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Full length paper

# Electronic nose technology for detection of invasive pulmonary aspergillosis in prolonged chemotherapy-induced neutropenia – a proof of principle study

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**Abstract**

Although the high mortality of invasive pulmonary aspergillosis (IA) in patients with prolonged chemotherapy-induced neutropenia (PCIN) can be reduced by timely diagnosis, a diagnostic test that reliably detects IA at an early stage is lacking. We hypothesized that an electronic nose (eNose) can fulfill this need. An eNose can discriminate various lung diseases through analysis of exhaled volatile organic compounds (VOC's). An eNose is cheap, non-invasive and yields results within minutes. Methods: **i**n a single-center prospective cohort study we included patients that were treated with chemotherapy expected to result in PCIN. Based on standardized indications a full diagnostic workup was performed to confirm invasive aspergillosis or to rule it out. Patients with *"no aspergillosis"* were considered controls and patients with *"probable/proven aspergillosis"* index cases. Exhaled breath was examined with a Cyranose 320®. The resulting data were analyzed using principal component reduction. Primary endpoint was the cross-validated diagnostic accuracy, defined as the percentage of correctly classified patients using the leave-one out method. Accuracy was validated by 100,000 random classifications. Results: we included 46 subjects that underwent 16 diagnostic workups resulting in 6 cases and 5 controls. The cross-validated accuracy of the eNose in diagnosing IA was 90.9% ($p = 0.022$; sensitivity 100%, specificity 83.3%). ROC analysis showed an AUC of 0.93. Conclusion: these preliminary data indicate that in PCIN patients with IA have a distinct exhaled VOC profile that can be detected with eNose technology. The diagnostic accuracy of eNose in invasive aspergillosis warrants validation.

## Introduction

The diagnosis of invasive pulmonary aspergillosis poses a significant challenge in clinical practice, due to the fact that symptoms and signs of invasive pulmonary aspergillosis are neither sensitive nor specific.(1-2) This also holds for conventional chest X-ray and cultures of sputum and/or broncho-alveolar lavage. Furthermore, a CT scan of the lungs is a sensitive but non-specific test.(3) The diagnosis is considered proven if a culture (from a normally sterile site that is clinically or radiologically abnormal) yields Aspergillus spp.(3) Unfortunately, this requires invasive procedures, such as percutaneous or transbronchial lung biopsy, which are rarely possible in the majority of patients with IA, i.e. hematological patients experiencing prolonged chemotherapy-induced neutropenia. This is due to concurrent thrombocytopenia and the risk of pneumothorax that is usually considered too large in these patients.(4)

Over the last 10 years a number of new tests have been introduced, most notably the Platelia Assay: a double-sandwich ELISA on galactomannan, a cell wall component of various molds including *Aspergillus spp.* When performed on serum the assay has a sensitivity and specificity of about 80% and, more importantly, a positive Platelia test can precede clinical manifestation by fever and other symptoms.(5) Recently, it was shown that when performed on broncho-alveolar lavage, the sensitivity and specificity of galactomannan is even higher.(6) However, broncho-alveolar lavage is not without burden or even risks, and often not feasible. In addition, galactomannan is not detectable in serum until accumulation of a considerable fungal burden.

As the mortality of IA is high (>50%) and can be reduced by a timely diagnosis, a diagnostic test that can reliably detect IA at an early stage remains one of the major goals in mycology and supportive care

in hematology.(7-9)

Exhaled air is known to contain thousands of volatile organic compounds (VOC's), derived from various metabolic pathways.(10) These VOC's can be used as biomarkers of lung disease, as has been demonstrated for bronchial carcinoma, infectious diseases, COPD and asthma.(11-16) Recent evidence indicates that a specific VOC, 2-pentyl-furan, could be a potential biomarker of IA.(17-18) However, the need for gas chromatography and mass spectrometry (GC-MS) in the assessment of individual volatile compounds precludes widespread on-site application in clinical practice.

