Chapter 8.

Volatile Metabolic Fingerprint of Ventilator-associated Pneumonia

*Published as Research Letter*
*Intensive Care Medicine 2014*

Abstract

There is urgent need for a better diagnostic approach of ventilator–associated pneumonia (VAP). Invading respiratory pathogens as well as patients produce volatile organic compounds (VOCs) that could serve as early biological markers for VAP. We hypothesize that volatile metabolic fingerprints can discriminate airway samples of patients with VAP from those of patients without VAP.

In this prospective cohort study, VOCs in headspace of tracheal aspirates from intubated and ventilated intensive care unit patients were analysed using electronic nose technology (eNose).

The analysis included 45 patients, of which 14 were classified as having ‘VAP’, 14 as ‘colonized patients without VAP’ and 17 as ‘non–colonized patients without VAP’. eNose analysis discriminated between VAP and patients without VAP with an area under the receiver operating characteristic curve (ROC–AUC) of 0.85 [95%–confidence interval (CI): 0.69–1.0]. Discrimination was not influenced by colonization of the airways in patients without VAP (p = 0.42). VOC fingerprinting of airway samples improved diagnostic accuracy for VAP on top of the clinical pulmonary infection score (net classification improvement: 1.4 [CI: 0.80–2.07, p<0.001]). Longitudinal analysis showed that the slope of the eNose signal was steeper in patients that went on to develop VAP (0.04 vs. 0.002, p=0.009).

VOCs are promising biological markers for the diagnosis of VAP. Diagnostic accuracy does not seem to be influenced by colonization of airways. Longitudinal analysis showed that the slope of the eNose signal is also different in patients that develop VAP.
Introduction

Ventilator–associated pneumonia (VAP) is a frequent complication in intubated and mechanical ventilated critically ill patients [1, 2]. The diagnosis remains complex as radiological confirmation of a new or progressive pulmonary infiltrate and symptoms of pulmonary inflammatory response are sensitive for VAP but a more definitive diagnosis requires positive microbiological cultures [3-5]. However, it may take days before the culture results become available and they could be false–negative [6]. Thus there is urgent need for a novel approach of VAP early diagnosis. Importantly, the diagnostic process should not only detect respiratory pathogens in the airways, but also differentiate infection from airway colonization, which is frequent in intubated patients [7].

In ancient times, physicians relied heavily on their senses for disease detection. Colour, taste, and smell were used to detect biological markers [8]. Bacterial strains are known to have a characteristic smell produced by volatile metabolic end–products of bacteria–specific biochemical pathways called volatile organic compounds (VOCs) [9-13]. The host interacts with bacteria through an oxidative and inflammatory response, which could even modulate other bacterial metabolic pathways. Certainly, this host response alters human metabolism in itself [14, 15]. VOCs, whether produced by invading pathogens or by the host, can be detected in the headspace of liquid airway samples (e.g., lavage fluids) or exhaled breath [16]. Such VOC–profiles have been shown to correlate with the frequently used clinical pulmonary infection score (CPIS) [17] and could be useful in discriminating between different pathogen species [18-20] but have not been compared to the consensus diagnostic criteria for VAP [21].

We hypothesize that VOC–profiles from airways samples can identify airway samples of intubated and ventilated patients with VAP. To test this hypothesis we included patients that did and did not develop VAP. VOC–profiles were detected in tracheal aspirates (TA) using a sensor based technology called electronic nose (eNose) [22]. This technology can be used at the bedside and provides a result within minutes [22]. We analysed whether identification of VAP by VOC–profiles depends on colonization of the airways, investigated the influence of the amount and Gram-status of bacteria in the tracheal aspirate, quantified the complementary value
of VOCs to CPIS and explored the development of the eNose signal over time.

**Methods**

*Design and setting*

This is a study within an international multi-center prospective observational cohort that evaluated the predictive value of biological markers for development of VAP. The study protocol was reviewed and approved by the Medical Ethical Committee of Parc Taulí, Sabadell, Spain (IRB: 2008/524).

