Diagnosis of pulmonary injury and infection by exhaled breath analysis
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Chapter 2.

Development of breath analysis in intubated and ventilated intensive care unit patients

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Abstract

Exhaled air contains a complex mixture of molecules that may change with disease. Breath analysis has shifted from detection of abundantly present molecules like carbon dioxide to monitoring of scanty volatile organic compounds, and from a focus on single molecules to a broader look at mixtures of biological markers. Breath analysis is now used to detect hundreds of exhaled metabolites, combinations of which have the potential to serve as markers of physiological and pathophysiological processes as they may reflect oxidative stress, inflammation, infection, and organ failure. While gas–chromatography and mass–spectrometry is used for discovery of volatile biological marker, this technique is impractical for daily practice since it is time–consuming and expensive. New techniques may, guided by gas–chromatography and mass–spectrometry discovery of volatile biomarkers, result in bedside, rapid diagnosis or monitoring of disease by a simple exhalation. Besides applications in the first line and on hospital wards, this may allow for continuous real-time monitoring in critically ill patients, especially when ventilated. This may result in a monitoring system for cellular and molecular processes like organ injury and infection that can momentarily only be extrapolated from alterations in macroscopic physiology.
Introduction

Most, if not all intensive care unit (ICU)–patients are under constant monitoring of physiological signs in an attempt to early detect deteriorations (mainly organ failure and infection) or responses to therapies. Unfortunately, presently used monitoring techniques are almost without exception limited to global parameters [1, 2]. Global parameters are the result of alterations in multiple molecular pathways and are therefore late and a–specific signs of dysfunction [2]. There is a growing need for assessment of molecular and/or cellular changes in order to interfere earlier and in a more homogenous group of patients [3, 4].

Assessment of molecular and cellular processes is difficult as it depends on off site biochemical analysis of blood or other bodily fluids. The fluid should be obtained and send to the lab for analysis. Sample collection, transport and analysis may cause delay in initiation or adaption of therapy; for example broncho–alveolar lavage cannot be performed at any given moment and it may take days before culture results are known. Delays are unwanted as therapy is most effective in an early phase of disease [5-8]. Ideally, monitoring should be continuous, rapid and online.

Physicians depended heavily on their senses before the era of technological innovation. Color, taste, and smell were their biological markers [9]. Although outdated, their sensing provided a quick, non–invasive and integrative view on biochemical processes without additional costs, processing and analysis. Several diseases are reported to induce a distinctive smell of breath (e.g. acetone during keto–acidosis and fetor hepaticus) [10]. Exhaled breath contains hundreds sorts of molecules in gas phase. The composition of this mixture can be altered in disease. Nowadays, the development of biochemical sensors and enhanced data–analysis allows for artificial smelling of exhaled breath [11]. Diagnosis and monitoring based on exhaled breath analysis may be attractive for the intensive care physician as it is non–invasive [12, 13].

In this chapter we discuss the emerging field of exhaled breath analysis for monitoring of patients on the ICU. Exhaled molecules of interest will be discussed from high to low abundance (figure 1); from known into unknown territory. Every molecule will teach a principle of exhaled breath
analysis that can be applied to novel techniques. We aim to provide an overview of the current status and future trends.

**Figure 1: Composition of exhaled breath**

![Composition of exhaled breath diagram]

**Carbon dioxide**

Carbon dioxide (CO2) is the only largely endogenous gas that is routinely measured in the breath of intubated and ventilated patients. Indeed, continuous capnometry has an important place in respiratory monitoring [1]. CO2 is the result of aerobic metabolism and transported to the lung by venous return. Therefore, the main applications of capnography are (1) monitoring airway patency and ventilation, (2) assessment of alveolar dead space and (3) during cardiopulmonary resuscitation [14]. The applications correspond to the three principles that are at the foundation of capnography: (1) the lung is the only compartment in which CO2 normally and continuously exits the circulation; (2) delivery depends on the amount and homogeneity of pulmonary blood flow and (3) without aerobic metabolism CO2 production ceases. The success story of capnography teaches us the potential of continuous monitoring.
Carbon monoxide