An alternative way of assessing VOC-mixtures is using electronic noses (eNoses). An electronic nose is an artificial olfactory system that discriminates complex odors using an array of sensors. When exposed to exhaled breath the sensors react in a promiscuous way to the different fractions of VOC's.(19-21) Each odor, which represents a unique mixture of VOC's, will result in a pattern of sensor signals unique to that odor. This is called a “breathprint” when it concerns exhaled air. Using pattern recognition algorithms, complex mixtures of VOC's can thus be discriminated at high-throughput without identifying the individual molecular components as such. eNoses are relatively cheap, mostly hand-held, easy to operate and yield a result within minutes. From a patient's perspective exhaled breath analysis is appealing, because it is non-invasive, safe, rapid, simple to perform and effort independent. Therefore, biomedical validation of eNoses is emerging.(19, 22)

We hypothesized that exhaled breath analysis using an electronic nose (eNose) can be used to diagnose IA. To test that hypothesis, we performed a prospective proof of principle study.

**Methods**

**Subjects –** Patients were included if they: 1) were 18 years of age or older, 2) had given written informed consent, and 3) were treated for a hematological malignancy with chemotherapy expected to result in severe neutropenia (<0.5 x $10^9$ neutrophils/L) of more than 7 days, e.g. hematopoietic stem cell transplantation or induction/consolidation treatment for acute myeloid leukemia. Patients were excluded if they were previously diagnosed with an invasive mycosis, or if they were unable to perform the breathing manoeuvre needed for eNose-analysis of exhaled air. The Medical Ethics Committee of the Academic Medical Centre approved the protocol of the study. The study was registered at ClinicalTrials.gov as study NCT01395446. All patients gave informed consent.

**Design** – The study was a single-center prospective cohort study. Based on standardized indications a full diagnostic workup was performed to confirm invasive aspergillosis or to rule it out. The results were classified according to the EORTC criteria, revised in 2008.(3) In the event of no possible, probable or proven aspergillosis and no sero-positivity, the patient qualified as neutropenic control. In the event of probable or proven aspergillosis a patient qualified as case. The breathprints of cases and controls were compared. Exhaled breath analysis was performed only once in each patient.

According to this design, patients with possible aspergillosis were excluded. We chose for this design because of the proof of principle nature of our study. Patients with possible aspergillosis can truly have invasive aspergillosis, but more often do not have invasive aspergillosis. Including patients with possible aspergillosis would make it harder to detect a breathprint associated with invasive aspergillosis.

**Antifungal prophylaxis** - All patients were managed identically and according to a standardized protocol based on recent guidelines with respect to the prevention, diagnosis and treatment of mycoses.(23) Except for analysis of exhaled air using the eNose every prophylactic, diagnostic and treatment-related procedure was according to standard care. Prophylactic antifungal treatment was started the same day as the chemotherapy. Patients received 500 mg amphotericine B every 6 hours orally until the peripheral neutrophil count exceeded 0.5 x $10^9$/L. Patients undergoing myeloablative allogeneic stem cell transplantation received 200 mg of fluconazole daily. If oral amphotericine B was not tolerated, no substitute was started. In principle, no antimycotics with activity against *Aspergillus spp.*, such as voriconazole or posaconazole, were administered prophylactically. If the treating physician judged that the administration of prophylactic anti-mold treatment was necessary, the patient was excluded from the study as eNose results could be influenced by anti-mold therapy.

**Diagnostic strategy** - From the start of chemotherapy onwards cultures of throat, nose, rectum and, if possible, sputum were performed weekly. From the start of neutropenia (<0.5 x $10^9$ neutrophils/L) a serum galactomannan assay was performed twice weekly. Both were continued until the peripheral neutrophil count exceeded 0.5 x $10^9$/L. A complete diagnostic workup was performed in case of a number of standardized indications for a diagnostic workup, based on international guidelines: 1) a rise of serum galactomannan above 0.5, or 2) five or more days of fever unresponsive to broad empiric antibiotic treatment and without alternative explanation, or 3) a new infiltrate developing under broad antibiotic coverage or highly dosed steroids, or 4) an abnormality on a chest X-ray consistent with invasive pulmonary mycosis, or 5) hyphae or molds found in a respiratory specimen, or 6) symptoms and/or signs considered by the treating physician to be possibly due to an invasive mycosis.(23)