*Inclusion and exclusion criteria*

Inclusion criteria were: (1) recruited to one of the participating ICUs, (2) intubated and ventilated for another reason than pneumonia, and (3) expectation that mechanical ventilation was needed for longer than 48 hours. Exclusion criteria were: (1) age less than 18 years, (2) expectation that withdrawal of treatment could happen within 72 hours, and (3) pregnancy or lactation. Furthermore we excluded patients who received antimicrobial therapy within the last 5 days before ICU admission (prophylactic antimicrobial therapy, e.g., for surgical procedures, was allowed), and patients with fulminant hepatic failure, pancreatitis, or disseminated cancer, since these all could effect levels of biomarker of interest in the original study. Finally, for the purpose of the present analysis we also excluded patients who developed pneumonia within the first two days in the ICU.

*Diagnostic definitions and patient selection*

Patients were classified into three groups (table 1). VAP was diagnosed using consensus criteria [21] (a new and persistent radiographic infiltrate plus at least 2 of the following criteria: a) temperature > 38°C or < 36°C; b) leucocytes >10 or <4 \( \times 10^3/\text{mm}^3 \); c) purulent tracheal aspirate [23, 24]) but always needed microbiological confirmation to fulfill the diagnosis of ‘VAP’ (> \( 10^3 \) or \( \geq 10^6 \) colony forming units (CFU)/ml in mini-bronchoalveolar lavage (BAL) fluid or tracheal aspirate (TA), respectively). Patients not fulfilling the abovementioned criteria for VAP but of whom
microbiological culture revealed presence of bacteria in mini-BAL or TA were classified as ‘colonized patients without VAP’. Patients not fulfilling the abovementioned criteria for VAP with negative cultures were classified as ‘non–colonized patients without VAP’.

Data and sample collection

Patient demographics, primary (and admission) diagnosis, SAPS II [25], APACHE II score [26] and ICU mortality were recorded for all patients. Tracheal aspirates (TA) were collected on Mondays, Wednesdays and Fridays and were sent for quantitative culture. Mini-BAL was performed on the day of clinical suspicion of VAP. Isolates were characterized by colony morphology and Gram stains. The remaining portions of TA samples were saved at −80°C (figure 1, bullet 1).

Figure 1: Plan for sample selection and statistical analysis
Headspace analysis

TA fluid samples were defrosted and subsequently analysed using an eNose (figure 1, bullet 2). For this, 0.5 ml supernatant (1500 rpm for 15 minutes at 4\(^\circ\)C) of TA fluid was transferred into a 5 ml headspace vial (MN–net N 20–5 DIN, clear with crimp top, Fisher Scientific, Landsmeer, the Netherlands), topped with a 20 mm headspace cap (Fisher Scientific, Landsmeer, the Netherlands) and warmed to room temperature. Two needles were inserted into the headspace cap before start of each measurement. One needle was placed into the sample and was purged with pure nitrogen gas (99.9999%, Linde Gas, Dieren, the Netherlands). The second needle was placed in the headspace for collection of gas (figure S1).

We used the Cyranose 320 eNose (Smith Detections, Pasadena, CA) containing a nano–composite sensor array with 32 polymer sensors (figure 1, bullet 3) [22]. This electronic nose relies on semi-selective recognition of VOCs as each sensor is cross-reactive to a variety of functional chemical groups. The combined response of the sensor array can be used for pattern-recognition and disease classification [27]. A full description of the chemical and statistical rationale for this approach is outside the scope of the present paper and has recently been discussed in an excellent review by Konvalina and Haick in Accounts of chemical research and is summarized in the online supplement [28]. Nitrogen gas was sampled for 30 seconds as a baseline-measurement, where after gas from the headspace was analysed for one minute using a low flow (40 ml/min). Afterwards, the eNose was purged to let the sensors recover to baseline. This was done in duplicate.

Statistical analysis

Differences between the groups were compared using the Mann–Whitney U test for continuous variables and chi–square for categorical variables. Data were summarized using the median and inter-quartile range for continuous variables and with count and percentage for categorical variables. All analyses were performed in R statistics using R studio [29]. P–values below 0.05 were considered significant.
Samples from patients with VAP were to be compared in time to those from patients who did not develop VAP. For this, we first determined the day the diagnostic criteria for VAP were met in patients who developed VAP. This was day 7 after ICU admission. We then selected patients who did not develop VAP, but who were still intubated and ventilated on day 7 after ICU admission to create the control group. To train the diagnostic algorithm ‘VAP’ patients were considered cases and both ‘colonized patients without VAP’ and ‘non–colonized patients without VAP’ were taken together as controls.

