The versatile use of exhaled volatile organic compounds in human health and disease

To cite this article: Agnes W Boots et al 2012 J. Breath Res. 6 027108

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The versatile use of exhaled volatile organic compounds in human health and disease

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Received 3 February 2012
Accepted for publication 1 May 2012
Published 23 May 2012
Online at stacks.iop.org/JBR/6/027108

Abstract
Exhaled breath contains thousands of volatile organic compounds (VOCs) of which the composition varies depending on health status. Various metabolic processes within the body produce volatile products that are released into the blood and will be passed on to the airway once the blood reaches the lungs. Moreover, the occurrence of chronic inflammation and/or oxidative stress can result in the excretion of volatile compounds that generate unique VOC patterns. Consequently, measuring the total amount of VOCs in exhaled air, a kind of metabolomics also referred to as breathomics, for clinical diagnosis and monitoring purposes gained increased interest over the last years. This paper describes the currently available methodologies regarding sampling, sample analysis and data processing as well as their advantages and potential drawbacks. Additionally, different application possibilities of VOC profiling are discussed. Until now, breathomics has merely been applied for diagnostic purposes. Exhaled air analysis can, however, also be applied as an analytical or monitoring tool. Within the analytic perspective, the use of VOCs as biomarkers of oxidative stress, inflammation or carcinogenesis is described. As monitoring tool, breathomics can be applied to elucidate the heterogeneity observed in chronic diseases, to study the pathogen(s) responsible for occurring infections and to monitor treatment efficacy.

(Some figures may appear in colour only in the online journal)

1. Introduction

1.1. The history of VOC recognition

If you were asked to smell the armpit of an unknown person, you probably would not do this, whereas you would say yes when asked to smell a baby. In other words, we are able to sense the different chemicals spread by an armpit or baby and can label them as either unpleasant or pleasant. The ability of humans to differentiate between these two smells is due to the recognition of a combination of specific volatile chemicals by a unique set of olfactory receptors [1]. This results in a multidimensional pattern that is integrated in our brain, enabling us to train ourselves in recognizing and memorizing smells. Indeed, even without knowing the chemicals, we are able to know how a bakery or a wet street or bad breath smells due to the presence of such algorithms in our head that are capable of identifying 4000 to 10 000 different smells [1].

Smelling can be seen as a chemical sense as we sample our environment for information and recognition. It has been told that in the medical setting nurses can recognize the condition of their patients by smell. In ancient times, Greek physicians were already aware of the relationship between the odor of a subjects’ breath and possible diseases associated with it. Indeed, they realized it could provide insight into physiological and pathophysiological processes in the body [2]. For example,
the sweet acetic smell of breath might indicate uncontrolled diabetes whereas a fishy musty reek of breath relates to liver disease and a urine-like smell is associated with kidney failure [3]. Apparently, there is something present in breath that might enable diagnosing certain diseases or providing the means to monitor metabolic processes in the body. Due to the great potential of applications in clinical diagnostics and its non-invasive nature, exhaled air analysis has become of increased interest in recent years. Over the last few decades, the technical advances in analytical analysis have been responsible for large developmental improvements both in diagnostics and understanding underlying metabolic and biological pathways. Together, these developments have led to the promise of new biomarkers in exhaled air that might characterize and identify diseases.

1.2. The best-studied volatile markers thus far

Exhaled air is a mixture of nitrogen, oxygen, carbon dioxide, inert gases, water vapor (up to saturation) and traces of volatile organic compounds (VOCs) [4]. Current analytical methods enable the detection of several hundreds of compounds in one single breath sample. Interestingly, several thousands of different compounds have already been identified in exhaled air overall, demonstrating the vast diversity of compounds available in breath. However, up till now only a few compounds detected in breath have demonstrated their value as biomarkers. One of the generally accepted exhaled biomarkers is nitric oxide (NO), of which increased levels are associated with the occurrence of pulmonary inflammation and oxidative stress in various chronic lung diseases including asthma and chronic obstructive pulmonary disease (COPD) [5–7].

Another well-studied volatile biomarker is carbon monoxide (CO), although its study results are rather ambivalent. For instance, Yamaya et al reported a significant relationship between exhaled CO concentrations and certain lung function markers as well as a clear correlation between exhaled CO and eosinophil count in sputum [8]. In contrast, other studies including that of Montuschi et al did not find any correlation between exhaled CO and lung function [9]. Besides the ambiguous results, the application of CO as a diagnostic marker is also limited due to the fact that exhaled CO levels are seriously affected by environmental CO. As the latter might fluctuate considerably and is easily influenced by active and passive smoking, using exhaled CO levels as a biomarker can be considered questionable at least [10]. Besides these small volatile biomarkers, research has also been performed regarding the use of organic compounds derived from biological processes such as lipid peroxidation including ethane and pentane (see also section 3.1.1). Already the possibility to analyze ethane and pentane levels in exhaled air as markers of in vivo lipid peroxidation has been demonstrated in the early 80s [11]. Additionally, elevated levels of exhaled ethane and pentane are reported in patients suffering from several chronic lung diseases including COPD, asthma and cystic fibrosis (CF) [12–15].

1.3. The development of breathomics

The use of individual VOCs as biomarkers of exposure or disease is seriously hampered by the fact that using a single compound is generally insufficient to monitor complex and heterogeneous situations including environmental exposures or chronic diseases. Consequently, exploring the total amount of exhaled VOCs, called the volatome, is expected to generate more adequate information regarding the complexity of processes underlying the pathophysiology of interest. Certainly, analyzing the volatome implies a more sensitive and specific discrimination between various conditions as it reflects changes in both exogenous and endogenous compounds.

The approach of measuring exogenous compounds comprises products derived from environmental noxes, bacteria and viruses whereas endogenously formed compounds are related to physiologic or pathologic biochemical processes. Such a systemic study of unique chemical fingerprints that specific cellular processes leave behind, thereby mapping the entire profile of metabolites in a single cell, tissue or organism, is called metabolomics [16]. The concept that individuals might possess a ‘metabolic profile’ reflecting the composition of their biological fluids and gases was first introduced in the late 1940s [17]. While the first disease-specific metabolic patterns were discovered in urine and saliva using paper chromatography, advanced technologies became rapidly available and further increased the interest in qualitative analysis of metabolic profiles in the late 1960s and 1970s. Indeed, new technologies led to the characterization of unique metabolic profiles, consisting of hundreds or thousands of chemical constituents, not only in biological fluids including blood and urine but also in the headspace of such fluids and in breath [18, 19]. Consequently, identifying discriminating compounds in breath with regard to exposure or disease is currently regarded as a way of delivering non-invasive biomarker profiles to monitor such conditions. The general concept of metabolomics on exhaled air, also called breathomics, follows steps starting from clinical and biological questions, supporting in vitro studies, measurements of volatile compounds by analytical platforms, data handling including statistical analyses, until identification of biomarker profiles that subsequently need to be clinically validated in independent patient studies (figure 1). In this paper we discuss issues around air sampling, the currently applied mass spectrometry (MS)-based methodologies, data (pre)processing and analysis, and examples of clinical applications.