Carbon monoxide (CO) is known as an exogenous intoxicative compound. The fact that CO is also produced endogenously by heme oxygenase in the process of heme degradation is less well known [15]. This process occurs mainly in the liver. However, heme oxygenase is also present in vascular endothelium and alveolar macrophages of the lungs and can be up-regulated by oxidative stress and inflammation [15]. CO is exhaled and may be a breath marker of inflammation. Indeed, CO was found in higher concentrations in respiratory infections, asthma and exacerbations of cystic fibrosis (CF) [15-18]. Smoking is a major confounder as it independently leads to very high CO concentrations in exhaled breath with a half-life of around 6 hours [15]. Interestingly, CO was found to negatively modulate inflammation in hyperoxic lung injury [19]. CO is thus associated with inflammation but is a mediator in the anti-inflammatory response [20]. CO teaches us that exhaled molecules can be of systemic or pulmonary origin and that volatile compounds may be biologically active.

Nitric Oxide

Exhaled nitric oxide (NO) is proposed as a diagnostic breath marker for the airway inflammation associated with asthma [21]. A major success of exhaled NO is that it predicts the clinical response to corticosteroid treatment in these patients [22]. NO is constantly produced by NO synthases (NOS) and acts as a modulator of arteriolar and bronchial tone, platelet aggregation and inflammation [23]. Endothelial cells have a relatively low output of NO while airway epithelial cells produce higher concentrations. The latter can be further induced through inflammation [24, 25]. Thiol-containing compounds may interact with NO and provide a temporal reservoir [26]. Importantly, the upper airways also contribute to the exhaled NO concentration.

An excellent recent review by Boshier et al. discussed the potential of NO as biological marker for acute lung injury [27]. One would expect a higher exhaled NO concentration as NOS activity increases with inflammation. However, predominantly lower exhaled NO levels are found in acute respiratory distress syndrome (ARDS) [27]. This may be explained by increased NO consumption by oxidative stress. NO reacts with superoxide
to produce peroxynitrate, leading to molecular damage, such as lipid peroxidation [28]. Besides, NO acts as an anti–oxidant by inhibition of lipid radical chain propagation [29]. Both lead to increased NO consumption and a lower netto exhalation. NO teaches us that exhaled molecules can be produced in different parts of the airways and that both higher and lower concentrations may be informative.

**Exhaled breath condensate**

Besides volatile water content, micron and sub–micron sized droplets emanate in exhaled breath [30]. It has been hypothesized that these droplets are blown from the epithelial lining fluid during turbulence or opening of collapsed airways [31]. Exhaled breath condensate (EBC) is a combination of droplets containing non–volatile molecules, diluted with condensed exhaled water content and substituted with water–soluble volatile organic compounds (VOCs) [31, 32]. Dilution, which can range from 20 to 30,000–fold, is a problem with EBC [33]. Three solutions are proposed: (1) standardize against a stable background molecule, such as urea [34], (2) use the ratio of multiple biomarkers (e.g. NO2–:NO3– or pH) and (3) use a qualitative test.

Several non–volatile biomarkers have been investigated in the EBC of mechanically ventilated ICU–patients. EBC levels of these markers should reflect concentrations in the epithelial lining fluid. Hydrogen peroxide (H2O2), isoprostane, NO2–, pH and lactate – markers of anaerobic metabolism and oxidative stress – are elevated in ARDS [35-38]. These markers may also be used to monitor treatment response [39, 40] and correlate well to values in broncho–alveolar lavage fluid (BALf) [41]. Inflammatory cytokines are also increased in the EBC of ARDS patients [42], but difficulties with the lower limit of detection and quantification complicate cytokine measurements in EBC [33].

Water–soluble volatile organic compounds can be found in high concentrations in EBC as these volatile molecules pass by during sample collection and dissolve into the water. High–throughput technology is nowadays available for the analysis of these small metabolites. Nuclear magnetic resonance (NMR) spectroscopy and liquid–chromatography and mass–spectrometry (LC–MS) can be used for liquid samples. For example,
NMR analysis of EBC showed good discrimination between patients with CF and controls and between stable and unstable CF [43]. One metabolite was not sufficient to discriminate. Rather, subtle changes in multiple metabolites reflected (unstable) disease. Since the underlying cellular and biochemical mechanisms of any disease are integrated into a complex network of interactions it is very probable that multiple biomarkers will outperform a single one. “Omics”–studies represent the integrated view of the biochemistry within a domain of complex organisms [44]. Metabolomics is the “global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity and physiological landscape” [9]. EBC teaches us how to deal with the problem of dilution and the potential of an “omics” approach to discover novel molecular markers.

