Exhaled breath analysis for the diagnosis of pneumonia

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GENERAL DISCUSSION
The diagnostic process of ventilator-associated lower respiratory tract infection (VA-LRTI) would benefit from a quicker, accurate and, ideally, non-invasive new test. "Diagnosis" is considered the core component of medical practice, since treatment and prognosis rely on an adequate and timely diagnosis. Before a new test is implemented in a diagnostic process, diagnostic accuracy and clinical utility have to be scientifically demonstrated. Comparing to existing methods, a novel test should either be (1) safer, cheaper, easier to interpret, less laborious for the clinician and/or delivering faster results; (2) more specific (excluding more cases of non-disease) and thus avoiding unnecessary treatment; or (3) more sensitive (detecting more cases of disease) and thus promoting more appropriate treatment. In the studies presented in this thesis, we have evaluated several possibilities for such a test, with a strong focus on breath analysis. The major advantage of breath analysis over contemporary diagnostics is that it is quicker and non-invasive.

Exhaled breath analysis may serve various purposes within the diagnostic process of pneumonia. It may become of use (1) for the detection (or exclusion) of the presence of pneumonia in a suspected patient; (2) the identification of the causative pathogen and/or (3) the monitoring of the treatment response after the initiation of antibiotics. This has been visualised in Figure 1 of chapter 2 and copied below. I will maintain this structure to further discuss the results of this thesis.

Figure 1.

The potential applications of exhaled breath analysis for pneumonia; ↑ = increase.
General discussion

EXCLUSION OF PNEUMONIA

Where are we now, based on the findings of studies presented in this thesis?

Breath sampling was shown to be feasible in invasively ventilated patients using a non-invasive procedure. Using this sample methodology, we recognised that the following molecules discriminated between the presence and absence of pneumonia: 1-propanol, carbon disulphide, hexafluoroisopropanol, 2-ethoxy-2-methyl-propane, acetone, cyclohexene and methylisobutylketone (chapter 5) and 4-methyl-octane, 2-5-dimethyloctane and tetra chloroethylene (chapter 6). A significant limitation is that these and prior studies were biased and did not show consistent results. This was an important motivation for the initiation of the BreathDx study, in which we complied with all criteria for diagnostic accuracy study\(^4\). The study delivered a breath test with a very high negative predictive value. The test showed the potential to save antibiotics in half of the patients with subsequent negative cultures in the BreathDx cohort. The involved specific VOCs have not yet been identified and further analysis will have to demonstrate the reproducibility of the previously identified VOCs. As a result, the biochemical origins of these VOCs are presently unknown as well. The described breath test gave promising results, but as a result of the use of gas-chromatography and mass-spectrometry (GC-MS), the test is time-consuming, expensive and laborious. There is no consensus regarding the ideal exhaled breath detection method.

A number of other discovered VOCs remained “unidentified” or “unknown”, because the mass spectrum was not unique for a single molecule. The mass spectrometer measures fragmentation patterns, which are sometimes very similar between molecules. If their retention time is similar as well, distinction is impossible using current techniques. Despite our present inability to name these compounds, they may be of diagnostic value. The combination of retention time and mass spectrum will enable future recognition of these markers in clinical studies, when GC-MS remains to be used. However, the development of alternative analytical methods will be severely hampered when identification of the VOC remains impossible.

The primary aim of the BreathDx study was to test whether exhaled breath analysis could be used to predict presence of infection. Initially, the BreathDx study solely focussed on patients suspected of ventilator-associated pneumonia (VAP). VAP has a very similar clinical presentation as ventilator-associated tracheobronchitis (VAT) and the same
microbiologic diagnostic criteria are used. The difference between the two diagnoses is the presence of a new and persistent infiltrate on the chest radiograph, which is required for VAP. When analysing the clinical data, it appeared that we captured a considerate number of VAT patients in our BreathDx study: 21% of the confirmed VA-LRTI patients. As of yet, it remains uncertain whether VAT patients should receive antimicrobial therapy\(^5\). However, VAT treatment has been shown to lead to less progression to VAP; reduce ventilator- and intensive care unit (ICU) days; facilitate weaning from the mechanical ventilator and may thus reduce healthcare costs\(^6\). Also in these patients it remains important to avoid antibiotic overtreatment, which is a recognized risk factor to the emergence of antimicrobial resistance\(^7\). A breath test would then benefit confirmed VAT patients as well. As a result, we broadened the scope of the BreathDx paper to VA-LRTI, as opposed to VAP alone, and I believe that this is the way we should approach diagnostic testing moving forward.