Sparse partial least square (SPLS) logistic regression was used to produce a diagnostic model (figure 1, bullet 4). SPLS analysis is a suitable form of regression that can select predictive variables and limit false discovery in situations were large number of independent variables are investigated in low numbers of individuals [30]. Two parameters are set in SPLS: K and eta. These were tuned using 10-times cross–validation [30]. SPLS logistic regression resulted in a predicted probability of VAP, which is called the “eNose signal” in this manuscript and was used for further analyses. Receiver operator characteristics (ROC) analysis was performed and the area under the curve (AUC) was reported (figure 1, bullet 5).

A sensitivity analysis was performed for ‘colonized patients without VAP’ and ‘non–colonized patients without VAP’ (figure 1, bullet 6). Secondly, we investigated the correlation between the eNose signal and to the number of colonizing forming units using Spearman’s correlation. Thirdly, the diagnostic performance of the eNose logistic regression function was compared to the CPIS and the net classification improvement of the combination of the two diagnostic tests was assessed [31]. Finally, the development of the eNose signal was displayed over time using a LOESS smoother and analyzed by means of mixed-model analysis. The period preceding VAP (7 days before diagnosis) was investigated (figure 1, bullet 7). The slope of the eNose signal was fitted using a linear mixed model with a random intercept and a random slope for the eNose signal per patient, as described previously [32]. The slopes were compared between patients with and without VAP and the area under the ROC-curve was calculated.
Results

Patients

Figure 2 shows the CONSORT diagram of the study; of 154 eligible patients, 10 development of pneumonia within 48 hours, and from 27 patients too much data was missing. Of the remaining 117 patients 14 patients fulfilled the diagnostic criteria for VAP (on median day 7); 103 patients never met the diagnostic criteria for VAP, and of them only 31 patients were intubated and ventilated for longer than 7 days. These patients served as controls in the planned analysis. Of the patients without VAP, 14 had colonized airways. Table 1 shows the patient characteristics and microbiological data. There were no missing physiological data (ventilator setting, hemodynamics, temperature) and follow-up for mortality was available for all patients in the database. None of the patients were treated with inhaled antibiotics or systemic corticosteroids.

Figure 2: Patient inclusion
### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>No VAP (n=31)</th>
<th>VAP (n=14)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non–colonized airways (n=17)</td>
<td>colonized airways (n=14)</td>
<td></td>
</tr>
<tr>
<td>Age, yrs N (%)</td>
<td>61 (33)</td>
<td>66 (27)</td>
<td>59 (31)</td>
</tr>
<tr>
<td>Male N (%)</td>
<td>15 (48)</td>
<td>7 (41)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>APACHE II median (IQR)</td>
<td>20 (10)</td>
<td>20 (10)</td>
<td>20 (13)</td>
</tr>
<tr>
<td>SAPS II median (IQR)</td>
<td>55 (20)</td>
<td>50 (15)</td>
<td>60 (19)</td>
</tr>
<tr>
<td>COPD N (%)</td>
<td>2 (6)</td>
<td>2 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Reason for MV N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>4 (13)</td>
<td>3 (21)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Shock N (%)</td>
<td>4 (13)</td>
<td>3 (21)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Low consciousness</td>
<td>17 (55)</td>
<td>8 (57)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Other N (%)</td>
<td>6 (19)</td>
<td>3 (21)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>No N (%)</td>
<td>17 (55)</td>
<td>17 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa.</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other gram negative</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6 (19)</td>
<td>0 (0)</td>
<td>6 (43)</td>
</tr>
<tr>
<td>Candida species</td>
<td>4 (13)</td>
<td>0 (0)</td>
<td>4 (29)</td>
</tr>
<tr>
<td>Tidal volume (ml)</td>
<td>446 (145)</td>
<td>445 (108)</td>
<td>447 (161)</td>
</tr>
<tr>
<td>Plateau pressure (cmH2O)</td>
<td>20 (8)</td>
<td>17 (6)</td>
<td>23 (8)</td>
</tr>
<tr>
<td>Positive end expiratory pressure (cmH2O)</td>
<td>5 (2)</td>
<td>5 (2)</td>
<td>5 (0)</td>
</tr>
<tr>
<td>CPIS (day measurement)</td>
<td>3 (4)</td>
<td>3 (5)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>WBC (x103)</td>
<td>10 (4)</td>
<td>10.4 (7.9)</td>
<td>10.5 (2.5)</td>
</tr>
<tr>
<td>28 day mortality N (%)</td>
<td>4 (13)</td>
<td>3 (21)</td>
<td>1 (8)</td>
</tr>
</tbody>
</table>

*: P-value for all patients without VAP vs. all patients with VAP.
†: Multiple organisms could have been cultured per patient.
Airway samples

175 airway samples were obtained. The median number of samples per patient was 4 [3 – 6] and 2 [2 – 3] in the VAP group and the groups of patients without VAP, respectively. One sample per patient was used for the cross-sectional statistical analysis (for patients with pneumonia: sample on day of diagnosis, for patients without pneumonia: sample obtained closest to day 7). The median day of sample collection was 7 [5 – 9] and 5 [4 – 8] for patients with VAP and patients without VAP, respectively.

