Diagnosis of pulmonary injury and infection by exhaled breath analysis
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Chapter 1.

General introduction and outline of the thesis

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Lieuwe DJ Bos
Abstract

Signs and symptoms of acute respiratory distress syndrome or pneumonia are preceded by local activation of various molecular pathways, which could provide diagnostic biological markers. This includes volatile metabolites that can be separated, identified and quantified by gas–chromatography and mass–spectrometry (GC–MS). Exhaled breath condensate can be used to investigate both volatile and non-volatile organic compounds. These techniques have recently been used to separate patients with acute lung injury from patients with uninjured lungs, to predict pulmonary infection, and even to discriminate between different respiratory pathogens. In this review, we pinpoint the aims, pros and cons, and perspective of currently available technologies on exhaled metabolic profiling in critically ill patients.

Although the first clinical studies on the diagnostic accuracy of exhaled breath analysis showed good results, methodological and biological validation is needed. First, methodological recommendations were not always followed strictly which might result in false–discoveries. Second, since metabolism is the end–product of gene–expression, protein function and physiological landscape, confounding factors may have biased patient studies so far. A more translational approach in breathomics research in mechanically ventilated patients is advisable, combining clinical trials with preclinical in–vivo and in–vitro experiments as well as combining metabolite discovery by GC–MS with disease diagnosis by pattern recognition with electronic noses. Clinical trials might use a combination of different technologies, including GC–MS and tailor made bedside analytical tools, in order to provide probabilistic evidence (positive– or negative predictive values) for clinical decision–making.
Introduction

Critically ill patients frequently develop acute lung injury (ALI) [1] or ventilator–associated pneumonia (VAP) [2]. The diagnostic assessment of these conditions is complex, leading to suboptimal clinical management. ALI is diagnosed according to the American–European consensus criteria, which include acute non–cardiogenic bilateral pulmonary infiltrates on chest radiography and hypoxia [3, 4]. VAP is suspected when sputum production increases or changes, in combination with new or changed pulmonary infiltrates on chest radiography and hypoxia [2]. Unfortunately, these clinical signs and symptoms are all far from specific and occur in a relatively late stage of disease, hampering accurate and early diagnosis [5, 6].

It is likely that signs and symptoms of ALI and VAP are preceded by local activation of various molecular pathways [7-11]. Monitoring of these so–called biological markers has the potential to improve the diagnostic process in many ways. Ideally a biological marker is sensitive to early pathophysiologic changes and specific for disease. Assessment of biological markers should preferably be rapid as well as non–invasive and cheap to allow for frequent monitoring [12]. While several biological markers were suggested to have the potential to assist in diagnosing ALI and VAP, unfortunately, most of them have been shown to have an unacceptable low diagnostic accuracy [13-16].

In the Middle Ages, physicians depended heavily on their senses. Color, taste, and smell were their biological markers [17]. Although outdated, their sensing provided a quick, non–invasive and integrative view on biochemical processes without additional costs, processing and analysis. The emerging possibilities of “smelling” hundreds to thousands of biological markers in exhaled air to diagnose pulmonary diseases in intubated and mechanically ventilated critically ill patients are spectacular, but research is facing huge challenges. In this review, we pinpoint the aims, pros and cons, and perspective of currently available technologies on exhaled metabolic profiling in critically ill patients. A translational roadmap for further biomedical and technological research is presented.
Use of biological markers

The medical usage of biological markers critically depends on the clinical objectives [18]. Pulmonary inflammation due to ARDS or VAP should not be missed, thus a rapid bedside test should at first be very sensitive. A relatively low specificity could still allow for clinical application because a high negative predictive value with an acceptable positive predictive value can exclude patients from treatment while adequately treat true-positive patients. The limitations of a low specificity are prescription of antibiotics in false-positive pneumonia, leading to increased bacterial resistance, and the usage of very low tidal volumes in false-positive ARDS, resulting in high carbon dioxide levels. A test with similar test-characteristics as the gold standard could add to the available clinical diagnostics if this test is more rapid, continuously available, less invasive or cheaper.