**Diagnostic workup** - The workup consisted of 1) analysis of sputum (using direct microscopy and

139 culture), 2) a high resolution CT of the thorax, 3) in case of abnormalities on the HR-CT consistent
140 with invasive pulmonary mycosis bronchoscopy and broncho-alveolar lavage. The BAL was examined
141 using direct microscopy; a PCR on *M.tuberculosis* complex, mycobacterial culture, PCR's on
142 respiratory viruses (human boca virus, parainfluenza 1 to 4, parechovirus, coronavirus, rhinovirus,
143 RSV, human metapneumovirus, enterovirus, influenza A and B, adenovirus, HSV, EBV, CMV), routine
144 culture and measurement of galactomannan were performed as well. In case a broncho-alveolar lavage
145 was not performed a throat gurgle specimen was examined using PCR's on the abovementioned
146 respiratory viruses. Sinonasal, ophthalmological and neurological symptoms and signs were actively
147 sought. On indication CT of the liver and spleen, CT or MRI of the brain and sinus, consultation of a
148 neurologist, otolaryngologist or ophthalmologist was performed.
149
150 **Exhaled breath analysis –** Every "diagnostic workup" was followed by exhaled breath analysis as
151 previously described.(11, 24) Patients were asked to breath through a mouthpiece for 5 minutes with
152 the nose clipped. Through a three-way non-rebreathing valve this mouthpiece was connected to an
153 inspiratory VOC filter (A2; North Safety, Middelburg, The Netherlands) as well as an expiratory silica
154 reservoir. Then, a deep inspiratory capacity maneuver was followed by the exhalation of a vital
155 capacity volume. The exhaled breath was collected in a 10 liter Tedlar bag which was connected to the
156 silica reservoir. Within 30 minutes the Tedlar bag was sampled using the electronic nose, a Cyranose
157 320® (Smith Detections, Pasadena, CA). This is a hand-held chemical vapor analyzer based on a nano-
158 composite sensor array with 32 polymer sensors.(19) The change in electrical resistance in each of the
159 32 sensors was stored as raw data for further analysis. Every sampling procedure was repeated after
160 which the first measurement was disregarded, as previously described because of deviant raw data at
161 first run.(11)
162

**Analysis –** As our primary analysis we compared the breathprints of cases and controls. We performed offline analysis of the raw data using R (version 2.11.1). First, data reduction by principal component analysis (PCA) was performed to reduce the original data of the 32 sensors to a non-predefined number of principal components, capturing at least 99.9% of the variance within the dataset. Secondly, t-tests (equal variance assumed depending on the outcome of an F-test) were used to assess which PCA factors discriminated between the two groups; a two-sided p-value of 0.10 was considered significant. Then, based on the differentiating PCA factors a categorical division was made using linear canonical discriminant analysis, assuming an equal chance of being a member of one of the two groups. The discriminant function was chosen to best distinguish between categories. Finally, the accuracy of this model was established. This was defined as the percentage of correctly classified patients, cases and control subjects combined. Cross-validation using the leave-one out method was used to calculate the cross-validated accuracy. The 95% confidence intervals (CI) were calculated using the exact binomial test. To calculate our p-value, we generated 100,000 random classifications of our subjects ("whether or not case or control") and determined the chance that a random classification would have led to a cross-validated accuracy identical to our primary outcome or better, constructing a new pattern recognition algorithm for each of the random classifications using the statistical method of the primary analysis.(25) Finally, ROC analysis was performed.