**What do we need from here?**

First and foremost, the discovered VOCs in the BreathDx study will have to be identified, as this is pivotal for all subsequent steps. Identification of the BreathDx VOCs enables us to investigate the origins of these molecules. If identified, we would still not have been able to report the identities of the discovered VOC in this thesis, since their importance comes with potential intellectual property interests. These will have to be assessed first, before publication is permitted.

Second, the biochemical origin of the VOCs needs to be evaluated. Several additional tests could be considered to obtain more insights into the complex interactions of microbiome and inflammation during lower respiratory tract infections. Bronchoalveolar lavage (BAL) cultures may not be the ideal gold standard in all VA-LRTI suspected patients, since some pathogens are hard to culture and culture results may be influenced by previous administration of antibiotics. For instance, polymerase chain reaction (PCR) on lower respiratory tract samples has shown to deliver exquisitely high NPV’s and sensitivities in suspected VAP patients\(^8,9\). It is therefore plausible that other methods may prove superior over BAL culturing to be used as reference test for the exclusion of pneumonia. Additionally, it has been hypothesized that the inflammatory host response might play a bigger role in VA-LRTI emergence than the actual bacterial disposition within the lung
microbiome\(^{10}\). Host defence mechanisms may initiate a positive feedback loop, thus advancing pathogenic growth and subsequent domination of the lung microbiome\(^{11}\).

Exploration of potential correlations with host response markers (e.g. inflammatory cytokines such as interleukin-1\(\beta\) and interleukin-8\(^{12}\)) and microbiome changes could lead to more insight into the origins of detected VOCs through investigation of the role of the host response separately to pathogenic invasion. When we better understand the biological origin of the VOCs and the links between VOC formation, host response and microbial composition and metabolism, we might learn more from specific signals in breath tests and interpret the results in a different light.

Additionally, when the identity of the VOCs is known, we might also consider different analytical platforms to GC-MS. In clinical practice, we need a breath test that can measure VOCs quicker or more easily than is realistically possible with GC-MS. Such a test probably needs to provide results within an hour to withhold antibiotics in a patient with suspected VA-LRTI. A test designed to specifically measure a (very limited) number of VOCs might provide quicker results. As the identity of the molecules of interest is already known, a more integrative view of the breathome is not necessary. Potential alternatives for such a new breath test are listed in Table 1.

**Table 1.**

<table>
<thead>
<tr>
<th>Potential for new breath test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compact-GC</td>
<td>- relatively quick (1 hour)</td>
<td>- some compounds are hard to be separated with gas-chromatography</td>
</tr>
<tr>
<td></td>
<td>- detects individual VOCs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- no sample transportation required</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- standalone tool</td>
<td></td>
</tr>
<tr>
<td>eNose</td>
<td>- quick (minutes)</td>
<td>- cannot measure individual VOCs</td>
</tr>
<tr>
<td></td>
<td>- standalone tool</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- no sample transportation required</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- continuous measurement and analysis</td>
<td></td>
</tr>
<tr>
<td>IMS</td>
<td>- quick (minutes)</td>
<td>- limited range of compounds that can be identified</td>
</tr>
<tr>
<td></td>
<td>- can detect individual VOCs</td>
<td></td>
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<tr>
<td></td>
<td>- no sample transportation required</td>
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</table>
Advantages and disadvantages of analytical platforms, visualising the potential for a new breath test targeting specific VOCs of interest. Potential alternatives to gas chromatography-mass spectrometry, which has been investigated. GC = gas chromatograph; IMS = ion mobility spectrometry; DMS = differential mobility spectrometry; SIFT-MS = selected ion flow tube-mass spectrometry; PTR-MS = proton transfer reaction-mass spectrometry; MS = mass spectrometry 

Following Table 1, compact-gas chromatograph (GC) seems most eligible as a new breath test for VA-LRTI. A prototype of this compact-GC is currently under investigation for octane in acute respiratory distress syndrome (ARDS). It is able to reliably measure octane in mechanically ventilated patients and has been validated in a proof-of-principle study (publication in progress). The device is small and will be available for bedside measurements. The BreathDx study results will have to be externally validated in a different cohort of VA-LRTI suspected patients. The compact-GC may be recalibrated for the identified molecule(s) for VA-LRTI and can thus be used for external validation of BreathDx.