Discrimination between samples of patients with VAP and without VAP

SPLS analysis of eNose data (K = 4, eta = 0.8) resulted in the selection of 26 sensors (for coefficients, see Table S1). Using the eNose model, VAP (median predicted probability: 0.53 [0.44 – 0.90]) and patients without VAP (colonized and non-colonized) (median: 0.14 [0.10 – 0.23]) could be discriminated with a ROC–AUC of 0.85 [CI: 0.69 – 1.0] (table 2).

Sensitivity analysis for colonization in patients without VAP

The eNose predicted probability was not different for patients without VAP, with and without colonized airways: 0.12 [0.09 – 0.22] and 0.14 [0.12 – 0.24], respectively (p = 0.42). VAP was well distinguished from non-colonized and from colonized patients without VAP: ROC–AUC of 0.84 [CI: 0.68 – 1.0] and 0.85 [CI: 0.68 – 1.0], respectively (table 2).

Correlation with bacterial growth

Bacterial growth was associated with a higher eNose signal (Spearman’s correlation coefficient: 0.56, p<0.001; figure S2). When the analysis was limited to samples with bacterial growth, the amount of CFU was not correlated with the eNose signal (Spearman’s correlation coefficient: 0.22, p=0.36, figure S2)

Comparison and combination with CPIS

The ROC–AUC for the CPIS by itself was 0.89 [CI: 0.80 – 0.99] for ‘VAP’ (table 2). The ROC–AUC was 0.95 [CI: 0.88 – 1.0] after combination of
CPIS with the eNose algorithm (table 2). The net classification improvement \[31\] was 1.4 [CI: 0.80 – 2.07, \(p < 0.001\)] for ‘VAP’.

**Longitudinal analysis**

Figure 3 shows the development of the eNose signal during ICU-stay. The slope of the eNose signal was 0.04 [0.02 – 0.05] for patients with VAP and 0.002 [-0.02 – 0.01] for patients without VAP (\(p=0.009\)). Using the slope of the eNose signal VAP and patients without VAP (colonized and non-colonized) could be discriminated with a ROC–AUC of 0.76 [CI: 0.56 – 0.96].

**Figure 3:** Breathprint over time
Table 2: Diagnostic accuracy

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control group</th>
<th>ROC-AUC [95% CI]</th>
<th>Cut-off</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNose</td>
<td>All controls</td>
<td>0.85 [0.69 – 1.0]</td>
<td>0.41</td>
<td>94%</td>
<td>79%</td>
<td>91%</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>Non-colonized controls</td>
<td>0.84 [0.68 – 1.0]</td>
<td>0.38</td>
<td>94%</td>
<td>79%</td>
<td>84%</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>Colonized-controls</td>
<td>0.85 [0.68 – 1.0]</td>
<td>0.41</td>
<td>93%</td>
<td>79%</td>
<td>81%</td>
<td>92%</td>
</tr>
<tr>
<td>CPIS (logistic regression)</td>
<td>All controls</td>
<td>0.89 [0.80 – 0.99]</td>
<td>0.45</td>
<td>87%</td>
<td>86%</td>
<td>72%</td>
<td>92%</td>
</tr>
<tr>
<td>CPIS + eNose</td>
<td>All controls</td>
<td>0.95 [0.88 – 1.0]</td>
<td>0.38</td>
<td>94%</td>
<td>86%</td>
<td>81%</td>
<td>92%</td>
</tr>
<tr>
<td>eNose slope</td>
<td>All controls</td>
<td>0.76 [0.56 – 0.96]</td>
<td>0.03</td>
<td>89%</td>
<td>75%</td>
<td>89%</td>
<td>75%</td>
</tr>
</tbody>
</table>

Discussion

The present findings suggest that volatile metabolic fingerprinting of TA fluid might discriminate patients with VAP from patients without VAP. Confirmation of these findings in a larger cohort of patients is needed. The diagnostic accuracy was not influenced by the presence or absence of airway colonization. The predicted probability increased towards the day of VAP diagnosis. These data suggest that volatile markers in airway samples can facilitate the diagnostic procedures for VAP before the availability of microbiologic results and warrant further clinical development of this technology.