2. Analytical techniques

Ever since breathomics first gained interest, the applied methodologies regarding breath analysis have evolved tremendously. In order to distinguish endogenous biomarkers of exposure or disease from contaminants originating from the sampling environment, reliable methods for sampling, analysis and data processing are obligatory [20–24]. However, to date no consensus has been established regarding a standardized and integrated operating procedure. Different research groups apply different methodologies regarding sampling, sample
2.1. Issues around the sampling of breath

Exhaled air comprises a mixture of dead-space air and alveolar air. The dead-space air consists of roughly 150 ml air from the upper airway where no gaseous exchange between blood and breath air is facilitated [4, 21]. Consequently, this part of the exhaled air displays a high resemblance with the previously inspired air. In contrast, alveolar air originates from the lower airways where gaseous exchange between blood and breath air results in concentrations of endogenous compounds that are two to three times higher compared to those observed in dead-space air. In short, there are three ways to sample exhaled air: (a) upper airway collection by sampling dead-space air only, (b) lower airway collection by sampling alveolar air only and (c) mixed air collection by sampling whole breath, i.e. a mixture of dead-space air and alveolar air [22]. In some breath tests, including exhaled nitric oxide (FeNO) measurements, different airway sites can be used to analyze the increased fraction of FeNO, since NO is directly released into both dead-space and alveolar air. In asthma, increased FeNO in both central as well as in peripheral airway sites reflects eosinophilic-mediated inflammatory pathways reasonably well and relates to increased inhaled and systemic corticosteroid responsiveness [5–7]. In contrast to FeNO, most VOCs are ideally not measured in the dead-space air. Systemically generated VOCs are known to be only present in breath due to their release from the blood and are therefore best analyzed in alveolar air or a mixed air collection. Additionally, VOCs generated during pulmonary physiologic and pathologic processes, including inflammation and lipid peroxidation, are best measured in the alveolar air as they will directly be transferred from the target organ. In general, it can be stated that endogenous VOCs display a higher concentration in alveolar air than in mixed air [4], although this largely depends on the fraction of dead-space air present in the latter.

In practice, measuring VOCs in alveolar air is often hampered by dilution of the required sample with dead-space air. Consequently, various solutions have been developed over the years to minimize the occurrence of such a dilution. The most efficient one samples the alveolar air by a CO2-controlled valve that uses the end-tidal CO2 concentration as a marker of the transition of dead-space into alveolar air [22]. A less subtle but more simple solution involves the breath-collecting apparatus that transports the alveolar air to a desorption tube using a reservoir filled with every exhalation [23]. In short, this reservoir enables the selective sampling of alveolar air by leaving behind the dead-space air provided that it is applied in an ideal situation where no diffusion is possible [23].

Measuring VOCs in mixed air implies sampling whole breath that consists of both dead-space air and alveolar air. Our group has adapted a whole breath collection method based on inflating a Tedlar bag of 5 l. Using this approach, it can be estimated that dead-space air comprises only 150 ml of the total 2.5–4 l of exhaled air in adults per exhalation. We have proven that the contribution of dead-space air to the total volume of whole breath does not lead to sensitivity issues in measuring VOCs by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) [24]. Additionally, an advantage of sampling 5 l instead of one single breath is a higher reproducibility and lower variability as it is still difficult to ensure that a single breath is representative for all subsequent breaths [4]. Finally, sampling whole breath instead of only alveolar air adds to the simplicity of exhaled air metabolomics as it increases the ease of use for the physician while decreasing the degree of discomfort for the patient.

2.2. Sample analysis techniques

In order to either quantitatively or qualitatively determine the compounds present in exhaled air, several advanced technologies are available. The next paragraphs are dedicated to the most important sampling analysis and data processing techniques currently employed with respect to breathomics. Additionally, the main characteristics of the various sample analysis techniques are summarized in table 1.

2.2.1. Gas chromatography mass spectrometry. A commonly applied methodology to accurately measure trace gases in
Table 1. Characteristics of the various sample analysis techniques currently used in breathomics.

<table>
<thead>
<tr>
<th>Sample analysis technique</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatography combined with mass spectrometry</td>
<td>(1) Complete profile recognition</td>
<td>(1) Time-consuming</td>
</tr>
<tr>
<td></td>
<td>(2) Single VOC identification, thus linkage to underlying processes possible</td>
<td>(2) No real-time measurements possible</td>
</tr>
<tr>
<td></td>
<td>(3) High sensitivity</td>
<td>(3) No real-time measurements possible</td>
</tr>
<tr>
<td></td>
<td>(4) Sample time and place independent of measurement</td>
<td></td>
</tr>
<tr>
<td>Ion mobility spectrometry</td>
<td>(1) High sensitivity</td>
<td>(1) No complete profile recognition</td>
</tr>
<tr>
<td></td>
<td>(2) Fast (compared to GC-MS)</td>
<td>(2) Time-consuming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) No real-time measurements possible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4) Limited number of components detectable, no differentiation of isomeric and isobaric ions</td>
</tr>
<tr>
<td>Proton transfer reaction mass spectroscopy</td>
<td>(1) No need for pre-concentration</td>
<td>(1) No complete profile recognition</td>
</tr>
<tr>
<td></td>
<td>(2) Real-time measurement possible</td>
<td>(2) No single VOC identification</td>
</tr>
<tr>
<td></td>
<td>(3) High sensitivity</td>
<td>(3) Total VOC concentration measurable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&lt;10 ppmv)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selected ion flow tube mass spectrometry</td>
<td>(1) Real-time measurement possible</td>
<td>(1) No complete profile recognition</td>
</tr>
<tr>
<td></td>
<td>(2) High sensitivity (&lt;ppb)</td>
<td>(2) No single VOC identification</td>
</tr>
<tr>
<td></td>
<td>(3) Water vapor quantification possible</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Schematic diagram of GC-TOF-MS (based on [25]). Breath sample is first injected into the separation column of the gas chromatograph. Separated molecules inlet to the TOF-MS where first ionization occurs. The time necessary to travel from an ion source to a detector is measured. Ions in the flight tube separate into groups or packets according to velocity.

complex mixtures such as exhaled air is based on gas chromatography mass spectrometry (GC-MS) [25]. In this method, schematically depicted in figure 2, the sample is introduced in a GC that separates the different compounds in the mixture based on differential specific interactions of the compounds with the mobile phase and the lining of the capillary column material. After their transport through the column, the compounds are introduced into the MS to detect and also to identify the separated VOCs. Within the ion source of the MS, the compounds are ionized leading to molecular ions and characteristic fragment ions. Different detectors are used of which the time-of-flight (TOF) spectrometer is the most widely applied [22, 25, 26].

In general, the GC-MS technique is proven to be highly sensitive and robust, two characteristics known to add to a high degree of reproducibility [22, 25]. It has been shown that GC-MS analyses result in highly accurate chromatograms, which are called breathograms when measuring exhaled air samples with this technique [22, 24, 27]. Figure 3 shows a typical example of such a breathogram containing all exhaled VOCs. In contrast to the previously described detector–GC–MS combinations, GC-TOF-MS is capable of delivering full mass spectral information of all compounds [23, 25, 45]. In other words, the GC-TOF-MS is equipped to detect specific VOCs linked to exposure or disease status and at the same time is also able to identify these unique VOCs. Therefore, it has been suggested that GC-TOF-MS is to be preferred as an excellent screening tool for new biomarkers in the monitoring of exposures and diseases [22, 24, 28].

This major advantage outweighs the drawbacks of this method, including the fact that it is rather time-consuming, needs sample pre-concentration and cannot take place in situ [29]. The latter implies that real-time measurements cannot be performed, which makes this technique more useful for analyzing stable physiological parameters than for monitoring rapid changes in VOC concentrations due to, for instance, changes in heart rate or ventilation [29]. However, if fast and continuous on-line monitoring is not required, the disadvantages of not measuring in situ and pre-concentrating the samples can easily be overcome by transporting the exhaled samples onto stainless steel two-bed sorption tubes, as they are shown to trap, concentrate and stabilize VOCs for at least 1 year [24]. Moreover, these disadvantages can turn into an advantage: in this case, the off-line sampling and storage on thermal desorption tubes means that samples from various locations and collected during a longer period of time can be measured on one instrument in a short period of time, thereby eliminating a good deal of instrumental variation.
2.2.2. Ion mobility spectrometry. Ion mobility spectrometry, or IMS, is an instrumental analytical method that is similar to TOF-MS. The IMS technique, schematically depicted in figure 4, separates (molecular) ions according to their mobility as they move through a so-called drift tube filled with a purified gas such as air or nitrogen [25, 30].