A second objective would be better phenotyping of patients with ARDS or VAP. In pneumonia, a very specific test (very high positive-predictive value) for a particular strain of bacteria is of added value because antibiotic therapy can be focused from broad- to narrow-spectrum. In ARDS, a specific test for a subtype of pulmonary inflammation might allow for phenotype–targeted therapy. In other words, if the test provides evidence for the involvement of a metabolic that is important in the pathogenesis of ARDS in some patients, this test could provide a target for intervention.

A third aim of biological markers is the monitoring of treatment response and adjustment of therapy. Antibiotic treatment might be stopped in pneumonia when biological markers return to normal, hereby limiting antibiotic usage. Furthermore, a different treatment strategy might be initiated rapidly after the primary therapeutic strategy failed to show a biological response.

Biological markers in blood

Numerous studies have tested the diagnostic accuracy of biological markers in blood for pulmonary complications in critically ill patients. Except for procalcitonin, which was found to have some value in the diagnosis and treatment response of VAP [19, 20], other candidate biological markers in blood were found to have an unacceptably low diagnostic accuracy [13-15]. This may not come as a surprise because blood levels of biological
markers may only partly, if at all, reflect pulmonary changes. Pulmonary inflammatory responses with ARDS and VAP are highly compartmentalized [7, 10, 11]. Indeed, while strong and early pro–inflammatory reactions are seen in broncho–alveolar lavage fluid (BALF) with ARDS and VAP, systemic levels of inflammatory mediators do hardly change before clinical manifestation.

Second, one or two systemic biological markers may not sufficiently capture the complexity of ARDS or VAP. The pathophysiology of ARDS and VAP compromises numerous biological processes, including but not restricted to inflammation, oxidative stress, coagulation and apoptosis [21, 22]. Traditional studies on single proteins or pathways therefore may have very limited potential because the complexity of pulmonary disease cannot be captured. In the light of the dynamics and adaptive capacities of all biological systems, supposed molecular or cellular “alterations” should probably always be seen in the context of state of the system as a whole [23].

Third, a single measurement of a biological marker in blood heavily disregards the rapid dynamics of (development of) pulmonary diseases [24]. While use of multiple biological markers may improve diagnostics the rapid dynamics of critical illness remain ignored. Also, while blood is relatively easily available, frequent blood sampling and analysis in patients with or at risk for ARDS or VAP are relatively expensive and time–consuming.

**Biological markers in lungs**

There is good evidence that the lungs, rather than blood, should be assessed when aiming for early and accurate diagnosis of ARDS or VAP. BALF can be obtained in intubated and mechanically ventilated patients using directed or non–directed techniques [25]. Directed BALF has the advantage of sampling biological fluids in specific compartments of the lung but requires specialized personnel, investment of time and is not without risk for the critically ill patient. Non–directed lung lavage is relatively fast and easy to perform but may be still too invasive for frequent assessment.

Different biological pathways should be investigated simultaneously to acquire a broad biological perspective [26]. Recently, proteins in BALF have
been profiled collectively using new analytical techniques (proteomics), allowing for biological marker discovery and better understanding of the host-response [27].

**“Omics” and systems biology**

“Omics”–studies represent the integrated assessment of the biochemistry within a domain of complex organisms (genomics, transcriptomics, proteomics and metabolomics). These techniques are purposely not hypothesis–driven (i.e., they are unbiased) and can be used to discover biological markers of pathophysiological pathways [28]. Systems biology focuses on combinations of different “omics”–domains seeking a deeper understanding in complex biological systems providing a top–down view of biochemical processes combined with mathematical and computational methods for modeling of structures and processes. Integrating these with clinical parameters by a multiscale analysis is now called ‘systems medicine’ [29].

Metabolomics was recently described as “the global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity and physiological landscape” [30]. The metabolome is very sensitive to physiological and pathophysiological changes because it is an end–product of the genome, transcriptome and proteome combined. This could possibly limit the specificity of the metabolome. Nuclear magnetic resonance (NMR) spectroscopy and liquid–chromatography/mass–spectrometry (LC–MS) can be used to identify hundreds of metabolites in any biological material. As with any “omics” technique, it remains a challenge to avoid false discoveries when applying metabolomics. Recommendations concerning bias, sample size, multiple testing and model fitting should be followed strictly [31, 32]. Since metabolism is an ancient and highly conserved biological mechanism, results can often be translated between mammalian species [33].