In the present study, we focused on the diagnostic accuracy of headspace analysis of airways samples with an eNose for the consensus definition of VAP [21]. Bacteria have a distinct metabolism, part of which results in the production of bacteria-specific volatile organic compounds that may be used for diagnosis of the presence or absence of specific strains [13]. Indeed, it has been reported that an eNose can discriminate between growth of Gram–positive bacteria and Gram–negative bacteria, and no bacterial growth in BAL fluid [19]. The number of patients with Gram–positive VAP in our cohort was too small to confirm this. We found that the discrimination between VAP and no VAP with electronic nose analysis was not influenced by bacterial colonization and we did not find any correlation with bacterial growth. Thus our results cannot be fully explained by bacterial
growth alone. Several other factors should be taken into consideration. For example, inflammation and bacterial ecology may modulate bacterial metabolism [33]. Therefore we may not detect bacterial presence per se, but the interaction with the host response.

Importantly, the metabolite fingerprint improved classification of VAP on top of the CPIS. This indicates that VOC detection may be complementary to clinical symptoms for the diagnosis of VAP. Previous studies correlated VOC–profiles to clinical, radiological or microbiological features of VAP alone [18, 20, 34]. These features may not be sufficiently representative for the disease entity VAP while adequate phenotyping of the included patients, thus using a well–defined group of cases, is highly important for teaching a diagnostic algorithm [35]. Therefore, the present study extends the previously observed correlation between VOC–profiles and CPIS, radiology and microbiology and leads to a more confident statement that the observed alterations in volatile metabolite profiles may be due to pneumonia.

The longitudinal data analysis of the period preceding diagnosis showed that the slope of the eNose signal was steeper in patients that were to develop VAP. This slope could be used to discriminate between patients with and without VAP with moderate accuracy. This may imply that the eNose signal can be used to monitor disease progression in an at-risk population.

The strengths of ours study seem to be adequate phenotyping, the combination of the eNose signal with the CPIS and longitudinal analysis. Nevertheless, several limitations should be noted. Firstly, all analyses were performed in a highly selected cohort of patients. It cannot be excluded that this selection promoted the strength of the discriminative signal between VAP and controls by electronic nose fingerprints. Therefore, the present work does not provide data on the diagnostic accuracy in all patients with suspicion on VAP. Generalization is further limited by the modest amount of patients with VAP that were included. We cannot exclude that the observed differences between patients with and without VAP may be due to other causes than the development of VAP. Secondly, tracheal aspirates can only be obtained when sufficient secretions are
present in the trachea. Therefore, we did not have airway samples from every subject at all time points. This may have led to sampling bias, which can influence longitudinal analyses. Finally, we could not identify the VOCs that differentiate between VAP and controls. Although we performed gas chromatography and mass spectrometry, we had difficulties with obtaining a reliable signal due to viscous matrix of the tracheal aspirates. This limits the translation of our findings to the development of new, VAP specific sensor arrays.

While volatile biological markers of airway samples promise to be helpful in distinguishing patients who develop VAP from those who do not develop VAP, the exact role of eNoses in clinical practice is yet uncertain. Even though previous studies suggest a good correlation between exhaled metabolic profiles, called breath–prints, and clinical symptoms or bacteriological growth [18, 20, 36], all these results require robust confirmation in future clinical trials in intubated and ventilated patients. Then it should be shown that eNose signals are complementary to those clinical parameters summarized in the CPIS. And then we can start performing studies in which we try to determine the clinical usefulness of a strategy that uses eNose signals, e.g., to see if use of eNoses leads to more timely use of antibiotics in patients who do develop VAP, or prevents overuse of antibiotics in patients who do not develop VAP.

In conclusion, volatile biomarkers can be complementary to clinical disease markers for improving the diagnosis of VAP. Diagnostic accuracy did not depend on colonization of the airways. Our data warrant the next step in establishing the diagnostic value of volatile organic compounds in VAP by performing longitudinal exhaled breath analysis in a larger population.

References


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