**Breath metabolomics**

It is not necessary to dissolve VOCs into water to perform metabolomics analysis. Volatile metabolites can be analysed directly in the exhaled breath, or absorbed onto a transport tube and analysed else–where. High–throughput analysis of exhaled breath is also called “breath metabolomics” or in short “breathomics”. Breath contains hundreds of VOCs, metabolites in gas–phase produced by both physiological and patho–physiological processes [45, 46]. Alteration of exhaled VOCs can be the consequence of changed systemic metabolism (see section on CO2) or due to pulmonary production (see section on CO and NO) [46]. Bacteria also produce volatile metabolites [47, 48]. Thus exhaled breath contains the composite signal of host–metabolism, as part of the host–response, and bacterial metabolism, which may interact [49]. Below we describe the two most important methodologies for VOC analysis that were used in this thesis; gas-chromatography and mass-spectrometry and electronic nose analysis.

**Gas–chromatograph and mass–spectrometry**

Gas–chromatography and mass–spectrometry (GC–MS) is considered the gold standard for the discovery of volatile organic compounds. In the following section, we will discuss the analytical principles and considerations in detail.
Trapping and pre-concentration

As the analytical machine is not available at the bedside, transport of the sample is required for the analysis of VOCs with GC-MS. VOCs can be contained in a glass syringe and injected into the gas-chromatograph, if done quickly [50]. VOCs cannot be stored over a longer period of time using this method as a syringe in not completely airtight and oxidation processes can occur, leading to loss and modification of the VOCs, respectively. Alternatively, VOCs can be trapped onto a sorbent material [51] such as Tenax (TA). However, all sorbent materials show selectivity towards certain types of VOCs. For example, Tenax is mainly hydrophobic and therefore polar compounds are not always trapped, although polar compounds are present if the air is humid, which suggest some trapping of water as well [51]. Volatiles with a very low boiling point show breakthrough (not trapped on the Tenax) if high concentrations are used [52]. Therefore, compounds that are polar and/or have a low boiling point cannot be determined quantitatively with trapping on Tenax. However, under the assumption that this bias is constant, semi-quantitative measurements can be performed. When VOCs are absorbed onto Tenax, they can be stored for at least two weeks at 4°C [53]. Volatile molecules can be released by thermal desorption of the tube filled with Tenax (figure 2). After re-focussing the breath sample onto a cold trap (with a lower mass, allowing for more rapid heatings and thus a shorter injection period) the sample can be injected into the gas-chromatograph through a heated liner.

Gas-chromatography

Gas-chromatography is used to separate the different VOCs within a breath sample so that they can be detected individually (see section on mass-spectrometry). For VOC analysis, typically a capillary column is used. In this long (i.e. 30m), thin (i.e..0.25 mm) column the molecules are carried by a gas (i.e. helium), which is called the “mobile phase”, towards the detector (figure 2). The time that a molecule travels through the column, called the retention time, is a representation of the chemical interaction of the molecule with the packing of the column, called the stationary phase. Many different types of stationary phases are available but for breath research a non-polar silicon polymer is mostly used (i.e.
dimethylpolysiloxane). Because chemical interaction alone results in insufficient separation and wide peaks (one type of molecule exits the column for a long period of time) temperature programs are used. This gives two advantages; better separation is obtained by a combination of chemical interaction with the stationary phase and boiling point; and the peaks can be narrowed as chemical interaction with the stationary phase rapidly become less likely as temperature increases.