Up till now, some research has focused on metabolomics in critically ill patients. A NMR–based metabolomic method in plasma was described to aid detection of the systemic inflammatory response syndrome (SIRS) versus multi–organ failure (MOF) based on abnormal metabolic
signatures [34]. It was possible to discriminate SIRS and MOF patients, suggesting that an NMR–based metabolomic approach can be developed to diagnose the disease progress of critically ill patients. In a rat-cecal ligation and puncture model metabolomics showed a 100% sensitivity and specificity when compared to sham-operated animals [35]. Similar results were found in a mouse model of inflammatory lung injury [36]. These results were further supported by a clinical study showing that NMR in plasma could generate quantitative data sets that revealed differences between patients with ARDS and healthy subjects on the level of several metabolites [37]. Importantly, some metabolites were associated with acute physiology scores and ventilator–free days. This study clearly demonstrates the feasibility of plasma NMR quantitative metabolomics since it yields a physiologically relevant metabolite data set that distinguished disease from health. Of note, several metabolites are volatile and thereby eliminated via the lung.

**Breath metabolomics**

Breath contains thousands of volatile organic compounds (VOCs), metabolites in gas–phase produced by both physiological and pathophysiological processes [38, 39]. VOC–patterns identified by smell have been used to diagnose disease and intoxication for ages (e.g. scent of acetone in diabetes mellitus) [40]. Alteration of exhaled VOCs can be the consequence of changed systemic metabolism (e.g., diabetes mellitus) or due to pulmonary metabolic shift [39]. Micro–organisms (e.g., bacteria) present in the airways also produce volatile molecules, which may be species specific [41, 42]. Thus the exhaled breath contains the composite signal of host–metabolism, as part of the host–response, and bacterial metabolism, which may interact [42]. It should be noted that due to the water and fat-solubility of VOCs the breath concentration is not a direct representation of the tissue concentration per se. A variety of techniques has been used to link disease to changed VOC composition of the exhaled air, including gas–chromatography and mass–spectrometry (GC–MS), Ion–molecule reaction mass–spectrometry (IMR–MS), and electronic nose techniques [43]. Both volatile and non–volatile metabolites can be studied in exhaled breath condensate (EBC) [44]. These techniques, including advantages and disadvantages, will be described on the following pages and are summarized in table 1. Head to head comparison of the diagnostic
value of the different techniques cannot be performed because of the low number of studies, differences in research aims and methodological inconsistencies.

Gas–chromatography and mass–spectrometry

GC–MS is considered the gold standard for the detection, separation and identification of large volatile organic compounds. With gas–chromatography molecules are carried with an inert gas (e.g., helium) through a column: molecules are separated by volatility and interaction with the stationary phase of the column. During mass-spectrometry components are fragmented into charged particles, carried though an electromagnetic field and quantitatively detected [45].

The lower limit of detection can be improved using pre–concentration. However, storage bears the risk of decomposition and/or loss of compounds. Considering the rapid dynamics of critically ill patients, test results should be available within minutes after measurement. This is not yet feasible, and storage and transport of air samples is a challenge. These factors still limit the clinical applicability of GC–MS in monitoring critically ill patients. Nevertheless, GC–MS remains essential for VOC identification and therefore for the understanding of pathophysiological pathways.

GC–MS has been used to study volatile metabolites in intubated and mechanically ventilated patients (table 2 [46, 47]). A decrease in isoprene production has been found in ARDS patients and an increased n–pentane/isoprene ratio during development of VAP [46]. Increased isoprene concentration is considered a marker of activation of neutrophils [48]. It was suggested that impaired cholesterol synthesis late in the course of ARDS could explain for the surprisingly low levels of isoprene. N–pentane is an end product of lipid peroxidation and could reflect an increase in oxidative stress. Although isoprene, pentane and acetone are about the most abundant VOCs in the exhaled breath, focusing on just these compounds disregards the potential benefit of composite information provided by “omics” strategies. All detected molecules should be presented to data reduction and classification algorithms in order to capture the complexity of the biological material and to obtain maximal diagnostic accuracy while limiting bias [31].
In short, GC–MS can be used to separate, identify and quantify volatile organic compounds in the exhaled breath. Clinical applicability is limited because it is expensive, slow, labor-intensive and requires specialized personnel. GC–MS will mostly be used to identify molecular targets in the exhaled breath.

