus infection in lung transplant recipients. J Heart Lung Transplant 2016;35:213-21. https://doi.org/10.1016/j.healun.2015.08.012.

11. Vital D, Hofer M, Benden C, Holzmann D, Boehler A. Impact of sinus surgery on pseudomonal airway colonization, bronchiolitis obliterans syndrome and survival in cystic fibrosis lung transplant recipients. Respir Med 2013;86:25-31. https://doi.org/10.1159/000339627.

12. Küppers L, Holz O, Schuchardt S, et al. Breath volatile organic compounds of lung transplant recipients with and without chronic lung allograft dysfunction. Article. J Breath Res 2018;12. https://doi.org/10.1088/1752-7163/aac5af.

13. van der Schee MP, Paff T, Brinkman P, van Alderen WMC, Haarman EG, Sterk PJ. Breathomics in lung disease. Chest 2015;147:224-31. https://doi.org/10.1378/chest.14-0781.

14. van de Kant KDG, van der Sande LJTM, Janssen Q, van Schayck OCP. Breath volatile organic compounds as a surrogate biomarker for classifying patients with asthma by atopy. J Allergy Clin Immunol 2020;146:1045-55. https://doi.org/10.1016/j.jaci.2020.05.038.

15. Abdel-Aziz MI, Brinkman P, Vijverberg SJH, et al. eNose breath prints as a surrogate biomarker for classifying patients with asthma by atopy. J Breath Res 2020;14:1045-55. https://doi.org/10.1088/1752-7163/aac5af.

16. de Vries R, Dagelet YWF, Spoor P, et al. Clinical and inflammatory phenotyping by breathomics in chronic airway diseases irrespective of the label. Eur Respir J 2018;51:1701817. https://doi.org/10.1183/13993003.01817-2017.

17. Moor CC, Oppenheim JC, Nakshbandi G, et al. Exhaled breath analysis by use of eNose technology: a novel diagnostic tool for interstitial lung disease. Eur Respir J 2020;57:2002042. https://doi.org/10.1183/13993003.02042-2020.

18. van der Sar IG, Wijbenga N, Nakshbandi G, et al. The smell of lung disease: a review of the current status of electronic nose technology. Respir Res 2021;22:246. https://doi.org/10.1186/s12931-021-01835-4.

19. Glanville AR, Verleden GM, Todd JL, et al. Chronic lung allograft dysfunction: definition and update of restrictive allograft syndrome - a consensus report from the pulmonary council of the ISHLT. doi: 10.1016/j.healun.2019.03.008 J Heart Lung Transplant 2019;38:483-92. https://doi.org/10.1016/j.healun.2019.03.008.

20. de Vries R, Brinkman P, van der Schee MP, et al. Integration of electronic nose technology with spirometry: validation of a new approach for exhaled breath analysis. J Breath Res 2015;9:046001. https://doi.org/10.1088/1752-7155/9/4/046001.

21. Cuperlovic-Culf M. Machine learning methods for analysis of metabolic data and metabolic pathway modeling. Metabolites 2018;8. https://doi.org/10.3390/metabo8010004.

22. Barker M, Rayens W. Partial least squares for discrimination. J Chemom 2003;17:166-73. https://doi.org/10.1002/cem.785.

23. Ruiz-Perez D, Guan H, Madhivanan P, Mathee K, Narasimhan G. So you think you can PLS-DA? BMC Bioinf 2020;21:2. https://doi.org/10.1186/s12931-021-01835-4.

24. Broadhurst DJ, Fell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. Metabolomics 2006;2:171-96. https://doi.org/10.1007/s11306-006-0037-z.

25. Determin M. Optimal algorithm for metabolomics classification and feature selection varies by dataset. Int J Biol 2015;7:100-15. https://doi.org/10.5539/ijb.v7n1p100.

26. Koutskouka A, Royer PJ, Antonietti JP, et al. Development of a multivariate prediction model for early-onset bronchiolitis obliterans syndrome and restrictive allograft syndrome in lung transplantation. Front Med (Lausanne) 2017;4:109-. https://doi.org/10.3389/fmed.2017.00109.

27. Verleden GM, Bos R, Vanaudenaerde BM, et al. Current views on chronic rejection after lung transplantation. Transpl Int 2015;28:1131-9. https://doi.org/10.1111/tri.12579.

28. Rohat F, Gautier B, Singh A, Lê Cao K-A. mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS Comput Biol 2017;13:e1005752. https://doi.org/10.1371/journal.pcbi.1005752.

29. Fleiss JL, Levin B, Paik MC. The measurement of interrater agreement. Statistical Methods for Rates and Proportions. Wiley Series in Probability and Statistics: 2003;598-626.

30. Sato M. Bronchiolitis obliterans syndrome and restrictive allograft syndrome after lung transplantation: Why are there two distinct forms of chronic lung allograft dysfunction? Ann Transl Med 2020;8:418-. https://doi.org/10.21037/atm.2020.02.159.

31. Bos R, Vanaudenaerde BM, Verleden SE, et al. A randomised controlled trial of azithromycin to prevent chronic rejection after lung transplantation. Eur Respir J 2011;37:164. https://doi.org/10.1183/09031936.00068310.

32. Rutten D, Verleden SE, Vandermeulen E, et al. prophylactic azithromycin therapy after lung transplantation: post hoc analysis of a randomized controlled trial. Am J Transplant 2016;16:254-61. https://doi.org/10.1111/ajt.13417.

33. Benden C, Haughton M, Leonard S, Huber LC. Therapy options for chronic lung allograft dysfunction—bronchiolitis obliterans syndrome following first-line immunosuppressive strategies: a systematic review. J Heart Lung Transplant 2017;36:921-33. https://doi.org/10.1016/j.healun.2017.05.030.

34. Kotecha S, Paul E, Ivulich S, et al. outcomes following atg therapy for chronic lung allograft dysfunction. Transplant Direct 2021;7:e681681. https://doi.org/10.1097/td.000000000001134.

35. Rutten D, Verleden SE, Demeyer H, et al. Montelukast in chronic lung allograft dysfunction after lung transplantation. J Heart Lung Transplant 2019;38:516-27. https://doi.org/10.1016/j.healun.2018.11.014.

36. Rutten D, Verleden SE, Vandermeulen E, et al. Montelukast for bronchiolitis obliterans syndrome after lung transplantation: a randomized controlled trial. PLoS One 2018;13:e0193564. https://doi.org/10.1371/journal.pone.0193564.

37. Bos S, De Sadeleer LJ, Vanstapel A, et al. Antifibrotic drugs in lung transplantation and chronic lung allograft dysfunction: a review. Eur Respir Rev 2021;30:210050. https://doi.org/10.1183/16000617.0005-2021.

38. Halloran K, Aversa M, Tinckam K, et al. Comprehensive outcomes after lung retransplantation: a single-center review. Clin Transplant 2020;34:e13281. https://doi.org/10.1111/ctr.13281.

39. Kovacs D, Bikov A, Losonczy G, Murakozy G, Horvath I. Follow up of lung transplant recipients using an electronic nose. J Breath Res 2013;7:017117. https://doi.org/10.1088/1752-7155/7/10/017117.

40. Beleites C, Neugebauer U, Bocklitz T, Krafft C, Popp J. Sample size planning for classification models. Anal Chim Acta 2013;760:23-33. https://doi.org/10.1016/j.aca.2012.11.007.