**Mass-spectrometry**

There are different techniques for detection of VOCs that exit the column. For quantitative detection flame ionization detection gives the best results. Basically, the molecules that exit the column are burned and the ions that are generated are measured. However, for breath research the molecules of interest are not known and discovery is an important aspect of the analyses. Therefore, quadrupole mass-spectrometry can be used to tentatively identify the molecules that are detected, while maintaining (semi-) quantification. When a group of molecules, presumingly of one molecular structure as they are separated by gas-chromatography, exit the column they are ionized by electron bombardment (electron ionization) (figure 2), although other forms of ionization are also available (e.g. chemical). Electron ionization results in a typical fragmentation pattern per molecule, which can be compared to a library of reference fragmentation patterns for known molecules (see identification). The ions are pushed in between the four parallel rods of the quadrupole mass-spectrometer that use an oscillating electrical field to selectively stabilize or destabilize the trajectories of certain masses of ions (figure 2). In other words, only ions with a certain mass-to-charge ratio (M/Z) pass through the quadrupole and can be detected electronically. Because the electrical field in the parallel rods oscillates very frequently a near-continuous measurement of all nominal masses within a certain range can be detected. Importantly, with adequate tuning of the machine, the semi-quantitative results of this mass-spectrometer are quite comparable over time.

**Processing of the GC-MS signal**

GC-MS analysis results in a three-dimensional matrix per sample (figure 2). The first dimension is the retention time and shows the separation of
the VOCs. The second dimension is the mass-to-charge ratio and displays the fragmentations of the VOCs. The third dimension is the intensity of the signal. After adjustment for background signals, it is key to reduce the number of dimensions to one to allow for statistical analysis. Ideally, one would like to create a list of concentrations per molecule per sample. The first step is to identify if a molecule is eluding from the GC by finding peaks in the different M/Z-windows of the MS. Under good chromatographical conditions, one compound exits the GC in such a way that it forms a Gaussian shaped pattern within the different M/Z-windows. That property can be used to detect peaks per M/Z by taking the first and second derivative; a well-separated peak passes zero in the first derivative and passes zero in close proximity on both sides in the second derivative.

The area under the peak, which is a semi-quantitative measure of the concentration, can be estimated by fitting a Gaussian function. This method eliminates one dimension; the retention time. The other dimension, the different M/Z windows, can be reduced quite simply by putting all peaks per M/Z in a wide format, resulting in a list of M/Z-peaks intensities, with a certain retention time. However, we cannot use these lists for statistical analyses because retention times can differ slightly between samples. Therefore, peaks first need to be matched across samples to allow for retention time correction. Portions of the chromatogram can be matched with the use of chemical standards and frequently present peaks that have a unique mass within a certain range of retention times. Information on the differences in retention times between the samples based on these matches can be used for retention time correction. Multiple iterations of linear and non-linear retention time corrections allow for optimal alignment of the samples and adequate grouping of the detected peaks. Additionally, it is plausible that ion-fragments meet the shape requirement in most samples but are not detected in a minority. With the information of the corrected retention time and the peaks that are detected in the other samples, the samples where the peak is “missing” can now be searched more sensitively for that ion-fragment. This limits the amount of missing data, which can be fatal for certain statistical classification methods (that for example require a normal distribution) and cross-validation methods (a peak classified cases in the training set, but is incorrectly missing in the cross-validation sample wherefore classification fails).
Figure 2: Gas-chromatography and mass-spectrometry

Breath analysis by means of GC-MS. From the upper left, clockwise: breath is collected and stored on an absorption tube that is desorbed to bring the molecules into the gas phase. The VOCs are separated by gas-chromatography and fragmented and detected by mass-spectrometry, which results in a three-dimensional matrix (mass over charge (M/Z), retention time and intensity (counts of ion-fragments). By means of filtering and retention time correction, a one-dimensional matrix of ion-fragments can be obtained per patient. The most relevant features are selected and used to predict the class of the patients. The identity of these selected compounds can be speculated upon by means of the mass spectrum but requires the injection of a chemical standard and comparison of the retention time and fragmentation pattern for proof.
Several programs are available to perform some of the before mentioned steps but most software is designed for targeted analysis of known compounds. An excellent program for untargeted metabolomics discovery is XCMS [54] that is available as a R-package and through an online interface. Now, a peak list per sample can be exported and used for statistical analysis; the peaks are now called features or predictor variables (figure 2). It should be noted that one compounds fragments to many ion-fragments, which are all detected and used as features for statistical analysis. This means this method results in multi-collinearity. The alternative, summing the intensities of the ion-fragments at every retention time, is presumably incorrect in complex samples such as breath because we cannot exclude co-elution of multiple compounds at a single retention time. In that case, the concentration of multiple molecules would be incorrectly summed. Maintaining the ion-fragment structure for statistical analysis allows for differentiation in predictive value between the M/Z values and can help in the identification of the co-elution, although identification of such compounds will remains very difficult (see section from feature to VOC).