How will we get there?

The breath test that we describe in this thesis is not directly clinically applicable, due to the impracticalities of use of GC-MS. The aforementioned new analytical platform may provide quicker results to allow for testing and future implementation on ICU. A new
study will have to be conducted to test the revised analytical technology. Exhaled breath samples will be analysed using both breath tests: the new test, in conjunction with GC-MS analysis. The latter will provide the gold standard measurements that can be used for external validation of BreathDx.

A new study investigating patients suspected of VA-LRTI, will encounter a significant obstacle. During the study period of BreathDx we faced a slower recruitment rate than expected. Over all centres, the occurrence of VAP was lower than calculated prior to the start of the study. A declining occurrence of VAP has been reported over the last couple of years. The reason behind this decreasing incidence is far from clear. It may in part be due to successfully applied prevention bundles. These preventive interventions include a 30°C to 40°C elevation of the head of bed; daily interruption of sedation and evaluation of readiness to extubate; drainage of subglottic secretion; silver-coated endotracheal tubes; and avoidance of scheduled ventilator circuit changes resulting in unnecessary manipulation of ventilator tubing. The use of oral chlorhexidine as a preventive measure for VAP has been questioned recently, as well as the use of gastrointestinal stress ulcer prophylaxis. Interestingly, it has been demonstrated that these VAP prevention bundles dramatically reduce VAP rates, but almost none of the investigated strategies have any impact on patients’ duration of mechanical ventilation, hospital length-of-stay or mortality. Over the last decade, mechanical ventilation rates have been decreasing on Dutch ICUs. In 2017, 44.9% of patients was under invasive ventilation within the first 24 hours of ICU admission, compared to 49.5% in 2013 and 51.6% in 2009. For the median duration of invasive ventilation a similar trend was shown. One can imagine that decreased use of invasive ventilation may result in less ventilator-associated complications such as VA-LRTI. In addition, lung-protective ventilation is applied more frequently on ICU. Ventilator-induced lung injury (VILI) may result in chest radiography abnormalities, fever and other inflammatory processes. At times, these are mistaken for pneumonia. A reduction of injurious ventilation may therefore contribute to the observed declining rates of suspected VA-LRTI. Furthermore, the host response to inflammation is suggested to promote the growth of bacteria. Less occurrence of VILI may therefore minimise chances for microbials to cause an infection.

Is it still necessary to investigate VA-LRTI at all, if the reported incidence is that low? Yes, because there seems to be a discrepancy between low officially reported VA-LRTI
rates on one hand, and a continued prevalence of clinical diagnoses and administration of antimicrobials on the other. This may be explained by the poor, nonspecific and subjective clinical definitions for VA-LRTI, which are associated with large inter-observer variability. A gold standard for VA-LRTI is not clinically available. It cannot be concluded that VA-LRTI is indeed disappearing. If we are able to diagnose VA-LRTI accurately, its incidence may remain clinically relevant. Unfortunately, the foreseen recruitment issues will remain as well.

Just as for the BreathDx study, the new study with the new breath test should aim for a very high sensitivity (>98%) with an acceptable specificity, in order to be able to rule out presence of pneumonia. If the diagnostic accuracy is confirmed in the external validation observation study, the next step would be to conduct an RCT in order to test the new breath test in ICU setting. VA-LRTI suspected patients will be randomised into two groups. First, all patients will undergo a breath test. For one group, the ‘intervention group’, the breath analysis results will be revealed to the ICU physician, to be used for subsequent clinical decision making. For the other group, the standard care group, the breath test results will remain undisclosed to the clinicians and the patient will be treated according to normal ICU practice. In the first group, if the breath test results are negative, the physician should withhold this patient from receiving antibiotics for suspected VA-LRTI. The subsequent impact on ventilator days, ICU- and hospital length-of-stay and ICU mortality will be evaluated to ensure the safety of withholding antibiotics. Also, the diagnostic accuracy of the test will be evaluated a third time, as the standard diagnostic procedures including a bacterial culture of BAL fluid will still be conducted.

