Diagnosis of pulmonary injury and infection by exhaled breath analysis

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Chapter 9.

Volatile Metabolites in Exhaled Breath of Ventilated Patients during Bacterial Colonization and Pneumonia

Not published

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Abstract

Exhaled breath of patients with pneumonia contains volatile molecules that are produced by invading pathogens and the host, which alone or in combination could be used as a marker for pneumonia. The aim of this study was to identify volatile molecules in exhaled breath of intubated and ventilated patients at the intensive care unit (ICU) that are associated with pneumonia, colonization of the respiratory tract and markers of inflammation.

Breath from intubated and ventilated ICU patients was analysed using gas chromatography and mass spectrometry during the first day of admission in a prospective design. Pneumonia was defined by clinical, radiological and microbiological criteria. Tracheal aspirates were cultured to determine the presence of bacteria in the lower airways. Levels of inflammatory markers were measured in plasma.

We analysed exhaled breath of 93 patients. 12 patients had probable/proven pneumonia and 25 patients had positive sputum cultures. The concentration of 1-propanol was significantly lower in exhaled breath of patients with probable/proven pneumonia than in patients without pneumonia and those with negative aspirate cultures (p-value: $10^{-4.1}$, fold change: -4.6). Concentrations of heptanal and 1-pentanol were significantly lower in patients with positive tracheal aspirate cultures as compared to those without (p-value: $10^{-3.4}$, fold change: -1.3, for heptanal and p-value: $10^{-3.4}$, fold change: -0.6 for 1-pentanol, respectively). Multiple individual VOCs correlated with circulatory markers of inflammation, but most profoundly with interferon-gamma.

Our data indicate that ventilated patients with pneumonia have a decreased concentration of 1-propanol in breath. Apparently, such decrease is not directly associated with positive sputum cultures or markers of systemic inflammation.
Introduction

Severe community- and hospital-acquired pneumonia (CAP and HAP), with admission to the intensive care unit (ICU) and need for mechanical ventilation, represents a major clinical problem associated with a high mortality [1, 2]. Currently, the diagnosis of pneumonia is based on clinical, radiological and microbiological criteria, but the spectrum of practice is heterogeneous [2] and might benefit from objective assessment by means of biological markers. Little is known about the diagnostic value of biological markers for the diagnosis of pneumonia [3, 4], especially in patients with severe pneumonia that are admitted to the ICU. Biomarkers for the diagnosis of pneumonia may detect the (pulmonary) inflammatory response or the presence of bacteria in the lung.

Bacterial metabolism can change the composition of volatile organic compounds (VOCs) in the exhaled breath, which may be used for sensitive detection of bacterial presence or the specific identification of pathogenic strains [5, 6]. Leukocytes are also known to produce VOCs [7] and since polymononuclear cells are a hallmark of pneumonia these could also contribute to exhaled VOCs [8]. VOCs may very well have biological functions. There is within-species and inter-species communication by the production of VOCs [9-11] and the bacteria may also use volatile molecules to interact with the host and vice versa [11].

The aim of this study was to identify volatile molecules in the breath of intubated and ventilated ICU-patients that are associated with pneumonia, colonization of the respiratory tract and markers of inflammation. We hypothesized that VOCs are present in different concentrations in (1) patients with confirmed pneumonia compared to patients without any signs of pneumonia and (2) patients with positive and negative cultures of tracheal secretions. Furthermore, we hypothesized that VOCs are correlated with specific systemic markers of inflammation (interleukin (IL)-1b, IL-6, IL-8, IL-10, IL-13, tumor necrosis factor (TNF)-alpha, interferon-gamma (IFN-g) and granulocyte-macrophage colony-stimulating factor (GM-CSF)). To that end, breath from newly admitted intubated and ventilated ICU patients was analysed using gas-chromatography and mass-spectrometry (GC-MS).
Methods

Ethical approval and informed consent

The institutional review board of the Academic Medical Center, Amsterdam, The Netherlands, decided that the study did not fulfil the criteria for medical research that requires ethics review according to the Dutch ‘Law on medical research’, because of the non-invasiveness and absence of burden of examining exhaled air (IRB: 10.17.0729). Therefore, it was judged by the institutional review board that exhaled breath can be analyzed without informed consent of the patient. The study was registered at the Dutch Trial Register (NTR 2750, www.trialregister.nl). Waste material from routine laboratory measurements in blood was collected, centrifuged at 1500g at room temperature for 15min and stored within 4 hours after blood draw at -80°C in all patients that were admitted to the ICU. Plasma storage was done within a multi-centre bio-bank study [Molecular Diagnosis and Risk Assessment of Sepsis (MARS): NCT01905033, www.clinicaltrials.gov]. An opt-out consent procedure was approved by the institutional review board.