**Results**

During the study period there were 53 eligible patients. As 5 refused informed consent and 2 had previously been diagnosed with an invasive mycosis, 46 patients were included. In 16 of these subjects one or more triggers for a diagnostic workup occurred. This resulted in 6 controls and 5 cases, see Table 1. Principal component analysis of the raw data resulted in 8 principal components (PC's) that described 99.9% of the variance. Of these 1 discriminated between cases and controls. Subsequent canonical discriminant analysis showed a cross validated accuracy value of 90.9% (95% CI 59% to 100%). The sensitivity and specificity were 100 (95% CI 48 to 100%) and 83.3% (95% CI 36% to 100%). Figure 1 shows the individual discriminant scores. ROC analysis of the discriminant scores revealed an area-under-the-curve of 0.933. In our simulation 2.2% of the 100,000 random classifications resulted in a cross-validated accuracy identical to or better than 90.9%. In all patients in whom both values were determined we calculated the correlation between the discriminant scores and BAL galactomannan values, currently the most accurate single test to diagnose invasive pulmonary aspergillosis. Although these were correlated, this was not statistically significant (unstandardized regression coefficient -0.23, 95% CI -0.54 to 0.08, R2 0.36), see Figure 2.

196 **Discussion**

197 Our study shows that patients with invasive aspergillosis have an exhaled VOC profile, distinctive from
198 controls, which can be established by eNose technology. The accuracy is high and, as shown by the
199 random classifications, is not a coincident finding. This implies that in the future exhaled breath
200 analysis could become a non-invasive addition to the diagnostic arsenal in invasive aspergillosis that is
201 cheap, fast and simple to perform.
202
203 eNose technology will hopefully enable us to detect invasive aspergillosis at an earlier point in time
204 than currently available diagnostic tools. At the time of the exhaled breath analysis in subject 4, he was
205 thought to have *no possible, proven or proven aspergillosis* based on the diagnostic workup according
206 to protocol. Two weeks later however, probable aspergillosis was diagnosed. In retrospect, very small
207 pulmonary lesions were already seen two weeks before at the locations where later aspergilloma
208 developed. Therefore, he was classified as being a case in our study. Out of interest, we also performed
209 a second exhaled breath analysis two weeks later, when we diagnosed probable aspergillosis. This
210 measurement was not used to derive the pattern recognition algorithm for our primary analysis, off
211 course. We compared the two exhaled breath analyses. Although the first signal (discriminant score of -
212 0.49) was less pronounced than the second (-1.37), it did already indicate IA.
213
214 To our knowledge, this is the first study examining the accuracy of exhaled air in the early diagnosis of
215 invasive aspergillosis. It is however in line with previous *in vitro* research, which already showed that
216 an eNose can reliably differentiate *in vitro* the most frequently encountered pathogens in pneumonia.
217 Moens et al. demonstrated that an eNose could differentiate the headspaces of various micro-organisms
218 after 17 hours of culturing with a diagnostic accuracy of 100%. They examined Gram negative bacteria
219 (*P.aeruginosa, E.coli, K.pneumoniae, E.aerogenes, P.vulgaris*), Gram positive bacteria (*S.aureus,*

*S.pneumoniae, E.faecalis*), a yeast species (*Candida albicans)* and a mold species, *Aspergillus fumigatus*.(26) Other groups confirmed that an eNose is able to differentiate the headspaces of various micro-organisms.(27-28) These results were already extended to an in vivo situation, i.e. ventilator-associated pneumonia (VAP). Hockstein et al. calculated pneumonia scores in 44 ventilated patients based on a number of clinical criteria.(29-30) An eNose could reliably differentiate between the 7 patients with a high pneumonia score and the 29 patients with a low pneumonia score. Our data thus support and extend the accumulating evidence that eNose technology can be used to diagnose pulmonary infections.

Our study has a number of strong points. It studied a prospective cohort in which the patients were followed according to a state-of-the-art diagnostic protocol, defining the timing of our exhaled breath analyses and characterizing our population well with respect to whether aspergillosis occurred. This also yielded a well-characterized control group.