*Ion–molecule reaction mass–spectrometry and other rapid detection methods*

Ion–molecule reaction mass–spectrometry (IMR–MS) has been proposed as a fast and sensitive alternative analytical method for the detection of molecules in gas–phase [49] With IMR–MS volatile molecules are ionized with a precursor ion and moved towards a detector (mass-spectrometer). Volatile molecules are thus detected based on reactivity with the precursor ion and mass [49]. IMR–MS can be divided into different technologies based on the type of precursor ion: H3O⁺ called proton–transfer reaction mass–spectrometry (PTR–MS), H3O⁺/NO⁺/O2⁺ called selected ion flow tube mass–spectrometry (SIFT–MS) and others using krypton mercury or xenon [50].

The fast reaction time (20 ms), on–site application and continuous registration bring IMR–MS closer to clinical applicability. However, at present IMR–MS is not yet an alternative for GC–MS analyses because IMR–MS does only detect gases which react with the precursor ion. Furthermore, only mass-to-charge ratios are insufficient for identification of specific VOCs. Roughly the same advantages and limitations apply to ion–mobility spectroscopy (I–MS). In this technology, the ionized gas is let into a tube at specific intervals, in which the ions collide with drift gas molecules travelling in the opposite direction. Based on size and shape, ions are decelerated resulting in different ion drift times [51]. A summary of the advantages and disadvantages of the different technologies can be found in table 1.

The potential of rapid mass–spectrometry based technologies for breath analysis in mechanically ventilated patients was illustrated through the continuous analyses of air from the ventilatory circuit for up to 120 minutes [50]. In this study, several VOCs were identified and quantified, but no patient groups were compared.
Another method to analyze these datasets is to use pattern-recognition software. This is based on the concept that diagnostic assessment does not require identification of individual molecular components, rather than being dependent on accurate pattern recognition [52]. All peaks and intensities are combined into one algorithm, which is subsequently used for diagnostic purposes. This approach was recently used in rats challenged with lipopolysaccharide [53]. In this study, exhaled breath profiling with I–MS discriminated extremely well between rats with inflammatory response syndrome from healthy controls.

SIFT–MS has previously been used to discriminate between different bacterial species in vitro. This could provide a rapid alternative to traditional, culture dependent, pathogen detection. Several papers showed differences in around twenty volatile organic compounds between several cultured bacterial species [54-56]. A single VOC did not sufficiently separate bacterial species, however a pattern-recognition based approach resulted in very good discrimination between potential pathogens [57]. The biological materials used in these studies remain confined to spiked blood samples and the results should be validated using prospectively collected patient material.

Shortly, IMR–MS and I–MS are rapid tests that can be used to identify some volatile organic compounds in the exhaled breath. Because IMR–MS and I–MS technique can be miniaturized and the analysis is continuous, it could be used as a diagnostic test. However, compared to GC–MS these techniques are more selective and unknown compounds cannot be identified with certainty, hereby limiting it’s applicability for pathophysiological research.
### Table 1: Advantages and disadvantages of different analytical techniques for breath.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC–MS</td>
<td>1. Identification of new compounds, thus allows for biomarker discovery</td>
<td>1. Slow and labor intensive</td>
</tr>
<tr>
<td></td>
<td>2. High sensitivity</td>
<td>2. Not available at bedside</td>
</tr>
<tr>
<td></td>
<td>3. Wide range of application</td>
<td>3. Need for pre-concentration and transport</td>
</tr>
<tr>
<td>IMR–MS</td>
<td>1. Identification of known compounds</td>
<td>1. Selectivity for reaction between VOC and precursor ion</td>
</tr>
<tr>
<td></td>
<td>2. Rapid measurement</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td></td>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td>PTR–MS</td>
<td>1. Identification of known compounds</td>
<td>1. Selectivity for reaction between VOC and proton</td>
</tr>
<tr>
<td></td>
<td>2. Rapid measurement</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td></td>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td>SIFT–MS</td>
<td>1. Identification of known compounds</td>
<td>1. Selectivity for reaction between VOC and precursor ion</td>
</tr>
<tr>
<td></td>
<td>2. Rapid measurement</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td></td>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td>I–MS</td>
<td>1. Identification of known compounds</td>
<td>1. High selectivity towards very volatile molecules</td>
</tr>
<tr>
<td></td>
<td>2. Rapid measurement</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td></td>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td>eNose</td>
<td>1. Rapid measurement</td>
<td>1. No identification of compounds</td>
</tr>
<tr>
<td></td>
<td>2. Portable and easy to use</td>
<td>2. Lower sensitivity with current sensors</td>
</tr>
<tr>
<td></td>
<td>3. New sensors development</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td>EBC</td>
<td>1. Liquid sample is obtained so traditional analyses can be performed</td>
<td>1. Collection and analysis are slow</td>
</tr>
<tr>
<td></td>
<td>2. Combination with NMR allows for profiling</td>
<td>2. Continuous analysis is impossible</td>
</tr>
</tbody>
</table>