As the different ions present in the sample are forced through the tube by means of an applied electric field, they will display different velocities based on their characteristics. Separating the ions can further be optimized by changing the drift length, drift gas, electric field strength, temperature and pressure [25, 30]. Combining this technique with GC improves its applicability.

2.2.3. Proton transfer reaction mass spectroscopy. Determination of the content of complex gas mixtures such as breath with proton transfer reaction mass spectrometry (PTR-MS) is based on chemical ionization of the target molecules by proton transfer reactions (figure 5) [31, 32]. Upon their chemical ionization by proton transfer reactions with H$_3$O$^+$ primary ions, the protonated molecules are accelerated followed by detection using an inline MS [33].

Advantages of this technique include the fact that samples can be readily analyzed as there is no pre-concentrating step or separation processes involved as is often the case for other analysis techniques [22]. Moreover, combining this ready-to-use sample analysis with very fast response times (in the order of 100 ms) even enables real-time measurement of samples with PTR-MS, which is of special importance in situations where rapid and sudden changes of VOC concentrations are expected [29]. Additionally, this method may display a slightly enhanced sensitivity due to a minimal fragmentation degree of the molecular ions compared to that observed in other analytical techniques [22, 32]. Unfortunately, PTR-MS is also a very selective technique that measures only a limited number of volatile compounds, as it can only detect compounds with a proton affinity higher than that of water [33, 34]. Moreover, PTR-MS cannot differentiate isomic and isobaric ions since they are all detected at the same nominal mass. Consequently, it can only be used to analyze a relatively small fraction of the total VOC profile present in breath and thus not to define a total breath-print. Moreover, chemical identification of the detected ions, required to pin down specific VOCs and/or underlying metabolic processes to exposure or disease status, remains very difficult since no fragmentation is detected. Finally, this method is also not applicable for rather concentrated samples as the total VOC concentration that can be analyzed should not exceed 10 parts per million per volume (ppmv).

2.2.4. Selected ion flow tube mass spectrometry. Selected ion flow tube mass spectrometry (SIFT-MS) is an analytical technique for the simultaneous real-time quantification of several gases (figure 6). This method is based on the formation of reactant ions (precursors) by electron impact or microwave discharge in a carrier gas in a separate ionization region. In short, the exhaled gases are led through a flow
Figure 5. Schematic representation of the PTR-MS (based on [31]). Reagent ions (H$_3$O$^+$) are created by the hollow cathode using water vapor. Next in the drift tube the proton trace reactions in moist air occur between H$_3$O$^+$ and any molecule whose proton affinity exceeds that of water. The ions then reach the detector. Abbreviations: N stands for neutral gas species while SEM for secondary electron multiplier.

Figure 6. Schematic diagram of the selected ion flow MS (based on [25]). Reagent ions (e.g. H$_3$O$^+$ or NO$^+$) are produced by moist atmospheric air and corona discharge (microwave gas discharge ion source). The reagent ions are selectively separated from other ions by the first MS. The selected ions react with molecules coming from the breath sample. The second MS is used as a detector.
desorbed, separated by GC (ThermoFisher Scientific, Austin, Texas, USA) employing an RTX-5ms capillary column (30 m × 0.25 mm, 5% diphenyl, 95% dimethylsiloxane capillary, film thickness 1.0 μm) and detected by TOF-MS (Thermo Electron Tempus Plus time-of-flight mass spectrometer, ThermoFisher Scientific, Austin, Texas, USA).

We have validated our approach regarding sampling reproducibility and intra- and inter-individual variation in exhaled VOCs [24]. Additionally, we have validated the instrumental reproducibility by analyzing two identical samples and determining the degree of similarity between the two measurements. A global chromatographic comparison correlation factor was applied presenting scores ranging from 0.96 to 0.99. A value of ‘1’ denotes identical samples and the lower the value the lesser the degree of similarity, implicating that our instrumental reproducibility was of high degree. The intra-individual variability, as determined by sampling the same subjects over a 1 week period, demonstrated larger variations (ranging from 0.80 to 0.99) and, as expected, the variability between different subjects appeared to be higher (ranging from 0.16–0.98). Usually four to five exhalations prove sufficient to inflate the bag. To explore the effect of different exhalation patterns on VOC profiles, volunteers were asked to inflate one bag by superficial exhalation and another bag through deep inspiration, i.e. a 5 s breath hold and subsequent total exhalation into the bag [24]. None of the detected compounds proved to be significantly affected by the exhalation characteristics (corrected for multiple testing using Bonferroni correction). Moreover, the intra-individual similarity between chromatograms was very high (degree of similarity above 0.95) proving that differences in exhalation patterns did not lead to different VOC profiles within an individual [24].

2.3. Data (pre)processing and analysis of GC-MS raw output

As an example, we will discuss how data generated by GC-MS have to be preprocessed properly in order to obtain more comparable data that can subsequently be used for data analysis. Similar to other analytical platforms, breathomic data typically involve a number of preprocessing steps including noise reduction, background (baseline) correction, alignment and normalization [4, 21]. If the preprocessing steps on raw GC-MS are done correctly, the chromatograms obtained from different measurements are transformed into such a format that they can be compared with each other. This is visually exemplified in chromatograms obtained by GC-TOF-MS before and after preprocessing (namely denoising, baseline correction and alignment) (figure 7).

2.3.1. Background corrections. Within breathomics, a correction for background VOC concentrations in inspiratory air should be made if absolute VOC concentrations or VOCs displaying inspired concentrations that are higher than 5% of expired ones are the endpoints. Such a background correction can be applied by correcting exhaled VOC concentrations for the inspiratory levels of these compounds or by calculating the so-called alveolar gradients in which negative and positive values are generated [4, 15, 24]. However, these correction methods do not include the complexity of pulmonary adsorption and exhalation of VOCs, which display a large inter-individual variability due to, for instance, the ventilation/perfusion rate of the lung that may be altered in many pulmonary pathologies [4, 57, 58]. Consequently, such subtraction methods are difficult to interpret and even lead to artificially generated statistical significance. The overall effect of one-dimensionally subtracted chemical background correction will be hampered due to introduction of more confounding factors, expressing presumable effects on breath, without being corrected for. In contrast to this background correction, the variation in the composition of background
air is expected to be randomly distributed between samples from various subject groups, implying that it will not exert any discriminatory power or interfere with the outcome of the analyses [24]. Additionally, one can argue that other possible confounding factors such as diet, exercise, smoking, age and whereabouts all comprise a certain influence on the VOC composition of breath for which it is difficult, if not impossible, to correct. Also in those cases, in analogy with the background air, potential confounding factors such as diet will be randomly distributed in the population. We believe that there is no reason to expect a different diet for people with or without a certain illness (except when the diet is adapted to the disease) and, as a result, putative confounding factors will not be selected in classification models. Consequently, although there still is no consensus regarding this issue in the literature, it is our opinion that in most set-ups there is no need to apply corrections for influencing variables such as diet, smoking behavior or background air. Indeed, we believe that the current advantages of the easy and non-invasive nature of sampling will be minimized if patients were to be sober, smoke free or resting for a certain period of time before the measurement or inhale through a VOC-filter in order to avoid or minimize the influence of these confounding factors.

2.3.2. Data (pre)processing. In order to minimize the degree of high frequency noise generated by the detector or other instrumental noise introducers, data need to be filtered prior to statistical analysis.