Design, subjects and setting

In this prospective single center cross-sectional cohort study, all subsequent patients were included within 24 hours of ICU admission if intubated and ventilated, between December 2011 and November 2013. Previous ICU admission and cardiopulmonary surgery were reasons for exclusion. Patients were categorized into two groups: pneumonia and without pneumonia. Exhaled air and peripheral blood samples were taken within 24 hours after ICU-admission.

Clinical diagnosis of Pneumonia

A team of trained clinical research fellows prospectively scored the presence of pneumonia based on adapted Center for Disease Control–criteria and a post–hoc likelihood of infection was scored (none, possible, probable or proven; see figure E1 and table E2 in the supplementary material). All assessors had attended meetings in which clinical case vignettes were discussed and had at least 6 months of work experience [12].
**Exhaled breath analysis**

Exhaled breath was sampled and analyzed by standardized methodology that was previously published [13]. In short, breath was collected through a disposable side-stream connection for 10 minutes and VOCs were stored on a sorbent tube. These tubes were analyzed by means of GC-MS resulting in multiple ion-fragments per VOC. The abundance of ion-fragments within a small window of retention times (+/- 5 seconds) were grouped if they were strongly correlated (correlation coefficient > 0.7) to limit collinearity of the predictor matrix (e.g. to get one intensity per patient per VOC) but still allow for differentiation between co-elutions.

**Microbiology**

Tracheal aspirates were obtained from all patients as part of standard surveillance cultures (in the setting of selective decontamination of the digestive tract [14]) and sent for semi-quantitative bacterial culture. Cultures were considered positive if a potential respiratory pathogen was “highly present” (around 10^5 CFU) [15].

**Host response**

The host response was investigated by the measurement of inflammatory mediators (interleukin (IL)-1b, IL-6, IL-8, IL-10, IL-13, tumor necrosis factor (TNF)-alpha, interferon-gamma (IFN-g) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) in plasma using a cytometric bead array (CBA) Flex Set multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendation. These markers represent different functional classes of cytokines [16]: pro-inflammatory mediators: IL-1b and TNF-a; anti-inflammatory mediators: IL-10 and IL-13; angiogenic cytokines: IL-6 and IL-8; chemokines: IL-8; colony stimulating factors: GM-CSF; T-helper 1 response and macrophage activation: IFN-g.

**Group allocation**

We divided the population into several groups to answer the three research questions. First, we used confirmed pneumonia (probable/proven, see
clinical diagnosis of pneumonia) patients as cases and patients without suspected pneumonia and negative sputum cultures from the tracheal aspirate as controls. Second, we used patients with positive sputum cultures, regardless of their pneumonia status, as cases with colonization and patients with negative sputum cultures as controls. Finally, we looked at the correlation of exhaled VOCs with inflammatory mediators in the whole patient population.

**Statistical analysis**

Differences between the groups were compared using the Mann–Whitney U or Kruskal–Wallis test for continuous variables and chi–square for categorical variables. Data were summarized using the median and 25–75th percentile for skewed variables and with mean and 95%–confidence interval (CI) for normally distributed variables and with count and percentage for categorical variables. VOC and inflammatory mediator concentrations were 10-log transformed to obtain a normal distribution. All analyses were performed in R statistics using the R–studio interface [17]. P–values below 0.05 were considered significant.

