Exhaled breath analysis for the diagnosis of pneumonia

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INTRODUCTION & OUTLINE OF THIS THESIS
Ventilator-associated lower respiratory tract infections

More than half of the patients on the Intensive Care Unit (ICU) receive invasive mechanical ventilation at some point during their stay. Ventilator associated-lower respiratory tract infections (VA-LRTI) are the most common complications in patients under invasive ventilation and the most common nosocomial infections in the ICU. VA-LRTI can be divided into ventilator-associated pneumonia (VAP) and ventilator-associated tracheobronchitis (VAT). VAP, defined as pneumonia that occurs more than 48 hours after endotracheal intubation, is characterized by the presence of a new or progressive infiltrate on chest radiography, fever, altered white blood cell count, increased quantity or purulence of tracheal secretions, and the identification of a causative pathogen. The definition of VAT overlaps with that of VAP, but without the presence of chest infiltrates. VAT is regarded an intermediate process between lower respiratory tract colonization and VAP. VAT progresses to VAP in approximately 30% of the patients.

The most important contributing factor for the development of VA-LRTI is the presence of the endotracheal tube. This invasive device hinders the natural anatomical barrier formed by the glottis and larynx, resulting in a reduced mucociliary clearance and cough reflex. Pathogenic bacteria are then more likely to migrate downwards into the lower respiratory tract. Also the application of positive pressure during invasive ventilation impedes elimination of pathogens, due to a hampered mucociliary clearance and decreased ability to cough up sputum. Intubation and positive pressure ventilation are thus associated with an alteration of the respiratory microbiome, with a more profound dysbiosis in patients who develop VAP. Another risk factor for VA-LRTI is the decreased gastric acidity often present in critically ill patients. This decreased gastric pH value is a result of administered stress ulcer prophylaxis or gastric nutrition in these ICU patients. The induced gastric colonization that follows, makes these patients at risk for VA-LRTI.

The clinical relevance of VA-LRTI is significant: VA-LRTI are associated with significant morbidity and healthcare costs, as well as mortality, an increased ICU length of stay (LOS) and hospital LOS and longer duration of mechanical ventilation.

Diagnosis of ventilator-associated lower respiratory tract infections

The interpretation of the aforementioned clinical symptoms (signs of systemic infection, sputum, infiltrates) is the first step of the diagnostic process of VA-LRTI. The clinical
pulmonary infection score (CPIS\textsuperscript{16}) is a scoring system that translates these clinical parameters into a numerical value that may help to predict VAP\textsuperscript{17}. Clinical examination can be used to alarm clinicians of the possibility of VA-LRTI development, but it is insufficient to establish a definite diagnosis\textsuperscript{17}. In the absence of a clinically available gold standard (i.e. confirmation via biopsy or autopsy), lower respiratory tract sputum samples are collected and subsequently cultured. Various techniques can be used to obtain these lower respiratory tract sputum samples. Endotracheal aspiration (ETA) is a non-invasive method for this, whereas bronchoscopic broncho-alveolar lavage (BAL), protected specimen brush, or blind bronchial sampling via miniature BAL are invasive techniques. Literature shows variability in microbiological cut-off thresholds (in colony-forming units (CFU)) for the confirmation of LRTI\textsuperscript{18}. For BAL, most commonly a cut-off of 10\textsuperscript{4} CFU/mL is used to define a positive culture\textsuperscript{19,20}, whereas for quantitative ETA cultures a threshold of 10\textsuperscript{5} CFU/mL\textsuperscript{21} or 10\textsuperscript{6} CFU/mL\textsuperscript{19} has to be reached. American\textsuperscript{22} and British\textsuperscript{18} guidelines recommend non-invasive sampling with semi-quantitative cultures for the diagnosis of VAP, although recent European\textsuperscript{15} guidelines suggest that ETA collection is less specific due to contamination by tracheal bacteria that are not pathogenic. It is strongly recommended to collect the bacteriological sample before starting or changing antimicrobials, aiming to limit the influence of administered antibiotics on subsequent culture results. A negative microbiology result enables immediate withdrawal of antibiotics, whereas antibiotic de-escalation is possible according to the cultured pathogens. VA-LRTI suspicion requires prompt initiation of broad-spectrum antimicrobial therapy\textsuperscript{2}, since delayed appropriate antibiotic treatment is associated with increased mortality\textsuperscript{23} and prolonged ICU stays\textsuperscript{24}. The microbiology culture results require days to become available and turn out to remain negative in approximately 40% of VAP suspected patients\textsuperscript{25}. As a result, broad-spectrum antibiotics are administered inappropriately, contributing to the development of antimicrobial resistance\textsuperscript{26–28}. A more promptly available – yet still reliable – alternative is highly required, which is able to deliver an on-the-spot diagnosis of VA-LRTI, thus avoiding unnecessary use of antibiotics.