Consequently, the first step of data (pre)processing usually concerns denoising. Techniques for denoising and filtering are applied in order to reduce the influence of the random variation. The application of transform functions, e.g. wavelets or Fourier transformations, allows reduction of data noise by an alternative representation of data. The original data are represented as a combination of a set of basis functions multiplied by transform coefficients. An overview of different denoising methods is given by Trygg et al [37].

The second step of data (pre)processing involves background correction. Proper background removal is important, since deformation in background may affect not only data analysis but also alignment and quantification. Different methods are available, e.g. asymmetric least squares (ALS) [38], B-splines, B-splines with penalization (i.e. P-splines) [39] or the use of orthogonal basis of the background spectra [40]. ALS uses the penalties least-squares approach to estimate baseline. B-splines are local basis functions, consisting of a number of polynomial pieces of a low degree connected in a special way, which are next used for baseline correction.

The third and last step of (pre)processing includes alignment, i.e. aligning the peaks across all samples in order to be able to perform a robust data analysis. The variations in peak position are mostly caused by column ageing, temperature differences and different sample composition. Misalignments may influence both the data analysis and the compound identification. Currently there are many methods and packages that are commercially or freely available for spectral alignment, namely dynamic time warping (the oldest warping technique), parametric time warping [41], correlation optimized warping (COW) [42], MetAlign [43, 44] and MZmine [45]. The most commonly used alignment techniques and methods are summarized by Jellema [46].

After data (pre)processing, the identification (or assignment) of VOC compounds can be performed. Upon decreasing the signals due to background and increasing the visibility of the signals of interest, the peak position and the occurrence of each compound have to be determined. This can best be done by analyzing the area under the curve (AUC) for each peak, as every peak represents—at least—one compound with an AUC that is related to the compound quantity(ies). Parameters such as retention time (RT) and mass spectrum are used to identify the compounds of interest and to build a database in which all compounds from all subjects are combined [24]. In order to find the specific patterns, these compounds can subsequently be analyzed by means of different statistical techniques.

2.3.3. Statistical analysis. Nowadays, the hunt for clinically relevant biomarkers is driven by the fast-paced technological advancements in high quality sensors and high throughput analyses combined with the current advances in bio-informatics and biostatistics. Additionally, the recent evolvement of data mining strategies and powerful computers enables researchers to handle the very large data sets acquired with for example breathomics. Indeed, as breathomics implies a relatively high number of interesting compounds compared to the number of subjects studied, advanced data mining routines are crucial in extracting only that information that is relevant to study possible biomarkers in the exhaled air.

In general, multivariate analysis or different data mining strategies can be applied to extract relevant information from breathomic data. It is important to mention that a success to proper statistical analysis is to have a good statistical validation as well as trustworthy biological interpretation of the results. The validation issue will be discussed later in this section. Typically, most of the statistical methods used in other omics-related investigations can also be utilized with respect to breathomic data.

As the first step in data analysis, unsupervised methods including principal component analysis (PCA) [47, 48] and hierarchical cluster analysis (HCA) [48] are performed. The main objective of these unsupervised methods is data exploration by visualization, outliers’ detection and identification of grouping trends.

PCA provides a reduced representation of the breathomic data by creating new factors, the so-called principal components (PCs), which are linear combination of original variables. PCs are constructed in such a way to express most of the variance within a data set and more importantly each consecutive PC explains the maximum amount of variance, but which was not described by the previous PCs. PCs are orthogonal and therefore independent of each other. PCA is also used for outliers’ detection. One has to keep in mind that PCA can detect only certain type of outliers, i.e. the so-called good leverage objects, while orthogonal outliers cannot be...
detected. Therefore for outliers’ detection robust version of PCA should be preferably used [49].

Another popular unsupervised method to analyze multivariate data is HCA [50]. In HCA, samples (i.e. individual chromatograms) are grouped according to their multivariate similarity. The measure of multivariate similarity is based on different types of distances (e.g. Euclidian, Minkowski or Mahalanobis distance) [50]. The calculated multivariate similarity can be represented as a tree called dendrogram. By deciding the similarity cut-off, which divides the dendrogram into separate clusters, HCA can be used for classification. However, the main disadvantage of HCA is that it does not deliver the information about the variables which are responsible for the certain clustering. Several applications of PCA and HCA to study VOC profiles can be found. Recently PCA was employed to analyze the VOC profile of patients with malignant pleural mesothelioma [51] and patients with lung cancer [52], while HCA was applied to validate the results obtained from discriminant analysis performed on expired air samples from healthy humans, fasting people, headspace air of urban waste disposal bins, air of bags with decaying human bodies and urban air samples [53].

PCA and HCA are very popular and powerful methods for data exploration; however since they belong to unsupervised techniques they do not use additional information, such as class information. Therefore they are unable to define a specific pattern capable of identifying a given class e.g. the disease patients. Moreover, due to highly complex data, unsupervised data are very often not sufficient enough for extracting class-related information (e.g. disease related). Therefore unsupervised analysis is very often followed by supervised analysis, where different classification techniques can be used. In the field of pattern recognition several linear and nonlinear techniques for data classification are available, for instance linear discriminant analysis (LDA) [50, 54], partial least-squares discriminant analysis (PLS-DA) [55], random forests and kernel-based methods [56, 57]. The first method, namely LDA, is fast, powerful and does not require any parameter tuning. However, it needs special adaptation before applying it to breathomic data, since it can only be directly used when the number of samples is much bigger than the number of variables. A possible solution is to use data compression by means of PCA (PCDA) beforehand. Then the number of PCs has to be optimized. The disadvantage of using PCA is the possibility of missing some class-related information. The use of LDA and several of its adaptations for breathomic data are described earlier [58–61]. PLS-DA similarly to PCA is a latent variable technique, but it uses a different set of new components calculated in order to maximize the covariance between spectra and the class information. Its ability to cope with collinear data explains the success of PLS-DA in many scientific fields [62]. However, a very important aspect of using PLS-DA is proper model optimization (i.e. number of latent variables) to avoid overfitting [63]. In the field of breathomics, Caldeira et al demonstrated the study where PLS-DA discriminated with a classification rate of 88% healthy from allergic asthmatic children [64].

The above described approaches belong to linear methods and make the assumption of a linear response. However, very often breathomic data display nonlinear parameter dependences. In such a situation the linear methods are bound to fail. Among many nonlinear techniques that have been developed in machine learning and pattern recognition area, we will discuss random forests and two kernel-based models, i.e. support vector machines (SVMs) and kernel-PLS-DA (K-PLS-DA).

The random forest method is an extension of decision trees [65]. In this technique bootstrap samples [66] are drawn with replacement from the training data, and trees using randomly selected subset of variables at each node are obtained for each bootstrap sample. Two tuning parameters, namely the number of trees and the number of random variables being used at each node, are crucial for proper accuracy and computational costs. Breiman has shown that using default values for the number of trees and the number of random variables gives an overall good outcome [65]. The random forest technique provides not only the predicted value for each sample but also variable ranking by computing a variable importance score for each input variable. Because of the averaging across different independent bootstrap samples and the low-bias nature of the trees, random forests tend to have the property to give low-bias predictions. Mazzone et al presented the application of random forests as a classification technique to VOC profiles of patients with different stages of lung cancer [67].