Table 1: Advantages and disadvantages of different analytical techniques for breath.
**Table 2:** Volatile organic compounds detected in exhaled air of mechanically ventilated patients, as described in literature [34] [50] [47]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Suspected origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Enhanced metabolism</td>
</tr>
<tr>
<td>2,3-Dimethylbutane</td>
<td>Unknown</td>
</tr>
<tr>
<td>2,4-Dimethylpentane</td>
<td>Unknown</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>Unknown</td>
</tr>
<tr>
<td>2-Methylbutane</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>2-Propenal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Smoking</td>
</tr>
<tr>
<td>Butanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Butane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>Smoking</td>
</tr>
<tr>
<td>Cyclohexanone</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Endogeneous or hospital air</td>
</tr>
<tr>
<td>Heptane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Hexanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hexane</td>
<td>Unknown</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Anesthesia</td>
</tr>
<tr>
<td>Isoprene</td>
<td>Cholesterol metabolism</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Methanol</td>
<td>Unknown</td>
</tr>
<tr>
<td>N-hexane</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Pentanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pentane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Propanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Propane</td>
<td>Unknown</td>
</tr>
<tr>
<td>Toluene</td>
<td>Smoking</td>
</tr>
</tbody>
</table>
Electronic nose

Electronic noses (eNose), named after their similarities with mammalian olfactory system, integratively capture complex VOC mixtures using an array of different sensors [43]. Humans, not even the most versatile smellers known to nature, are able to discriminate 1 trillion olfactory stimuli with just 400 receptors [58]. Chemical sensors have individual sensitivities and specificities for multiple VOCs. The composite signal of all sensors can be analyzed using pattern-recognition algorithms. eNose analysis of breath results in a unique fingerprint of exhaled metabolites, called a breath-print. Subsequently, these breath-prints can be used for diagnostic and monitoring purposes, which do not require identification of individual molecular constituents.

Metal oxides, conducting polymers, optical and infra-red spectroscopy have been used as sensors. Electronic noses can be miniaturized and might allow for continuous analyses. Data are available in real-time and electronic noses are relatively easy to use. Indeed, eNoses are very attractive from a clinician point of view [39]. Identification and quantification of specific compounds is not necessary for diagnosis and monitoring as long as patterns are diagnostic for particular conditions. Although promising, several technical issues are to be considered regarding eNoses, including the fact that sensors in use at present have a limited sensitivity and specificity for VOCs, are not interchangeable between devices and could “drift” over time. Sampling techniques should be adapted to the clinical setting and the disease of interest.

Several volatile compounds have been linked to metabolic activity of relevant bacterial species including *Pseudomonas aeruginosa* and *Staphylococcus aureus* [39]. Assessment of a large quantity of VOCs combined with pattern recognition software leads to good discrimination between bacterial species [59]. Different species of bacteria can be discriminated *in vitro* based on integrative analysis of volatile metabolites using an electronic nose [60-62]. Interestingly, distinct metabolic alterations have been reported for methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* as compared to antibiotic susceptible bacteria of the same species [63].
Patterns of VOCs in exhaled breath of intubated and mechanically ventilated patients undergoing surgery are associated with the clinical pneumonia infection score, a sensitive marker for VAP [64]. Real–time pathogen detection has been reported in mechanically ventilated critically ill patients with pneumonia, showing good in vivo discrimination between *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinobacter baumanii* and *Acinobacter lwoffi* [65] (figure 1). Air was acquired through a suction catheter for the purpose of this study, compromising the non–invasiveness of breath sampling and the possibility of continuous analysis. Although GC–MS analysis of exhaled breath was not performed (i.e., the specific compounds remained unknown), these studies illustrate the diagnostic potential of volatile metabolites generated by airway pathogens and/or host response.