Feature selection and classification

The questions in breath studies that use GC-MS analysis can be split into the pathophysiological angle: "Which VOCs are associated with this disease" and the clinical angle: "What is the diagnostic value of breath analysis for this disease". To translate the second question to a clinical application, it is recommendable to obtain a concise list of biomarkers that can be detected with targeted assays in validation trials. Therefore, identification of the few key-biomarkers is important in GC-MS research. Additional reasons for reduction of the number of predictor variables are to increase prediction accuracy by limiting the variance (but potentially increasing bias) and to allow for interpretation of the model.

Several criteria could be applied to select the most appropriate markers. Traditional measures such as p-value and fold-change can describe a breath profile and can be used to show that there are features that are different between the disease states but have a limited value with regard to diagnostic applications. Receiver-operating characteristics analysis can be used to quantify the discrimination between the disease states per
variable and is more closely related to the final application, but still relies on the simplistic view that the combination of the best individual biomarkers also gives the best composite signal. Alternatively, several methods are available that allow for selection using the information of all features. In logistic regression techniques the Akaike Information Criteria (AIC) are frequently used to select the most relevant features. However, in breath research the number of predictor variables is typically an order higher than the number of subjects and this may limit the use of the AIC. Alternatives are LASSO and sparse-partial least squares (SPLS) analysis [55]. Both methods allow for selection of the most relevant features even when the number of predictors is larger than the number of patients. Importantly, whatever method is chosen, a high number of predictor variables per number of included patients frequently induces over fitting (in other words bias) and thus cross-validation is essential to tune the settings for the model and estimate the discrimination accuracy. Furthermore, it should be noted that external validation of the diagnostic accuracy of the selected features is very important, especially in metabolomic discovery studies [56-58].

**From feature to VOC**

Statistical analysis has led to the selection of the most relevant features and the evaluation of their discrimination accuracy. In order to identify the VOC that resulted in those ion-fragments the raw GC-MS data should be consulted. But first, it is important to verify that all ion-fragments with that retention time show the same association with the disease state as the selected ion-fragment; this supports the hypothesis that it is indeed one VOC that eludes at that retention time. If not, co-elution of multiple compounds is highly suspected and identification will be considerably more difficult. In the raw GC-MS data, deconvolution software can be used to obtain as pure mass-spectra as possible of the target compounds. These spectra can than be compared to reference libraries, such as that of the National Institute of Standard and Technology (NIST) (figure 2). This method will lead to a short list of VOCs that have a high matching factor and to tentative identification of the compound.

However, for most molecules it is impossible to obtain certainty about the molecular identity, as isomers are frequently difficult to distinguish by
mass-spectra alone. To confirm the identification, pure standard of the compounds that are on the short list should be injected into the column and the molecule of interest will have the exact same retention time as the identified ion-fragment. (figure 2) Incidentally, two isomers may have the same retention time because of insufficient chromatographic separation and thus the conclusion should be that one of the two, or both compounds are different between the target conditions. Identification completes the GC-MS evaluation for breath biomarker discovery but it should be noted that the process of identifying the biochemical pathways that lead to the formation of the identified VOCs in disease is now just starting.

**Electronic nose analysis**

Electronic noses (eNose), named after their similarities with mammalian olfactory system [11], integratively capture complex VOC mixtures using an array of different sensors [11]. Sensors can be utilized through two mutually exclusive routes: (1) very specific sensors that follow a “lock-and-key” principle that have a very high sensitivity and specificity or (2) semi-selective, cross-reactive sensors that are less sensitive and less selective but an array of which can be used to characterize unknown complex samples [59]. Mamallians rely on a semi-selective “sensor system” and are able to classify approximately 1 trillion different olfactory stimuli [60]. Since the VOCs that should be detected to classify disease most accurately are unknown and most biological samples are highly complex, that approach is used currently most frequently used.