As their names indicate, kernel-based models (here K-PLS-DA and SVMs) involve a kernel transformation which maps objects into feature space. This results in obtaining a kernel matrix of size \( n \times n \), where \( n \) is the number of objects, i.e. measured samples. Kernel transformation has to be selected by a user. The obtained kernel matrix must be positive semi-definite (i.e. eigenvalues are positive) and nowadays there are many kernel functions that complete this assumption [68]. Unfortunately, the kernel transformation causes that the information about the original variables is lost. This means that direct interpretation of the kernel-based models is not possible. It is, however, possible to obtain variable ranking by applying recursive feature elimination [69]. This approach is implemented with SVM and ranks variables on the basis of the smallest change in a cost function minimized in the SVM algorithm. It is worthwhile to note that a recent proposal from Krooshof et al permits to trace back the influence of the original variables on the model even after the kernel transformation [70]. SVM and K-PLS-DA are both very powerful and popular binary classifiers [56, 71]. K-PLS-DA is beneficial in comparison to SVM in terms of simplicity and speed calculation. Moreover, K-PLS-DA has the possibility of visualization in the latent variable space. Nevertheless, K-PLS-DA and SVM perform similarly in terms of prediction and can thus be utilized interchangeably. While SVM has been employed to breathomic data [61, 72], a direct application of K-PLS-DA as a classification technique to VOC profiles has not been yet performed. However, Moorhead et al have shown a method utilizing kernel density estimate as a classification technique [73].

Breathomic analysis generates large numbers of variables (compounds) and such data do not fit to the assumption of having more samples ‘n’ than variables ‘p’. This bears
2.3.4. Customized data-acquisition and analysis. In our approach, we have automated the data-acquisition, and the analysis of the data output files from the GC-TOF-MS is performed in successive steps. In short, the first step is to perform peak detection and baseline corrections on all analysis output files. Normalization of the calculated peak areas is performed using an area scaling factor based on the cumulative area under the detected peaks; since all chromatograms display rather similar profiles this method of normalization proved most robust. RT of all samples are corrected for chromatographic drifting. Applying the correction for RT is very effective and easy to perform. We do not use an added internal standard; instead already available compounds that demonstrate availability in a large number of samples are used, adding to the straight-forwardness and robustness of the methodology. Finally, the output files are merged by combining corresponding compounds based on RT and on similarity of the corresponding mass spectra. The degree of mass spectra similarity is calculated using a match factor (MF) based on the similarity index as described by Stein et al [75]. These MFs are only determined for compounds within a selectable RT-window. MF-threshold values are determined based on a variety of complementary compounds manually combined. To determine which compounds in the database are of interest regarding the classification of diseased versus controls, we have applied SVMs because of their ability to select those compounds that provide the best performance as implemented into a classifier and to construct predictive models with large generalization power even in the case of large dimensionality of the data or when the number of observations available for training is low [76].

3. Application possibilities

3.1. In vitro mechanistic applications

Until now, the utility of breathomics to examine ongoing physiologic and/or pathologic processes in vitro has hardly been explored. However, analyzing VOCs excreted by in vitro systems mimicking these processes might provide interesting and useful information that can be extrapolated to various in vivo clinical settings. There are already a few reports available demonstrating the usefulness of breathomics with regard to studying specific processes including oxidative stress, bacterial inflammation and carcinogenesis [12, 26, 77].
Figure 9. Process of lipid peroxidation, started by the radical R. A ROS abstracts a hydrogen atom from the lipid, thereby producing a fatty acid radical. This phase is followed by a process called propagation in which the previously generated fatty acid radical almost instantly reacts with molecular oxygen. This reaction creates a peroxyl radical that is capable of forming a lipid hydroperoxide as well as a new lipid radical by subtracting a hydrogen atom from a second PUFA. In this way, a propagating chain reaction will be generated that continuously passes an unpaired electron from one PUFA to another one, thereby giving rise to more and more fatty acid radicals. This cycle continues until a termination reaction occurs that will lower the overall reactivity of the mechanism. Examples of such a termination reaction are (i) the consumption of one of the two reactants, (ii) the formation of a relatively unreactive radical or (iii) the reaction between two reactive radicals that produces a non-radical.

Volatile products formed during lipid peroxidation include ethane, pentane, hexanal, octanal, nonanal, propanol and butanol [88, 89]. Ethane was one of the first VOCs demonstrated not only to be measurable in exhaled air but also to be elevated in the breath of COPD patients compared to controls [13, 104]. Moreover, a correlation between ethane levels and the degree of airway obstruction, smoking habits and FEV1 could be observed [12]. However, the analysis of single compounds deriving from lipid peroxidation in exhaled air is currently still hampered by a relatively low sensitivity and specificity. Consequently, it might be of interest to investigate the availability and importance of these compounds as markers of oxidative stress by measuring them in the headspace of in vitro systems mimicking this process. Unfortunately, this exciting new field of applying breathomics has, to our knowledge, not been addressed thus far.

3.1.2. VOCs and inflammation. An important feature of most chronic diseases is the occurrence of inflammation, a process designed to recognize, attack and kill invading microorganisms [90]. Interestingly, inflammation and ROS are tightly intertwined: on the one hand, inflammatory mediators and activated inflammatory cells released into the circulation can induce ROS generation whereas on the other hand ROS can stimulate pro-inflammatory signaling via activation of for example the nuclear factor kappa B (NF-κB) pathway [86, 90]. Consequently, it is not surprising that VOCs characteristic of inflammatory processes are often compounds generated during processes induced by excessive ROS production such as lipid peroxidation (see section 3.1.1). Indeed, it has been shown that among the variety of VOCs produced by micro-organisms various fatty acids and their derivatives including hydrocarbons, alcohols and ketones are found [77]. Additionally, inflammation has also been associated with typical inflammatory markers such as nitric oxide, nitrate and nitrogen- and sulfur-containing VOCs [77].

Aside from detecting VOCs characteristic of the underlying damaging processes of oxidative stress and inflammation, breathomics can also be applied to examine volatile compounds produced by specific species or strains of bacteria. Indeed, VOCs found in infections are not only host-derived but are also often related to metabolic processes occurring in the infectious organism. It is known that all organisms generate VOCs as part of their normal metabolism and certain infections are longtime known to be accompanied by a distinct smell in vivo as well as in vitro [89, 106]. Recent studies have shown the usefulness of VOC analysis instead of the current automated blood culture system with regard to evaluating bacterial growth in human samples or in vitro cultures [91, 92]. To this end, blood samples from healthy donors were in vitro infected with five bacterial strains commonly involved in bacteremia, i.e. Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli and Neisseria meningitides. Next, the headspace air of these samples was collected and analyzed for growth and species identification by means of SIFT-MS. Positive results were recorded for 88.3% and 96.6% of the cultures at respectively 8 and 24 h, indicating that VOC profiling is indeed a sensitive method for bacterial detection [92]. The presence of significant distinctions between both the presence and relative concentrations of individual VOCs for each of the
test species allowed bacterial identification as well. Moreover, as co-incubating E. coli and S. aureus with antibiotics resulted in a decreased VOC production, VOC analysis might also be useful for the determination of antibiotic susceptibility [91].

Ultimately, applying VOC profiling in vitro to quickly identify micro-organisms encloses the potential of developing a marker of the presence or absence of specific microbial growth in both environmental and clinical settings [93]. Additionally, based on the reports that VOCs can accurately identify bacterial species within a shorter timeframe than the existing traditional culture methods, VOC analysis might also be used as a diagnostic tool [91, 93]. Characterization of different microbial infections by means of VOC production can be performed by analyzing either individual VOCs or unique VOC profiles. Until now, various individual VOCs have already been identified for specific bacterial species. For instance, indole production is reported to be elevated in E. coli cultures [93] whereas dimethyl sulfide and ammonia are increasingly produced by S. aureus [91]. Moreover, headspace air analysis of P. aeruginosa cultures revealed high levels of hydrogen cyanide gas (HCN) [109]. Indeed, the HCN concentration was significantly higher above P. aeruginosa cultures than above other bacterial growth and was proven to be a sensitive (68%) and very specific (100%) biomarker of this micro-organism [94]. Interestingly, HCN was also shown to be elevated in the headspace of another gram-negative bacteria, namely the Helicobacter pylori reference strain NCTC 11637 [95], suggesting that single VOCs do not possess enough discriminative power to distinguish between specific species and/or strains of micro-organisms. Indeed, the production of specific single VOCs is not necessarily limited to one bacterium and therefore it can be anticipated that VOC profiling will be a more effective approach to discriminate between a wide range of microbial species. The usefulness of this approach has recently been shown by Thorn et al as their study revealed that significant differences can be measured in VOC profiles obtained from various bacterial cultures in vitro [93].