**Figure 1: Discrimination between pathogens in vivo**

![Discrimination between pathogens in intubated and mechanically ventilated pneumonia patients by electronic nose analysis. The X– and Y–axis represent vectors obtained by data reduction. Each point represents an infected patient, infected with: □, Acinobacter baumanii; x, Klebsiella pneumoniae; ○, Pseudomonas aeruginosa; Δ, Staphylococcus aureus; ◊, Acinobacter lwoffi; +, normal pharyngeal fauna; •, centroids (Reprinted from Sensors and Actuators B: Chemical, 148(1): Shih et al. Real–time electronic nose based pathogen detection for respiratory intensive care patients, 156, [65] Copyright (2009), with permission from Elsevier).](image-url)
During a preliminary proof of concept study we recently showed the potential of eNoses to discriminate ARDS patients from critically ill patients without lungs injury (figure 2) [66]. These results are very encouraging since the patient groups were small and highly heterogeneous, but validation is very much needed. In this study, air was collected for one minute via a disposable T-piece connector placed between the endotracheal tube and the heat–moist exchanger. This methodology allowed for fully non-invasive and continuous sampling, suggesting electronic nose technology may already be very close to clinical applicability.

**Figure 2:** Discrimination between ARDS and controls

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*Discrimination between acute lung injury patients and controls by electronic nose analysis. The X- and Y-axis represent principal components obtained by data reduction. Each point represents a critically ill patient: □, controls; ∆, acute lung injury patients. Each group is connected to a centroid.*

eNoses can provide rapid analysis of complex volatile mixtures as the breath. The devices are portable, easy to use and sensors can be tailor-made. Furthermore, in intubated and mechanically ventilated patients,
continuous exhaled breath analysis is possible. Therefore, electronic noses are potentially suitable as diagnostic and/or monitoring instruments. However, electronic noses cannot identify volatile organic compounds making them less important for understanding the molecular mechanism altered in disease.

**Exhaled breath condensate**

Exhaled breath condensate originates from lining fluid in the upper or lower airways [44]. EBC therefore contains a large number of peptides and metabolic products. Concentrations of diverse peptides and other molecules (e.g., interleukines, isoprostanes and cytokeratins) in EBC have found to be altered in acute lung injury [67-69]. One study showed leukotriene B4 in EBC to be increased in children with community-acquired pneumonia [70].

The association between pneumonia, ALI and metabolite concentrations in EBC has not yet been investigated. In respiratory research, metabolomic profiling of EBC fluid by NMR–spectroscopy showed excellent results in distinguishing asthma, COPD, and cystic fibrosis patients from controls. In a similar way stable from unstable cystic fibrosis patients could be separated [71-73]. NMR–spectroscopy records the interaction of radiofrequency electromagnetic radiation within the nuclei of atoms in a strong magnetic field. The nuclei within a biomolecule can hereby be detected and used to determine the molecular structure, conformation and dynamics. Identification might be possible, as with GC–MS, by comparing spectra with a reference library.

A major advantage of EBC analysis is that the more traditional, well-understood biomarkers can be measured non-invasively. It is therefore a diagnostic tool with great potential for pulmonary disease, also in critically ill patients. However, collection of EBC remains challenging in mechanically ventilated patients. For instance, air should not be artificially humidified to obtain a meaningful signal. Therefore, humidifying–systems should not be used, which may be unwanted. One other disadvantage of EBC collection is that collection systems are rather large. In addition, continuous analysis of complex mixtures does not seem possible.
Perspective

Volatile organic compounds in the exhaled breath can be separated, identified and quantified by gas–chromatography and mass–spectrometry. This remains the gold standard for pathophysiological research. Several other technologies can be considered as diagnostic tools because they are rapid and easier to handle: IMR–MS and I–MS can be used for pattern recognition as well as VOC detection while electronic noses rely on pattern recognition only. Non-volatile organic compounds can be investigated using exhaled breath condensate.