**IDENTIFICATION OF PATHOGENS**

Where are we now, based on the findings of studies presented in this thesis?

The molecules 4-methyl-octane, 2,5-dimethyloctane, hexadecane, 2-,4-dimethylhexane, 1-methoxy-2-propanol, 2-methyl-nonane and 2-,4-dimethylheptane were found to be associated with Streptococcus pneumonia, whereas 2-ethylhexyl ester 2-propenoic acid was linked to Pseudomonas aeruginosa (chapter 6). No consistency was seen with results of other animal studies, and only partly for data from in vitro studies. The VOCs from the BreathDx study have not been identified yet (as discussed in the previous section). The animal study described in chapter 6 showed that VOC patterns may provide
more diagnostic accuracy than detection of individual VOCs regarding identification of pathogens.

Results of in vitro and animal studies were not uniform either. As opposed to human studies, animal models enable the investigation of one single bacterial infection without the presence of co-morbidities or coexisting microorganisms. A controlled environment can be created, free of genetic or behavioural influences, thus limiting the risk of contamination of the breath signal. Also, post-mortem histology – the true gold standard for pneumonia – can be used for diagnosis. A matter of debate has always been to what extent animal studies are translatable to the human situation. This is especially true for animal studies that try to predict effectiveness of treatment strategies\textsuperscript{29,30} or investigate a preventive intervention\textsuperscript{31}. Animal studies are also subject to a high risk of publication bias: neutral or negative results may hinder the studies from publication. Despite this, we do expect that the results from our animal study were translatable. Breath was sampled successfully in the controlled laboratory environment. As a result, the identified changes in VOC emissions between groups were likely to reflect the infection itself and less likely to be attributed to confounding factors. Emitted breath VOCs can either derive from the host response to infection or from the causative pathogen, as a product of its metabolism. The VOCs originating from the host response may indeed be different in the breath of animals compared to humans. Thus, the translatability of these specific results may be questionable. However, the VOCs that are directly linked to a causative pathogen, are likely to be similar in the human and the animal situation.

**What do we need from here?**

We need to understand why VOCs are produced by certain pathogens and we need a better understanding of the biochemical processes that result in VOC formation. If we learn the pathophysiological process the molecule is linked to, we can make better predictions based on the presence of a specific VOC and test those predictions accordingly. As discussed above, we rely on the identification of the pathogen specific VOCs that were found in the BreathDx study, which we hope to link to specific pathways of bacterial metabolism. Also, as mentioned before, we need the results of the BreathDx study to be externally validated.
The breath test for pathogen detection can be used in two ways. In this thesis we followed contemporary guidelines for VA-LRTI, which recommend initiation of combination therapy when *Pseudomonas aeruginosa* or *Klebsiella pneumonia* infection (in the setting of multi-drug resistance) is identified\(^2,3\). Adequate detection of these pathogens by a highly specific breath test would allow the clinician to commence the appropriate therapy without culture delay. For this purpose, a breath test would need to selectively identify VOCs that are only produced by one type of bacteria. As a result, we would require analytical method such as the compact-GC, which is able to separate between the molecules with high accuracy. As this test is less time demanding than a test that will withhold antibiotics, conventional GC-MS in a laboratory setting might be an alternative.

Alternatively, prompt recognition of the causative pathogen at the start of clinical symptoms of a new VA-LRTI episode, may enable prescription of specific antibiotics and thus limit the use of broad-spectrum antibiotics. Here, a breath test with very high sensitivity would be required, in order to decide not to prescribe broad-spectrum or empirical antibiotic therapy. It can be postulated that pattern recognition would work better for this purpose. Following Table 1, the eNose might be the best candidate to qualify for alternative new breath test. It is quick, easy to use and provides real-time analysis.