3.1.3. VOCs and carcinogenesis. An intriguing application of breathomics comprises the analysis of VOCs for cancer screening and disease monitoring. Although it is generally known that cancer survival rates largely depend on early detection, preferably in a stage where no metastasis has yet occurred, such early diagnosis is often not the case in clinical practice [96]. For instance, diagnosing lung cancer in an early stage is still relatively difficult, occurring in approximately 25% of all cases [111, 112]. Consequently, there is an urgent need for innovative, early biomarkers that can be used for detecting the presence and observing the course of various forms of cancer, including lung cancer. In this context, breath analysis has already been suggested and tested as a potential method.

Until now, most research regarding VOCs and carcinogenesis has focused on their possible relationship in vivo in lung cancer patients. As described in more detail in section 3.2, several groups have demonstrated that a combination of different VOCs can differentiate lung cancer patients from healthy controls [26, 97–101]. Besides discrimination based on profiles, the usefulness of single VOCs as possible biomarkers has also been shown in lung cancer [118–121]). Interestingly, most of these studies have revealed that VOC concentrations can be increased or decreased depending on the specific compound and clinical status in question, which makes it rather difficult to extrapolate these findings to biomarkers suitable for general use. Indeed, it can be anticipated that the VOC profile excreted by a given tumor will change due to various factors including staging, the ratio between untransformed and malignant cells and the presence of immune cells or even pathogens [102, 103]. Therefore, validation of appropriate biomarkers should ideally be performed in vitro on cellular and molecular origin [103]. However, to our knowledge only one study has examined the correlation between in vivo exhaled and in vitro excreted VOCs thus far. The group of Chen et al has studied the VOCs excreted by lung cancer patients and compared them with those emitted by four different types of lung cancer cells (i.e. squamous cell, bronchioloalveolar, non-small cell and adenocarcinoma) isolated from lung tissue from cancer patients [122]. The VOC profiles of the in vitro samples were not only markedly different from those excreted by medium controls but also displayed four universal VOCs, which could be regarded as metabolic products of lung cancer cells and thus as biomarkers of lung cancer at cellular level. Interestingly, two of these general VOCs (isoprene and undecane) could also be detected in the in vivo samples as well, underlining the usefulness of in vitro VOC analysis in the search for new early biomarkers [122]. However, the fact that the in vivo exhaled VOCs also revealed multiple differences with those excreted by the lung cancer cells in vitro indicates that more research regarding the correlation and possible extrapolation between these two approaches is mandatory.

Over the last few years, a handful of studies have been performed to detect and identify tumor-derived VOCs in vitro. To our knowledge, these studies were merely focused on volatile compounds excreted by various lung-tumor-derived cell lines. Overall, it can be stated that the in vitro VOC studies have revealed the same pattern as their in vivo counterparts, i.e. increases or decreases in VOC levels depending on the studied compound and cell line [102, 104–108]. For instance, in the headspace of the human non-small cell lung cancer cell-line CALU-1, increased concentrations of 2,3,3-trimethylpentane, 2,3,5-trimethylhexane, 2,4-dimethylheptane and 4-methyloctane were observed, whereas other compounds including acetaldehyde, acrolein, 2-butane and hexanal showed decreased levels [106]. Employing VOC profiling to another non-small lung cancer cell line, i.e. NCI-H1666 derived from a broncho-alveolar carcinoma, also revealed the consumption of several aldehydes as well as of two ethers and n-butyl acetate. Interestingly, no unequivocal release of VOCs was observed [103]. Yet another cell line, the NCI-H2087 derived from a metastatic lung adenocarcinoma, showed lower levels of acetaldehyde as well as of 2-methylpropanal, 2- and 3-methylbutanal and butyl acetate [108]. This cell type also revealed a significantly increased concentration...
cells, suggesting a lower production and branched hydrocarbons and alcohols were seen increased cells [105]. Perhaps the most striking observation was that observed to be preferentially associated with the transformed primary bronchial epithelial cells, different VOC groups were epithelial cell line A549, with those excreted by untransformed cell line, i.e. the adenocarcinomic human alveolar basal VOC concentrations in the headspace of another lung cancer of important biomarker characteristics.

**Table 2.** Testing the usefulness of VOCs as biomarker on the basis of important biomarker characteristics.

<table>
<thead>
<tr>
<th>Biomarker characteristic</th>
<th>Applying to VOCs</th>
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<tr>
<td>High sensitivity and specificity</td>
<td>++</td>
</tr>
<tr>
<td>Fast and accurate</td>
<td>+</td>
</tr>
<tr>
<td>Methodological simplicity</td>
<td>++</td>
</tr>
<tr>
<td>Interpretive simplicity</td>
<td>+/-</td>
</tr>
<tr>
<td>Thorough validation</td>
<td>+/-</td>
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<tr>
<td>(Patho)physiological link to either disease or exposure</td>
<td>+/-</td>
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3.2. In vivo diagnostic applications

Over the last few decades, the analysis of exhaled air as non-invasive and fast diagnostic application for a variety of diseases has gained mounting interest. As various VOCs present in exhaled air are derived from physiological as well as pathological processes, they can be used as potential predictive biomarkers of both exposure and disease. However, before they can actually be used as such, diagnostic VOC profiles have to be proven to exhibit relevant distinctive characteristics (table 2).

At first, in order to ensure a fast and accurate methodology as is required within diagnostics, biomarkers should demonstrate a high sensitivity and specificity combined with a short processing time. Additionally, the actual analyzing procedure should be cost effective and ideally display low appliance and operating costs [109]. With regard to volatile biomarkers, current advances in both sampling and analyzing techniques have enabled fast breath sampling, although the translation from the existing procedures to a ready-to-use clinical device has not taken place yet. Moreover, recent studies have proven that distinct VOC profiles can discriminate healthy controls from patients suffering from various diseases with a high sensitivity and specificity (a.o. [4, 27, 110–114]). Indeed, we have shown that a profile of only eight VOCs can distinguish asthmatic children from healthy controls with a sensitivity of 89% and a specificity of 95% [110]. The compounds of interest were identified as: (branched) hydrocarbon (C_{13}H_{28}), carbon disulfide, 1-penten-2-one, butanoic acid, 3-(1-methylethyl)-benzene, unsaturated hydrocarbon (C_{15}H_{26}), benzoic acid [110]. For COPD, we were able to achieve a correct classification of 91% with 100% sensitivity and 81% specificity using a profile of only six distinct VOCs [24]. The compounds of interest were identified as: isoprene; an unidentified C_{16}-hydrocarbon; 4,7-dimethyl-undecane; 2,6-dimethyl-heptane; 4-methyl-octane and hexadecane. Additionally, the group of Basanta et al also demonstrated that a specific VOC profile could differentiate COPD smokers from asymptomatic smokers with 88% sensitivity and 81% specificity [112]. With regard to CF, we could correctly classify 92% of healthy and diseased subjects with high sensitivity and specificity using only ten VOCs [130]. Several other groups have revealed that a combination of VOCs can differentiate patients with lung cancer from the control group (consisting of either healthy controls, disease controls or subjects at risk) with a sensitivity and specificity ranging from respectively 71–100% and 80–100% [113, 116, 117, 119, 121, 133, 134]. Moreover, it has recently been observed that exhaled VOC markers are sensitive enough to distinguish patients with pulmonary tuberculosis from healthy controls with 95.7% sensitivity and 78.9% specificity [114]. Interestingly, pilot studies have revealed that breathomics can also be used to diagnose non-pulmonary diseases, including breast, ovarian and hepatocellular cancer and coronary heart diseases, with relatively good sensitivity and specificity [115–121]. Moreover, breathomics has recently been shown to be useful in assessing occupational or environmental exposures [122, 123].