On the other hand, our study is subject to two major limitations. First of all, the sample size was small due to the low incidence of IA. This precluded external validation of our results. As the aim of the study was to detect However, our 100,000 random classifications indicated that the chance of false-positive discovery was only 2.2%. It also Eventually, according to guidelines on stepwise assessment of diagnostic accuracy of novel tests, the confirmation of our results in a separate group of subjects that was not involved in generating the pattern-recognition algorithm will be required to definitively establish the ability of an eNose to detect IA.(31) Such external validation has already been provided for the differential diagnosis by eNose between COPD and asthma.(32)

Secondly, eNose technology, albeit applicable for medical applications, does not allow identification of

the individual VOC's that drive the signal. It is unknown which VOC's enable the detection of IA by eNose technology. First, these could be VOC's produced by *A.fumigatus* itself. In the literature a number of potential candidates have been suggested. One such compound is 2-pentylfuran, which was reported by a research group from New Zealand to be *A.fumigatus*-specific, being exhaled by subjects with colonization as well as invasive disease caused by *A.fumigatus*.(17-18) However, differences in the composite molecular signatures as captured by breathprints may arise from other sources rather than A.fumigatus, such as the host response. The presence of *Aspergillus spp.* in the airways triggers an immune response. In a number of patients this even leads to the clinical entity called allergic broncho-pulmonary aspergillosis (ABPA).(33) Notably, inflammatory airway diseases, such as asthma and COPD, can be discriminated at a high level of accuracy through eNose technology, in which the signals by eNose as well as GC-MS are significantly associated with cellular and molecular markers of airways inflammation.(34-35) Such inflammatory host responses could have played a major role in our study, augmenting the difference in exhaled VOC profiles and aiding in early detection. Invasive aspergillosis induces a major immune response, despite neutropenia.(36)

The implications of our results are potentially wide. Exhaled breath analysis could increase the accuracy of the diagnostic workup of a patient suspected of having invasive aspergillosis. It could also decrease the mortality of invasive aspergillosis, for example through a reduction of the diagnostic delay by monitoring patients with prolonged chemotherapy-induced neutropenia twice per week. And lastly, if it were to improve the diagnostic accuracy enough, it could obviate the need for bronchoscopy, thereby making the workup less invasive. Furthermore, if further translational research would unravel the molecules involved in the generation of the specific breathprint, eNoses could be "tailor-made" to detect these VOC's to improve the diagnostic accuracy even further.(16)

In conclusion, this study shows the potential of eNose technology in the detection of IA in patients experiencing prolonged chemotherapy-induced neutropenia through analysis of exhaled breath. This warrants the next step in testing diagnostic accuracy by performing a large-scale validation study in order to determine how much diagnostic delay can be prevented by adding twice weekly exhaled breath analysis using eNose to a state-of-the-art diagnostic strategy in invasive aspergillosis.(31)

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**Legenda of figures and table**

Table 1. Subject characteristics.

AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; R-VIM: ritxumab/ etopside/iphosphamide/methotrexate; n.p. not performed; P: positive as a clinical EORTC criterium; N: negative as a clinical EORTC criterium; induction: induction chemotherapy

Figure 1. Individual discriminant scores derived from exhaled breath profiles of patients with and without invasive pulmonary aspergillosis.

Figure 2. Correlation of galactomannan on BAL and discriminant scores based on exhaled breath profiles in subjects in whom bronchoscopy was performed. The fit line based on linear regression and the 95% mean prediction intervals are shown.