For the VOCs, we calculated p-values (with the Mann-Whitney U test), fold-change and area under the receiver operating characteristics (ROC-AUC) between cases (pneumonia or positive sputum cultures) and controls. Subsequently, the label (case/control) was permutated and these analyses were repeated for 1000 times. The lowest p-value that was obtained in less than 5% of these random scenarios was calculated and used to identify significantly altered VOCs using the actual label. For correlation with inflammatory markers the p-values were obtained using Spearman’s correlation and the optimal cut-off was set at the highest value for alpha where the false discovery rate was below 5% (thus on theoretical basis, not on simulations as before). All results were displayed in volcano-plots and significant VOCs were manually identified using library matching (National Institute of Standards and Technology) and the injection of a pure chemical standard.
Table 1: Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control negative cultures (N=47)</th>
<th>Control; positive cultures (N=13)</th>
<th>Possible pneumonia (N=21)</th>
<th>Probable pneumonia (N=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59 48-70</td>
<td>64 43-79</td>
<td>63 55-71</td>
<td>61 45-72</td>
<td>0.93</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>28 59.6</td>
<td>8 61.5</td>
<td>15 71.4</td>
<td>5</td>
</tr>
<tr>
<td>APACHE IV Score</td>
<td>80 55-97</td>
<td>76 56-89</td>
<td>77 57-103</td>
<td>66 59-83</td>
<td>0.74</td>
</tr>
<tr>
<td>Admission type</td>
<td>Medical</td>
<td>31 66</td>
<td>8 61.5</td>
<td>20 95.2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Elective Surgery</td>
<td>1 2.1</td>
<td>0 0</td>
<td>0 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Emergence Surgery</td>
<td>12 25.5</td>
<td>5 38.5</td>
<td>1 4.8</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory comorbidities</td>
<td>2 4.3</td>
<td>0 0</td>
<td>6 28.6</td>
<td>2 16.7</td>
<td>0.010</td>
</tr>
<tr>
<td>Malignancy</td>
<td>4 8.5</td>
<td>3 23.1</td>
<td>4 19</td>
<td>4 33.3</td>
<td>0.18</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4 8.5</td>
<td>3 23.1</td>
<td>2 9.5</td>
<td>2 16.7</td>
<td>0.55</td>
</tr>
<tr>
<td>Maximal inspiratory pressure (cmH2O)</td>
<td>17 14-22</td>
<td>16 13-17</td>
<td>21 18-24</td>
<td>24.5 22-28</td>
<td>0.004</td>
</tr>
<tr>
<td>Positive end expiratory pressure (cmH2O)</td>
<td>5 5-5</td>
<td>5 5-5</td>
<td>8 5-10</td>
<td>9.5 5-10</td>
<td>0.002</td>
</tr>
<tr>
<td>Minute ventilation (L/min)</td>
<td>9.6 8.3-11.3</td>
<td>8.9 7.7-10.9</td>
<td>10.5 9-14.2</td>
<td>12.05 9.8-13.8</td>
<td>0.023</td>
</tr>
<tr>
<td>PaO2/FiO2 (mmHg/%)</td>
<td>280 243-377</td>
<td>349 294-467</td>
<td>300 233-368</td>
<td>240 189-306</td>
<td>0.06</td>
</tr>
<tr>
<td>Gram Positive isolation tracheal aspirate</td>
<td>0 0</td>
<td>6 46.2</td>
<td>1 4.8</td>
<td>2 16.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gram Negative isolation tracheal aspirate</td>
<td>0 0</td>
<td>8 61.5</td>
<td>2 9.5</td>
<td>7 58.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as median (25th to 75th percentile). Categorical variables are expressed as number (percentage). Differences between groups (possible/probable pneumonia) are tested using Kruskall Wallis or Chi-square test. APACHE II: Acute Physiology and Chronic Health Evaluation II.
Results

Subjects

300 patients were screened of whom 140 did not meet the inclusion criteria, mostly because they were not mechanically ventilated (figure 1). Of the 160 eligible patients, 59 met exclusion criteria (42 were previously mechanically ventilated and 17 were missed) thus 101 patients could be included. Eight additional patients could not be used because the GC-MS results were too deviant (as defined by >3 SD in principal component analysis) to allow for meaningful statistical analyses. The clinical and microbiological data on these patients are summarised in table 1.

Figure 1: Inclusion chart
**Volatile organic compounds**

1246 ion-fragments were found in all breath samples. Grouping of these ion-fragments based on retention time and correlation coefficient resulted in the separation of 140 VOCs.

**Probable pneumonia vs. control patients with negative tracheal cultures**

12 patients were classified as probable/proven pneumonia and 47 as patients without pneumonia and without positive sputum cultures. Figure 2A shows the volcano plot. 1-Propanol was significantly lower in the breath of patients with probable/proven pneumonia than in patients without pneumonia and without positive sputum cultures ($p$-value: $10^{-4.1}$, fold change: -4.6, AUC-ROC: 0.85). Figure 3 shows the abundance of 1-propanol in patients in all patients, stratified per likelihood of pneumonia.