Additionally, a biomarker should excel in simplicity in both a methodological and an interpretive way. Methodologically, breathomics already fits the biomarker prerequisite of displaying a rather non-invasive character that combines a low degree of discomfort for the patient and a high clinical accessibility [124, 125]. Furthermore, most applied methodologies already combine a high reproducibility with a low instrumental variability. However, at the interpretive level, useful biomarkers are expected to generate easy-to-interpret results that leave little room for interpretation. As described in section 2.3.3, the current lack of consensus regarding breath sampling, analyzing and normalizing still hampers correct validation and thus clinical usefulness of volatile biomarkers [125].

Biomarkers should be thoroughly validated before being clinically applied to determine the real value of their predictive and/or discriminatory capacities. By performing such a validation test in one or more separate validation set(s), the real value of specific biomarkers can be evaluated outside the test set in which they were initially selected [126]. This approach has already been successfully applied within breathomics, demonstrating the usefulness of specific VOC patterns as selective biomarkers for various diseases including COPD and asthma [45, 129].
Finally, a good biomarker displays a clear relationship with either the exposure or the disease it was designed for. Until now, this relationship has merely been the focus in studies exploring the use of individual VOCs to predict or classify various diseases. Indeed, especially products of lipid peroxidation, a damaging process described in section 3.1 that is highly associated with various chronic diseases, have been widely studied as possible volatile biomarkers. For instance, higher exhaled pentane levels are reported in patients with acute asthma [15], CF [13], acute respiratory distress syndrome [127], ventilator-associated pneumonia [128], obstructive sleep apnea [15] or lung cancer [120, 145]). Other volatile lipid peroxidation products studied as possible biomarker include ethane, hexanal, octanal, nonanal, propanol and butanol [14, 15, 118, 145]. Interestingly, more than half of the VOCs included in the discriminating profiles designed for asthma, CF and COPD by our group was identified as hydrocarbons and/or possible lipid peroxidation products as well. Moreover, due to their low solubility in blood, lipid peroxidation products such as ethane and pentane are exhaled within a few lung passages and can therefore serve not only as a pulmonary but also as a systemic marker of inflammation and oxidative stress [4, 146]. For instance, pentane and/or ethane are enhanced excreted in the breath of patients with sepsis, systemic inflammatory response syndrome, ischemic heart disease, myocard infarction, cardiopulmonary bypass and allograft rejection following organ transplantation [147–151].

Ideally, a biomarker should not only be linked to exposure or disease in general, but also to different stages of disease including development, severity and progression. Interestingly, this has already been shown for exhaled ethane as this VOC is higher not only in asthmatics compared to healthy controls but also in severe compared to mild asthma [13]. Moreover, a recent study by Philips et al suggests that VOC patterns can be used to distinguish between active and non-active pulmonary tuberculosis albeit with a relatively low accuracy ranging from 65% to 85% [129]. However, associating volatile biomarkers with the physiology of either exposure or disease is still in its infancy due to the fact that the (patho)physiological meaning of specific VOCs is often not known. Consequently, more research regarding the exact identity and biological role of individual VOCs is urgently needed. Unfortunately, this area of research is hampered by the fact that compounds originating in breath can very well be biochemically altered before their excretion, implying that their volatile appearance does not necessarily display a direct relation to exposure or disease. Consequently, more studies in clinical as well as in vitro settings are necessary to elucidate the biochemical origin, physiological meaning and exhalation kinetics of selected VOCs. Nevertheless, even without this knowledge, volatile compounds can already be valuable as a predictive tool in a clinical setting.

3.3. Potential future applications

Until now, breathomics has merely been applied for diagnostic purposes and it is therefore not surprising that the current focus within this exciting field of research mainly lies at developing easy-to-use devices for clinical use that can detect volatile biomarkers specific for various diseases. Interestingly, recent developments have been made regarding the screening for lung cancer as it was shown that low-dose CT scanning could significantly reduce lung cancer mortality [130, 131]. Since there are some difficulties in generalizing these results to the community, it has once again been suggested to combine this screening method with other new testing techniques including VOC measurement to develop a successful screening algorithm for lung cancer [130].

The analysis of exhaled air, however, encloses far more intriguing promises such as elucidating the (clinical and pathological) heterogeneity observed in several chronic diseases, studying the exact pathogen responsible for occurring (respiratory) infections or monitoring treatment efficacy.

3.3.1. Heterogeneity in diseases. Various chronic diseases have already been recognized as rather heterogeneous conditions, displaying different phenotypes based on variances in disease development and progression. These heterogeneous pathologies comprise pulmonary diseases, including COPD and asthma, as well as non-pulmonary conditions such as IBD, Crohn’s disease (CD) and ulcerative colitis (UC).

In COPD, significant heterogeneity of clinical presentation and disease progression leads to different phenotypes [153, 154]. Moreover, various pathologic conditions including chronic airway inflammation, bronchitis, emphysema and airway wall thickening have been reported in COPD with intra-patient differences [155, 156]. Although part of this heterogeneity might be explained by misdiagnosis, it most likely also reflects reversibility occurring in these patients. Consequently, phenotyping of COPD patients is needed to identify patient groups with unique prognostic or therapeutic characteristics, as this will provide information regarding underlying mechanisms, risk factors, natural history, monitoring and clinical outcome [132]. To this extent, the following new definition of a COPD phenotype has been introduced by Han et al: ‘a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes’ [133]. Up till now, defining such COPD phenotypes, by means of either radiography, physiology or (cellular or molecular) fingerprinting, represents one of the biggest challenges with respect to the management of this disease. Ultimately, this information could lead to treatments specifically targeted for defined phenotypic groups rather than for COPD in general as is currently the therapy standard [134].

Asthma can roughly be divided into mild, moderate and severe asthma. Interestingly, only the latter variant displays an urgent need for phenotyping as the other two forms respond to simple treatments and therefore do not require great subtlety [135]. Severe asthma, also known as refractory asthma, comprises less than 5% of the total asthma population but accounts for up to 50% of asthma-related health care costs as it is the only variant with poor medical control [136, 137]. Within the subgroup refractory asthma, a considerable inflammatory heterogeneity occurs as people suffering from this severe type of asthma display large differences in their eosinophil and
neutrophil population [138–140]. Interestingly, the different inflammatory phenotypes of refractory asthma present different clinical outcomes as eosinophilic inflammation favors severe exacerbations whereas increased neutrophil counts are correlated with fixed airway obstruction [136]. Moreover, these phenotypes also influence treatment efficacy as the eosinophilic variant can still effectively be treated by increased corticoid doses while its neutrophilic counterpart is rather corticoid-insensitive. Determining the appropriate phenotype of refractory asthma may contribute to earlier treatment with steroid alternatives such as macrolide antibiotics, tumor necrosis factor-α inhibitors, cytokine receptor antagonists and bronchial thermoplasty [141].