**Biomarkers**

Biomarkers are objective, quantifiable characteristics of biological processes\textsuperscript{29} and could be used for diagnostic purposes. The inflammatory mediators pulmonary interleukin-1β (IL-1β) and interleukin-8 (IL-8) measured in BAL fluid samples have been shown to be eligible as markers for VAP\textsuperscript{30}. Previously soluble triggering receptor expressed on
myeloid cells (sTREM-1) had been suggested as biomarker for VAP\textsuperscript{31}. However, when measured in BAL and exhaled breath condensate samples, sTREM-1 failed to differentiate between VAP positive and negative patients\textsuperscript{32}. Also C-reactive protein (CRP) and procalcitonin (PCT) have been investigated for the diagnosis of VA-LRTI\textsuperscript{33–35}, as well as for the differentiation between VAT and VAP\textsuperscript{3}. As of yet, none of these biomarkers have shown strong diagnostic benefit or anyhow failed to be widely implemented in the clinical setting\textsuperscript{36–38}.

As opposed to the measurement of a single or a couple of (targeted) biomarkers, the ‘omics technologies’ aim at measuring molecular patterns. This omics term has been used to define studies of genomes (genomics) and gene expression (transcriptomics), lipids (lipidomics), proteins (proteomics) and metabolites (metabolomics)\textsuperscript{39}. Relatively new within the metabolomics field is the study of exhaled breath metabolomics, referred to as breathomics.

Exhaled breath contains hundreds of volatile organic compounds (VOCs)\textsuperscript{40,41}. Exogenous VOCs originate from previous or current environmental exposures\textsuperscript{42}. Endogenous compounds are produced through internal metabolic processes, either occurring in the host during inflammation\textsuperscript{43}, oxidative stress or carcinogenesis\textsuperscript{44}, or processes taking place within microbes\textsuperscript{45}. VOCs in exhaled breath can derive from processes locally in the lungs. Also VOCs that are produced peripherally in the body can transported via the pulmonary circulation and excreted in the breath. In particular these endogenous VOCs are regarded as potential non-invasive metabolic biomarkers, since they may reflect pre-clinical signs of various diseases\textsuperscript{46–51}. As a result of the close interaction of breath and the respiratory tract, it seems only logical that exhaled VOCs are ideal markers to reflect pulmonary diseases\textsuperscript{52} and lower respiratory tract infection in particular\textsuperscript{45}. As a result, the field of breathomics has increasingly become of interest, due to its huge potential as a new diagnostic tool for future clinical practice. The first modern breath gas research paper already stems from 1971\textsuperscript{53}. Nonetheless in the following decades the pace of breathomics research was rather slow. This was due to technological difficulties associated with the complexity of breath collection and the initially limited analytical capacity. Also there was an inability of the used chemical detectors to achieve sufficient sensitivity and specificity for VOCs with low abundancy\textsuperscript{54}. Detection platforms and sampling methodologies have improved
significantly over the past years, provoking a renaissance in breath research that is yet ongoing.

**Analysing breath samples**

The complexity and variation of organic vapours in breath has led to the development of a multitude of breath analysis techniques, either detecting individual VOCs or focussing on the recognition of patterns (or so-called breathprints). Examples of methods that analyse individual VOCs are gas chromatography-mass spectrometry (GC-MS) and selected ion flow tube mass spectrometry (SIFT-MS)\(^{55}\), whereas electronic nose (eNose) technology\(^{56,57}\) and secondary electrospray ionisation-mass spectrometry (SESI-MS)\(^{58,59}\) deliver breathprints.

Gas chromatography-mass spectrometry (GC-MS) is the current gold standard for VOC separation, detection and identification\(^{45}\). Transportation of the breath sample is required, since the GC-MS technique is laborious and the machine is not available at the patient’s bed side. Again, a variety of methods have been described for the collection and transport of breath samples, including but not limited to glass syringes\(^{60}\), Tedlar® bags\(^{61}\), or the use of sorbent tubes/traps\(^{62,63}\). The research that is covered in this thesis, mainly involves thermal desorption (TD) GC-MS (see section Thermal desorption GC-MS). Breath analysis using TD GC-MS requires pre-concentration of the sample, since most VOC abundancies in exhaled breath fall within the nmol-pmol/L (ppbv-pptv) range\(^{64}\), while the analytical range of the system requires µmol-mmol/L concentrations. Pre-concentration can be established by adsorbing VOCs onto sorbent traps, coated fibres (used in Solid Phase Microextraction) or cryofocussing using liquid nitrogen\(^{65}\). Since VOCs have different boiling points, a careful selection of sorbent traps is required in order to avoid breakthrough of highly volatile compounds. Described sorbent traps include organic polymers (e.g. Tenax®), activated charcoal, graphitised carbon (e.g. Carbograph or Carbopack X) and carbon molecular sieves (e.g. Carboxen™)\(^{64}\).