**Patients with positive vs. negative tracheal cultures**

25 patients had positive and 68 patients had negative sputum cultures, irrespective of the suspicion of pneumonia. Figure 2B shows the volcano plot. Heptanal and 1-pentanol were significantly lower in patients with positive sputum cultures ($p$-value: $10^{-3.4}$, fold change: -1.3, AUC-ROC: 0.74 for heptanal and $p$-value: $10^{-3.4}$, fold change: -0.6, AUC-ROC: 0.74 for 1-pentanol, respectively).

**Correlation with markers of systemic inflammation**

None of the VOCs passed the threshold for statistical significance for the correlation with IL-1b, IL-6, TNFa and GM-CSF. Table 2 shows the significant correlations between IL-8, IL-10, IL-13, IFN-gamma and individual VOCs.
Figure 2: Volcano plots

Figure 3: 1-Propanol per group
**Table 2:** Correlation between inflammatory markers and VOCs

<table>
<thead>
<tr>
<th>Inflammatory marker</th>
<th>VOC</th>
<th>Correlation coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>Methyl-cyclopentene</td>
<td>0.44</td>
<td>$10^{-4.6}$</td>
</tr>
<tr>
<td>IL-10</td>
<td>Methyl-cyclopentene</td>
<td>0.46</td>
<td>$10^{-5.1}$</td>
</tr>
<tr>
<td>IL-10</td>
<td>2-Methyl-2-Propanol</td>
<td>0.38</td>
<td>$10^{-3.6}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>Unknown</td>
<td>-0.35</td>
<td>$10^{-3.0}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>Unknown</td>
<td>-0.36</td>
<td>$10^{-3.3}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>à-Pinene</td>
<td>-0.39</td>
<td>$10^{-3.8}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Cyclohexanone</td>
<td>0.36</td>
<td>$10^{-3.1}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>1-Pentanol</td>
<td>0.68</td>
<td>$10^{-3.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Heptanal</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Hexanal</td>
<td>0.35</td>
<td>$10^{-3.1}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Propenoic acid, 2-methyl-, ethyl ester</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Pentanone, 3-methyl-</td>
<td>0.34</td>
<td>$10^{-2.9}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.34</td>
<td>$10^{-3.0}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.32</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>C9-alkene</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Butanol</td>
<td>0.37</td>
<td>$10^{-3.4}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown cyclic compound</td>
<td>0.34</td>
<td>$10^{-2.9}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.31</td>
<td>$10^{-2.4}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Benzene, (1-methylethyl)-</td>
<td>0.31</td>
<td>$10^{-2.4}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>C9-alkene</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
</tbody>
</table>
Discussion

The data presented in this study indicate that pneumonia is associated with a decrease in the concentration of 1-propanol in breath of ventilated intensive care unit patients. A positive culture of tracheal aspirate with a respiratory pathogen was associated with a decrease in 1-pentanol and heptanal. Inflammatory mediators in plasma were correlated with multiple VOCs. The most profound correlation was found for interferon-gamma. These results warrant validation of the diagnostic value of VOCs for pneumonia in clinically relevant patients populations on the intensive care unit. The association between the presence of pneumonia and a decreased concentration of 1-propanol represents a novel observation. It has previously been suggested that breath analysis could be used to diagnose pneumonia in ventilated patients [18, 19], but the VOCs that differentiate have never been identified before. 1-propanol has been linked to bacterial metabolism \textit{in vitro} [5]. In contrast to our results, \textit{in vitro} studies observed higher concentrations of 1-propanol during bacterial growth [5]. This suggests that it may not be bacterial metabolism that is detected by this breath test, but another feature of pneumonia reflecting host-pathogen interaction. The latter hypothesis is further supported by the fact that the breath concentration of 1-propanol was not different between patients with and without positive sputum cultures.

Positive sputum cultures were associated with lower concentrations of 1-pentanol and heptanal. In line with the observation in the previous paragraph, 1-pentanol is found in higher concentration during bacterial growth \textit{in vitro}. However, it should be noted that bacterial metabolism is highly dependent on the availability of specific substrates [5]. Our data suggest that bacteria consume instead of produce 1-pentanol during growth \textit{in vivo}. Alternatively, positive sputum cultures essentially reflect overgrowth of one specific bacterial strain, which inhibits the metabolism of the “normal” lung microbiome and thereby decreases the concentration of specific bacterial metabolites.