The two most important IBD are CD and UC, two chronic diseases of the gastrointestinal tract that are both characterized by flairs and remissions due to a global immune defect [142, 143]. The golden standard for diagnosing IBD in general and for differentiating between CD and UC in particular comprises histological evaluation performed during endoscopic procedures. As this approach is very time-consuming and highly invasive, alternative diagnostic markers that can be measured in the blood or stool are currently being developed [142, 144]. The development of such discriminating markers is of special importance as both UC and CD are highly heterogeneous and display a disease history and progression that are strikingly variable per patient [145, 146]. Indeed, there is an urgent need for non-invasive markers that can adequately (1) differentiate between CD and UC, (2) determine whether and when a relapse may occur once the disease is officially in remission state and (3) distinguish between the various IBD phenotypes that display different progression as well as diverse responses to treatment [145, 146].

Most heterogeneous chronic diseases, including the ones described above, are defined by damaging processes such as excessive inflammation and oxidative stress (described in section 3.2). Consequently, it is not surprising that specific VOC patterns characteristic of these processes are suggested to be useful non-invasive markers to discriminate between the various phenotypes of these diseases. Although it appears obvious to analyze these components in breath for pulmonary conditions, exhaled air analysis has also been used before to determine damage to intestinal organs, as occurs in IBD. Actually, in the 1990s it was already shown that tissue damage in UC could be detected by ROS-induced lipid peroxidation products measurable in breath [147]. Additionally, active IBD has already been shown to be correlated with increased levels of breath alkanes [148]. Due to the lack in specificity and sensitivity in breathomics back in the 1990s, these initial studies were never followed up in larger, clinical studies to examine whether the different IBD phenotypes can indeed be successfully diagnosed using VOC profiling. Interestingly enough, breathomics has also not been utilized yet in discriminating between various phenotypes of pulmonary diseases such as COPD and asthma, even though the literature suggests that differentiation could be performed based on different levels of inflammatory or oxidant-derived products.

In summary, heterogeneous diseases are characterized by pathological and clinical characteristics, ultimately leading to various phenotypes that display different reactions toward therapy and thus variation in clinical outcome. Consequently, accurately differentiating the various phenotypes underlying the heterogeneous pathology of interest by means of VOC profiling will largely contribute to optimizing treatment based on personalized medicine.

3.3.2. Respiratory infections. The occurrence of detrimental respiratory tract infections embodies a great burden not only for patients and our health care system but also for our economy [149, 150]. In particular the sudden worsening of symptoms, also referred to as acute exacerbations (AE), in various chronic lung diseases including COPD and CF is known to largely depend on such microbial infections. In general, AE are associated with poor quality of life as well as with increased morbidity, mortality and health care costs [149–152]. Among the factors influencing the occurrence of AE are age, gender, lung function, history of previous exacerbations and the use of inhaled corticoids. Additionally, although the exact cause of AE is still unknown, they are associated with pulmonary and systemic inflammation due to a change of the pulmonary microbial balance [153, 154].

AE require hospital admission where treatment often consists of intravenous antibiotics [151, 155]. In general, broad-spectrum antibiotics are used since the exact microbial cause of an exacerbation is often not detectable. Moreover, accurate and fast treatment of exacerbations is also hampered by a poor standardization of both the definition and detection of AE in most lung diseases [156, 157]. Recognizing AE appears to be difficult for both the patients and care takers as exacerbation profiles vary enormously between individuals. Current diagnostics include clinical criteria such as changes in symptoms and lung function but they often occur when an exacerbation is already manifest. Additionally, sputum induction or bronchoscopy accompanied by sampling broncho-alveolar lavage fluid can be applied for quantitative laboratory microbiological analysis in order to detect the exact microbial cause [158–160]. However, the usefulness of these techniques is seriously hampered by their invasiveness but mostly by their long duration of more than 48 h before results become available. In other words, to date it is still not possible to predict or quickly diagnose the occurrence and microbial cause of an exacerbation and thus prevent severe lung damage and deterioration on the long term. Therefore, there is an urgent need for non-invasive fast diagnostic tools that will contribute to a rapid diagnosis of AE in general and of the underlying micro-organism(s) in particular, thereby leading to an earlier start of appropriate antibiotic therapy and a more favorable health outcome. The analysis of exhaled air, combined with the adequate interpretation of selected VOC levels, might provide such a new and promising diagnostic tool for early identification and closer monitoring of patients with high risk profiles for AE. Indeed, monitoring high risk patients and early identifying the worsening of pulmonary symptoms might allow adequate therapeutic interventions with oral antibiotics and/or physiotherapy to prevent AE and thus improve the quality of life in these patients [161, 162].
3.3.3. Treatment efficacy. Once VOC profiling has been proven to be a successful diagnostic tool for specific chronic diseases, their various phenotypes and/or underlying pathogens, it can be anticipated that VOC patterns can also be used to monitor disease progression and therapy efficacy. Until now, treatment effects in several chronic lung diseases including asthma and COPD have only been monitored using clinical symptoms such as lung function [163, 164]. However, these symptoms only partially correlate with, and are thus not completely representative for, the underlying damaging processes including oxidative stress and inflammation. Consequently, within the fields of guiding treatment strategies and predicting therapy outcome, novel and more accurate biomarkers are needed [164]. Until now, the development of these new biomarkers has mainly been focused on inflammatory markers such as the presence of specific inflammatory cell-types [165, 166], the identification of inflammatory mediators [167, 168] or the detection of the fraction of nitric oxide in exhaled breath (FeNO) [169, 170]. However, none of these novel inflammatory biomarkers has been proven adequate to use in routine clinical practice due to several pitfalls and shortcomings (extensively reviewed in [164]). Therefore, VOC profiling has recently been suggested as a new and more successful approach to monitor disease management and treatment efficacy. It can be anticipated that the detection of disease-specific VOCs in large study populations will ultimately enable monitoring therapy effectiveness by measuring breathograms.

4. Summary and perspective

Whether novel biomarkers provide useful information for disease diagnosing, monitoring or risk prediction has been the focus of intense study over the last years [171, 172]. Proving the usefulness of such new biomarkers, however, has been hampered by a variety of factors including inadequate statistical power, the lack of measures such as calibration and reclassification and the lack of external validation [173, 174]. It can, on the other hand, also be argued that the proven successful application of such new biomarkers in some studies has been overestimated due to the use of homogeneous sample populations or inappropriate endpoints.

An intriguing point of discussion regarding the usefulness of novel biomarkers is the importance given to their statistical significance. In other words, statistically significant changes in predicted probabilities might not be relevant to clinical care, whereas reclassification of individuals into new, clinically relevant risk categories based on the application of new biomarkers is. Therefore, breathomics might be the new tool that provides the means to differentiate on a physiological basis between clinically related disease profiles or sub-stages. Interestingly, it has already been suggested that exhaled air analysis might be extra valuable in clinical practice due to its superior accuracy compared to the reported sensitivity and specificity of current methodologies [4].

In conclusion, it can be stated that breath analysis holds the promise to be of huge interest in clinical practice. It has already been proven that in some disease profiles, breathomics can generate quality biomarkers and thus serve as a sensitive and non-invasive methodology. However large (prospective) cohort studies are necessary in order to validate such selected biomarkers linked to different disease profiles. Future research regarding more specific and highly sensitive sensors will provide the means for these biomarkers to be highly cost effective and very simple to use [175, 176].

At present, a wide range of sensors is under research including metal-oxide sensors and polymer-based sensors. Both sensors are capable of registering the absorption of specific VOCs by means of either resistance or acoustic variations. At present, such sensors are already capable of accurately detecting low concentrations of volatiles as well as other techniques and are under development to be used as bedside tests. At the same time, parallel approaches comprise the detection of disease-related VOCs by means of miniaturized GCs in line with highly sensitive and specific sensors such as the MS. Improved sensitivity and specificity followed by implementation of these technologies into small ‘fool-proof’ handheld devices, combined with advanced signal processing modules and an easy-to-apply breath collection procedure, will lift breathomics right into clinical practice.

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