**How will we get there?**

A combination of *in vitro* and *in vivo* research is necessary in order to discover the biochemical origin of VOCs. It has to be investigated which VOCs are emitted by which pathogen, and in which process of the disease. An *in vitro* model based on pulmonary epithelial cells can be used to study VOCs that emerge from metabolic processes of these human cells. Subsequently, several common influential factors encountered in clinical practice can be tested using this model. The influence of the host response can be investigated by adding isolated neutrophils to the bronchial epithelial cells. The subsequent addition of lipopolysaccharide will mimic a pro-inflammatory response, resulting in the formation of reactive oxygen species. The amount of induced inflammation and oxidative stress can be quantified by measuring the production of IL-1β and IL-8. Hydrogen-peroxidase (H\(_2\)O\(_2\)) can be added to the epithelial cells to induce oxidative stress. Bacterial metabolism can be investigated by adding isolated colonies of particular bacteria to the epithelial cells model. A joint state of bacterial metabolism under host
response can be studied through co-culture of bronchial epithelial cells, bacteria and neutrophils. The influence of antibiotic treatment can be assessed as well by repeating the bacterial metabolism experiments in the presence of an antibiotic that is often used in VA-LRTI, e.g. ceftazidime. The next step is to compare these in vitro results to patient data obtained in previous in vivo studies. This will enable validation of the relation between the identified VOCs and the pathophysiological processes of interest, i.e., culture, microbiome, metabolome, inflammation, oxidative stress. Since the samples from the in vitro experiments and the breath samples are analysed on the same platform, direct matching of volatile metabolites is possible. Even without exact identification of the molecule, we can still find the same compound in the breath dataset, based on retention time and fragmentation pattern. Instead of evaluating hundreds of candidate markers in untargeted analyses, only a limited set can be analysed now. This will limit the chances of false discovery and reduce the need for correction of multiple comparisons.

The VOCs linked to Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella spp. in the BreathDx study will have to be identified. These study results will then have to be externally validated in a new clinical study. The new breath test (as described in the previous section) may be recalibrated for the identified pathogen specific VOCs. I propose to use a combination of a more targeted breath test and GC-MS, as mentioned previously as well. This will enable external validation of the BreathDx results. This combination also gives the opportunity to investigate whether identification of VOC patterns provides better diagnostic accuracy as opposed to individual VOCs. Two approaches could be followed to integrate the new breath test for identification of causative pathogens into clinical practice after external validation has been established. On the one hand, a substudy of the aforementioned RCT – investigating the new breath test for exclusion of pneumonia – could be initiated using the breath test to further target antibiotic therapy. Alternatively, the breath test may be directly applied into clinical ICU practice after external validation.

**EVALUATION OF TREATMENT EFFECT**

**Where are we now, based on the findings of studies presented in this thesis?**

The evaluation of the treatment effect has not been investigated in this thesis.

Despite the lack of research on this topic, there would be a great benefit of a breath test that can be reliably used to evaluate effect of antibiotic treatment. Antimicrobial
resistance, unnecessary use of antibiotics and antibiotic waste are great global problems\textsuperscript{33}. Due to inadequate antibiotic stewardship, patients with pneumonia may receive antibiotic treatment longer than necessary, resulting in overuse of antibiotics\textsuperscript{22,34}. A reliably test could help physicians to de-escalate or stop antibiotics in a timely manner.

**What do we need from here?**

Again, we need to know much more about the biochemical origin of VOCs. Specific VOCs have been linked to particular pathogens, but how do the abundancies of these VOCs change over time? We want to know whether we can relate the shedding of the pathogen to the course of the breath abundancy of a particular VOC.

We need a breath test that helps us to de-escalate or discontinue antibiotics as soon as the breath signal changes. We do not know whether we need a test that can identify individual VOCs, or VOC patterns.

**How will we get there?**

*In vitro* and animal studies will have to teach us more about how the course of bacterial infection, linked to the potential changes in emission of pathogen specific VOCs. It needs to be investigated whether the clearance of the bacteria is associated with a similar trend of the abundance of a VOC or VOC pattern.

In order to investigate the relationship between shedding of a pathogen and the course of VOC emission in a patient setting, it may be useful to obtain daily BAL samples for PCR as reference standard in an observational cohort study. The major challenge of such a clinical study is the diversity of causative pathogens between patients.

**FINAL CONCLUSIONS**

Exhaled breath analysis can be used to predict the presence of pneumonia, by differentiating patients with positive cultures from those with negative cultures. The clinical BreathDx study gave promising results, yet needs external validation and identification of specific VOCs. A breath test may also enable discrimination of particular causative pathogens of VA-LRTI. The diagnostic performance of exhaled breath analysis may improve when combined with clinical scoring systems or biomarkers. A future additional application of a breath test is to guide duration of antibiotic treatment.
REFERENCES