**Thermal desorption GC-MS**

Breath VOCs trapped onto Tenax® can be stored at 4-7°C for two weeks without affecting diagnostic accuracy\(^{66}\). Thermal desorption releases the compounds, after which a carrier gas (often helium) carries them through the GC column. The retention time reflects the time it takes for a molecule to travel through the column and indicates how the molecule
interacts with the inner lining of the column: highly volatile compounds will leave the GC column relatively quick. Complementarily temperature programs are used in order to improve separation of the compounds and avoid wide peaks on the chromatogram. When the separated molecules exit the GC column and enter the quadrupole mass spectrometer, again different methods are available, of which one is electron ionization: the molecules are ionized after electron bombardment. It follows the principle that every molecule has a typical fragmentation pattern and ions are distinguished according to their mass-to-charge (m/z) ratio.

**Breath sampling in invasively ventilated patients**

As of yet there is not one standardised or ideal technique for collecting breath samples in general, nor for sampling in invasively ventilated patients. The choice of methodology depends on the application, taking into consideration issues related to breath sampling, e.g. effect of humidity, correction of environmental inspired VOCs, co-morbidities or types of collecting materials. Multiple breath sampling techniques have already shown to be successful in intubated critically ill patients. Before sampling methods like these can be clinically implemented, standardisation and external validation in multicentre studies is required.

**The BreathDx Consortium**

In 2014 the BreathDx Consortium was constituted as a result of a Marie Curie Action Industry Academia Partnership Programme: an initiative that pursues to enhance cooperation between industry and academia, by stimulating research training, career development and knowledge sharing. The Consortium initially consisted of the Amsterdam University Medical Centres – location Academic Medical Centre (the Netherlands), the University of Manchester (UK), Philips Research (Eindhoven, the Netherlands) and the University of Twente (the Netherlands). Later an expert panel was added to the BreathDx study, comprising members from Portugal, Ireland and Spain. The BreathDx collaboration involved the establishment of the multicentre clinical BreathDx study investigating the diagnosis of VA-LRTI by analysis of VOCs in the exhaled breath.

**Aims and outline of this thesis**

In this thesis we primarily investigate the use of exhaled breath analysis for the diagnosis of VA-LRTIs, studying whether (1) breathomics can differentiate patients with confirmed
VA-LRTI from patients with negative cultures and (2) different causative pathogens can be distinguished using exhaled breath analysis. We aim to assess the diagnostic value of breath biomarkers, but also other existing biomarkers or clinical scoring systems for pneumonia. Also different methods of collecting breath samples from invasively ventilated patients are studied, aiming to gain insight in what is required for breath sampling from a ventilator circuit.

In chapter 2 we systematically review the existing literature for the use of exhaled breath analysis for the diagnosis of pneumonia. In chapter 3 we aim to compare two previously sampling methods that had been previously shown to lead to successful breath sample collection. For this we construct a laboratory-based ventilator circuit, aiming to mimic patient setting on ICU. Chapter 4 describes the protocol for the BreathDx study: a multicentre observational clinical cohort study, for which patients suspected of VA-LRTI were recruited at the ICUs of four hospitals in Manchester and Amsterdam. In chapter 5 we search for VOCs that can be used to identify mechanically ventilated patients with (either community- or hospital acquired) pneumonia on ICU. Also distinctive concentrations of VOCs in patients with solely colonized airways – and no pulmonary infection – are studied, searching for markers associated with bacterial presence and growth. In chapter 6 we aim to find distinctive VOCs for Streptococcus pneumoniae and Pseudomonas aeruginosa: common causative pathogens for VA-LRTI. A rat model is used to initiate pneumonia, 24 hours later followed by intubation, mechanical ventilation and subsequent breath sampling. In chapter 7 we aim to confirm the predictive value of soluble urokinase plasminogen activator receptor (suPAR) for VAP. SuPAR is an inflammatory biomarker measurable in blood, which is deemed to have certain prognostic value in critically ill patients with systemic infection. By presenting the results of the BreathDx study, in chapter 8 we aim to find distinctive VOCs that enable discrimination between patients with and without VA-LRTI, as well as compounds that can be linked to causative pathogens. Chapter 9 and 10 contain a summary and a discussion of the results.
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Introduction


