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Exhaled breath analysis by electronic nose in airways disease. Established issues and key questions.

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Abstract

Exhaled air contains many volatile organic compounds (VOCs) that are the result of normal and disease-associated metabolic processes anywhere in the body. Different omics techniques can assess the pattern of these VOCs. One such omics technique suitable for breath analysis is represented by electronic noses (eNoses), providing fingerprints of the exhaled VOCs, called breathprints. Breathprints have been shown to be altered in different disease states, including in asthma and COPD.

This review describes the current status on clinical validation and application of breath analysis by electronic noses in the diagnosis and monitoring of chronic airways diseases. Furthermore, important methodological issues including breath sampling, modulating factors and incompatibility between eNoses are raised and discussed. Next steps towards clinical application of electronic noses are provided, including further validation in suspected disease, assessment of the influence of different comorbidities, the value in longitudinal monitoring of patients with asthma and COPD and the possibility to predict treatment responses. Eventually, a Breath Cloud may be constructed, a large database containing disease specific breathprints. When collaborative efforts are put into optimization of this technique, it can provide a rapid and non-invasive first line diagnostic test.

Introduction

Using exhaled breath as a diagnostic test is an age old concept. Already in ancient times it was recognized that breath can provide information about health and disease. For example, a fishy smell of breath could indicate liver disease, the sweet acetonic smell of breath is

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related to uncontrolled diabetes, a urine-like odor to kidney disease, and the smell of grapes to *Pseudomonas* infections [1,2].

The primary opportunity of using exhaled breath as a source of biomarkers is the ease with which breath can be collected for subsequent analysis. Patients are generally only required to exhale into a device or container. For small children and sedated ICU-patients, breath collection methods have been developed that require no cooperation of the patient, making breath analysis techniques readily applicable in virtually any clinical setting [1,3,4]. Despite these advantages of exhaled breath testing, its use in clinical practice is still limited. The main reason being a lack of thoroughly validated techniques in different disease states. The aim of this review is to provide a comprehensive update on the strengths and limitations of exhaled breath assessments by electronic noses (eNoses) in the diagnosis and monitoring of respiratory diseases.

Constituents of exhaled breath

Exhaled breath is a mixture of nitrogen, oxygen, carbon dioxide, water, and inert gases such as nitric oxide and carbon monoxide. In the 1970s it has been shown that exhaled breath also contains a complex mixture of volatile organic compounds (VOCs) in very low concentrations [5,6]. The VOCs in human exhaled breath represent various mixtures of hydrocarbons, such as formaldehyde, methanol, ethanol, hydrogen sulphide, benzene, acetaldehyde, propanal, acetone, dimethyl sulphide, isoprene, toluene, phenol, xylene, and many others [6]. These VOCs can originate from virtually any metabolic process inside the body, either physiological or related to a disease process, either from human metabolism or from an environmental (*e.g* infectious) agent. When produced locally in the airways these gaseous molecules can be excreted directly into the exhaled breath. VOCs that originate

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from other organs are transported by the bloodstream, to the alveoli, where diffusion into the exhaled breath takes place [6]. This means that VOCs present in exhaled breath samples are most often the result of metabolic fractioning of larger molecules. Oxidative stress is a common pathway resulting in the breakdown of larger molecules which occurs in many diseases, including COPD and asthma (Figure 1). Analysis of exhaled biomarkers is therefore a very attractive approach for non-invasive exploration of various biochemical pathways operative in health and disease [4,7].

Over 3000 different VOCs have been identified in human exhaled breath [7-11]. Thus far, only a small proportion of those VOCs has been identified as disease-related [12]. Most (respiratory) diseases are not characterized by an increase of a single chemical compound. The challenge therefore is to capture the mixture (profile) of exhaled VOCs, which can be seen as a signature related to particular underlying conditions. Therefore, eNoses may serve as a cheap and rapid alternative to chemical analytical methods such as GC-MS for clinical application. This requires careful consideration of methodological issues as well as strict validation in the clinical diagnostic setting.