Heptanal is produced during peroxidation of n-9 unsaturated fatty acids in humans [20], which suggests that oxidative stress is decreased during bacterial growth. However, an increase in lipid peroxidation may have
been expected during bacterial colonization as oxidative stress is part of the innate immune response [21]. We could speculate that patients with a decreased oxidative response to pathogens have increased bacterial growth and therefore a decrease in heptanal was observed, although we have no data to support that hypothesis. Moreover, the positive correlation between heptanal and interferon gamma supports the biological plausibility of our findings, suggesting that inflammation (increased T-helper 1 response and macrophage activation) is associated with lipid peroxidation. An alternative explanation may be that the amount of n-9 unsaturated fatty acids is lower in patients with positive sputum cultures. However, to our knowledge there is currently no evidence that links bacterial growth to decreased concentrations of n-9 unsaturated fatty acids. Thus the association between positive bacterial cultures and decreased heptanal concentrations remains to be explained.

Several VOCs were correlated with markers of inflammation. For example, methyl-cyclopentene positively correlated with IL-8 and IL-10. IL-8 is a chemo-attractant and leads to the accumulation of inflammatory cells on site whereas IL-10 is anti-inflammatory. A pro-inflammatory response is typically followed by an anti-inflammatory counter reaction and therefore the two contra regulatory processes may be correlated in vivo. The biochemical link between methyl-cyclopentene and inflammation is unclear and has not been described in literature. That also holds for all other VOCs that are linked to inflammation in this study. Pre-clinical studies are mandatory to clarify the relationship between inflammation and the observed individual VOCs in this and previous studies [22].

We believe this study has several strengths. We used an unbiased approach for the identification of VOCs that are associated with pneumonia. Furthermore, we did not limit the analysis to the clinical syndrome consensus diagnosis of pneumonia, but also used more objective biological measures such as bacterial growth and inflammatory response. However, several limitations should also be noted. First, the included groups are easily differentiated based on other characteristics and there is no clinical need for a biological marker in this patient population. Second, even though we took all statistical measures to suppress false positive results, the sample size was limited. This may hamper the generalization
of the results and awaits independent validation. Furthermore, the limited sample size prevented us from analyses between different individual bacterial strains. The latter is obviously important in view of clinical usage of breath tests in pneumonia. Third, systemic inflammation was assessed but inflammation within the pulmonary compartment may be of more interest in pneumonia [8, 23, 24]. Finally, univariate analysis was performed to identify potential biological markers for pneumonia, bacterial growth and inflammation but composite markers were not investigated. It is well established that markers that show good univariate classification are frequently outperformed by combinations of markers that show moderate univariate classification [25]. Because of the high dimensionality of the predictor matrix, the limited number of cases and the absence of an external validation cohort multi-variate analysis was of limited added value in this proof-of-concept study, but should definitely be considered in any larger validation cohort, especially if the classification paradigm is the main objective of the study.

We identified several potential breath markers for pneumonia and positive sputum cultures. However, as stated before, our results merely provide a first identification of the VOCs that could be of diagnostic value in pneumonia. Validation would require the use of extensive diagnostic procedures to obtain a gold-standard reference diagnosis for pneumonia in a population that has a high clinical suspicion of pneumonia. Possibly, the most clinically relevant patient population is those suspected of ventilator-associated pneumonia. Breath analysis could thereby assist to determine which patient should and should not be treated with antibiotics, very similar to cytokine analysis of BAL-fluid [26, 27], but without the need for an invasive diagnostic procedure.

**Conclusion**

Our data suggest that pneumonia in ventilated patients is associated with a decreased concentration of 1-propanol in breath. Lower concentrations of 1-pentanol and heptanal in breath were associated with positive sputum culture of a respiratory pathogen. Inflammatory mediators in plasma were correlated with multiple individual VOCs. The most profound correlation was found for interferon-gamma. These results warrant validation of the
diagnostic value of VOCs for pneumonia in clinically relevant patients populations on the intensive care unit.

References

12. Klein Klouwenberg PMC, Ong DSY, Bos LDJ, de Beer FM, van