Momentarily, there are two large gaps in the exhaled breath research: firstly there is little consistency in research aims and methodology between basic research and clinical trials, secondly biomedical researchers and the developers of new technologies are not in close contact. Future research on exhaled breath analysis in intubated and mechanically ventilated patients should approach volatile biomarker discovery using translational biology and translational technology (from bench to bedside and vice versa) (figure 3).

VOCs can be discovered in vitro, as demonstrated by head–space analysis of bacteria [41]. The head–space analysis of pulmonary fluids (e.g., BALF) also investigates (part of) the host response. These findings can be extended using “clean” in vivo animal models (e.g., models of pneumonia or lung injury), in which two variables are added: the physiology of the respiratory system and the systemically produced volatile organic compounds. Clinical trials following STARD–guidelines are then necessary to validate the diagnostic accuracy of breath metabolomics in patients with co–morbidities and exposure to exogenous VOCs [74].

Translational technology should focus on matching technology to research aim. Fundamental research should use GC–MS for biomarker discovery and detailed study of (patho–) physiological processes. Clinical trials might use a combination of different technologies, including GC–MS and tailor made bedside analytical tools, in order to provide probabilistic evidence (positive– or negative predictive values) for clinical decision–making [32]. Tailor–made eNoses and/or diagnostic algorithms for SIFT–MS are to be produced when VOCs associated with disease have been identified and
validated [75].

No head-to-head comparison between the available analytical technologies for complex breath samples is available. The hereby proposed purposes for each technology imply that rapid analytical tools (for diagnosis and monitoring) should not be compared to GC–MS (pathophysiological research) but should be compared to each other. However, the problem with defining the gold-standard remains and might imply that we need more objective end-points.

**Figure 3: Perspective**

*Perspective for the development of breath analysis as a diagnostic test for pulmonary disease.*
Conclusions

Exhaled breath analysis has potential as a diagnostic and monitoring tool in intubated and mechanically ventilated critically ill patients especially so because it is fully non-invasive and can be performed continuously. There is accumulating evidence for the diagnostic accuracy of breath analysis in several pulmonary diseases. Additionally, several VOCs have been linked with disease processes. However, there is still room for improvement in trial design and the use of the right technology for the research aims of the future studies. Special attention should be given to the coupling of biochemical pathways with the observed alterations in exhaled VOCs.
Outline of this thesis

The general aim of this thesis is to obtain more insight into changes of volatile organic compound composition during ARDS and pulmonary infection.

- In **Chapter 2**, we give an introduction in the analysis of exhaled breath in ventilated ICU-patients. Special consideration is given to the previously studied volatile compounds and the techniques of measurement that are used for the experiments that are described in this thesis.
- In **Chapter 3**, we describe a new, simplified method for exhaled breath collection in ventilated patients. This method was used in all subsequent chapters.
- In **Chapter 4**, in an attempt to discriminate between patients with and without ARDS, we integratively measure the VOCs in exhaled breath using a commercially available electronic nose.
- In **Chapter 5**, we investigate the alterations in exhaled volatile organic compounds during the development of systemic and local inflammation. For this we use a rat model with intravenous or intratracheal lipopolysaccharide injection and mechanical ventilation.
- In **Chapter 6**, we identify VOCs that may be used for the diagnosis of ARDS. We aim to find a small panel of biomarkers and externally validated the accuracy of these volatile metabolites.
- In **Chapter 7**, we review the literature for potential volatile biomarkers for bacterial presence.
- In **Chapter 8**, we describe the *in-vitro* examination of volatile organic compounds analyzed by electronic nose for the diagnosis of ventilator-associated pneumonia.
- In **Chapter 9**, we measure VOCs in the exhaled breath of ventilated ICU-patients and analyze their association with pneumonia.
- In **Chapter 10 and 11**, we summarize and discuss the results from the previous chapters.
References


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Bacteria with a Disposable Colorimetric Sensing Array. Journal of the American Chemical Society 133: 7571-7576