Metal oxides, conducting polymers, optical and infra–red spectroscopy have been used as sensors. Peaks and intensities obtained by mass-spectrometry can also be presented to pattern-recognition algorithms, hereby virtually converting every detected mass into a “sensor” [61]. The composite signal of all sensors in an array can be analyzed using pattern–recognition algorithms. The composite signal of eNose analysis results in a unique fingerprint. Subsequently, these fingerprints can be used for diagnostic and monitoring purposes [61] (figure 3). It should be noted that eNose technology is not designed to tell something about the details of the molecular composition of a sample but is used as a pragmatic method for clinical classification, based on quantitative, probabilistic evidence. In other words, the eNose may be used to confirm or exclude diagnoses rapidly.
and non-invasively but without providing the doctor with information on what molecular criteria this diagnoses is based. Therefore, the eNose can be seen as the modern exemplification of empirical medicine. Because the patho-physiological background of a well-understood biochemical assay is ignored with the eNose approach, it is even more important that the training and validation in biomedical research is as rigorous as possible.

**Figure 3:** Electronic nose

*Breath analysis by means of eNose. From the upper left, clockwise: semi-selective sensors react with the VOCs in the breath. The properties of the sensor change as the VOCs bind (e.g. the electrical conductance changes) and that can be measured. All sensors in the array have a different functional layer and react differently to the composition of the breath. Most commonly, the proportion of most variance is captured by dimension reduction with for example principal component analysis. These composite variables can consequently be used for classification.*

The statistical analysis of electronic nose signals is closely related to that described in the section on GC-MS. However with eNoses it is less important to perform feature selection because no molecular information can be obtained from the individual sensors and thus it does not add to the interpretability of the algorithm. Several types of statistical methods have been used in the literature [62], but most involve a form of dimension reduction followed by a classification algorithm (figure 3). Unsupervised dimension reduction methods such as principal component analysis
are frequently used [63], but are easily disturbed by, for example, co-
morbidities to capture other large variations in the data than the disease of interest [64]. Therefore, other groups have relied on supervised methods for dimension reduction and classification [65], which can be combined in partial least square regression [66]. The major advantages of both principal component analysis and partial least square regression is that they perform well when the predictor matrix has more variables than observation (hence the dimension reduction) and when there is multi-
collinearity (as with semi-selective sensors) by reducing the predictor matrix into a projection of variance. As with GC-MS data, over fitting of the model to the training data is easy and cross-validation should always be performed. External validation is eventually required, especially in clinical studies where disease diagnosis is the main objective.

Current electronic nose technology thus relies on semi-selective sensors, pattern recognition and empirical disease classification. However, tailored sensors that have chemical interactions with pre-specified VOCs following a "lock-and-key" principle could set the stage for different approaches [59]. If gas-chromatography and mass-spectrometry would allow for the selection of a limited panel of volatile biological markers, eNose technology might be used for rapid bedside detection. In this way, hybrid form eNosess could be constructed that do allow for “black box” empirical classification but also provide specific information concerning the biochemical background. Such a combined approach may result in the integration of the best of both worlds, within a device that still can be used for disease recognition at the bedside and for continuous monitoring in ventilated patients.

**Conclusion**

Breath analysis has shifted from detection of abundantly present molecules like carbon dioxide to monitoring of scanty volatile organic compounds, and from a focus on single molecules to a broader look at mixtures of biological markers. Breath analysis is now used to detect hundreds of exhaled metabolites, combinations of which have the potential to serve as composite markers of physiological and pathophysiological processes as they may reflect oxidative stress, inflammation, infection, and organ failure. While gas–chromatography and mass–spectrometry is used for
discovery of volatile biological marker, this technique is impractical for daily practice since it is time-consuming and expensive. New techniques such as electronic noses may, guided by gas-chromatography and mass-spectrometry discovery of volatile biomarkers, result in bedside, rapid diagnosis or monitoring of disease by a simple exhalation. Besides applications in the first line and on hospital wards, this may allow for continuous real-time monitoring in critically ill patients, especially when ventilated. For the first time, this may provide in a monitoring system for cellular and molecular processes like organ injury and infection that can momentarily only be extrapolated from alterations in macroscopic physiology.

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