Table 1. Subject characteristics

| | subject | age | sex | diagnosis | therapy | HR-CT of the lungs | serum galactomannan | BAL galactomannan | cultures positive for *Aspergillus spp.* | EORTC classification | remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **probable/proven aspergillosis** | | | | | | | | | | | |
| | 1 | 62 | F | AML | induction | P | 0.1 | 2.3 | no | probable | concurrent influenza (H1N1) |
| | 3 | 35 | F | AML | induction | P | 0.2 | 7.4 | no | probable | |
| | 4 | 47 | M | AML | induction | P | 1.9 | 7.6 | no | probable | |
| | 8 | 70 | M | ALL | induction | P | 0.3 | 8.1 | no | probable | |
| | 9 | 56 | M | Waldenström | R-VIM | P | 1.2 | n.p. | no | probable | |
| **means** | | **54.0** | **40% female** | | | | **0.7** | **6.4** | | | |
| **no aspergillosis** | | | | | | | | | | | |
| | 2 | 53 | F | AML | induction | N | 0.1 | n.p. | no | no | |
| | 5 | 51 | F | AML | induction | N | 0.2 | n.p. | no | no | |
| | 6 | 65 | F | AML | induction | P | 0.1 | 0.3 | no | no | bacterial pneumonia |
| | 7 | 46 | F | AML | induction | N | 0.1 | n.p. | no | no | left-sided pleural fluid |
| | 10 | 63 | M | AML | induction | N | 0.1 | n.p. | no | no | bacterial pneumonia |
| | 11 | 74 | M | AML | induction | N | 0.1 | n.p. | no | no | bilateral pleural fluid |
| **means** | | **58.7** | **67% female** | | | | **0.1** | **0.3** | | | |

**Figure 1. Individual discriminant scores derived from exhaled breath profiles of patients with and without invasive pulmonary aspergillosis**

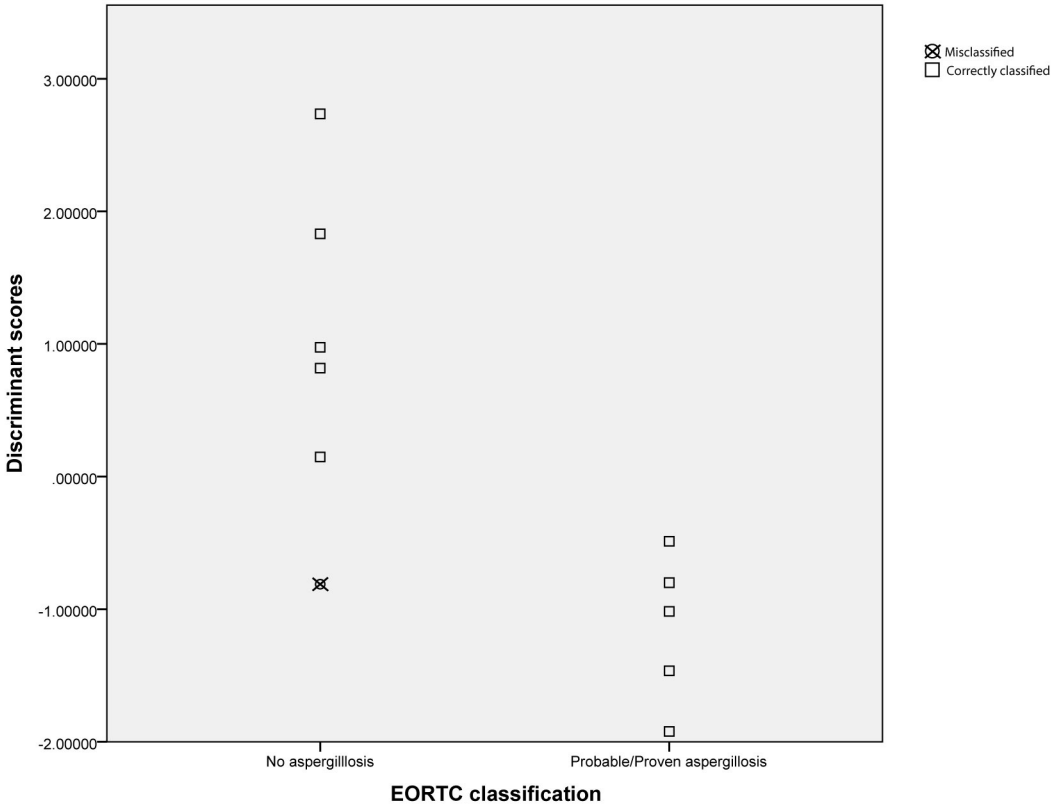

**Figure 2. Correlation of galactomman on BAL and discriminant score based on exhaled breath profiles in subject in whom bronchoscopy was performed**