Methods of breath analysis

Gas chromatography – mass spectrometry (GC-MS)

Several techniques are used for the analysis of VOCs, each exhibiting its own merits and limitations. The gold-standard test is considered to be gas chromatography coupled to mass spectrometry (GC-MS) or related techniques such as PTR-MS, SIFT-MS, GC-TOF etc. Using this technique both the composition and concentrations of individual components of a gas mixture can be determined [6,13]. Interestingly the focus in GC-MS based research has also slowly shifted towards the analysis of the total pattern of volatiles rather than a single

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biomarker. The relative limitations of GC-MS technology in the medical setting are the lack of standardized collecting methods and difficulties in detecting VOCs that are present in nanomolar (10^{-9} mol) or picomolar (10^{-12} mol) concentrations [6,9,14]. In addition this technology is not ready yet for doctor's office application because it is laborious and costly.

GC-MS has initially been used for assessment of lung cancer [15-18]. Subsequently, it has been employed in the study of inflammatory lung diseases, including asthma [19-23], cystic fibrosis [24,25], and COPD [26-28]. The strength of these measurements is that it allows broad spectrum individual VOC identification, which can aid in the discovery of yet unidentified pathophysiological pathways and new targets for therapy.

Electronic noses (eNoses)

Electronic noses (eNoses) follow an essentially different approach which closely resembles mammalian olfaction [29], by measuring the whole spectrum of VOCs without identification of the individual components [30,31]. In general the output of eNoses represents a signature of the total expired VOC-profile, which is called a 'smellprint' or 'breathprint'. Individual VOCs cannot be identified, but breathprints can be analyzed by modern pattern recognition algorithms in order to discriminate different VOC mixtures and thereby potentially diseases and disease phenotypes.

There are several principles for eNose sensors [31], including conducting polymers [32-34], metal oxide [35], surface or bulk acoustic waves or quartz microbalance [14,20], optical sensors [36], colorimetric sensors [37,38], infrared spectroscopy and gold nanoparticles [39-41]. Most of these eNoses have been developed for a broad range of applications [30-33]. The suitability of the instrument for detection of specific disease states depends on the selection of sensors in relation to the gas mixture of interest and the choice of pattern

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recognition algorithms [42]. Unlike GC-MS and other chemical analytical techniques, eNoses do not contribute to the discovery of biomarkers that are specific for a disease, or targets for therapy. However, because of the cheap and readily implementable nature of this technology it is well suited for use in clinical settings (Table 1) [30,31,43-47]. In Table 2, the pros and cons of eNoses and GC-MS are compared.

E-Nose in chronic airway diseases (asthma, COPD)

To our knowledge, nine studies have tested the value of electronic nose breath measurements in the assessment of obstructive airway diseases. Of these studies, three were conducted in asthma patients [19,20,48], three in COPD patients [49-51], and three in both patient groups [52-54]. An additional four studies were performed in healthy subjects [55-58]. Details of these studies are summarized in Table 3.

From these cross-sectional eNose studies it can be inferred that asthmatics can be discriminated from healthy controls with cross-validated accuracies between 86-100% [19,20,52,53]. Furthermore COPD patients can be discriminated from asthmatics [52,53] as well as from patients with lung cancer [49].

All these data in obstructive lung diseases except the study by Fens *et al* [52] are based on cross-validation procedures in so-called training sets. The critical step in these diagnostic assessments is external validation [59,60] to test the robustness of the acquired diagnostic algorithm in a newly recruited population. The first data based on including validation sets of newly recruited patients with asthma and COPD are showing successful identification and differential diagnosis amongst these two diseases [52].

Several of the previously mentioned studies have addressed the issue of reproducibility of eNose measurements [48,53,57] in patients with asthma, COPD and in healthy smoking and

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non-smoking controls, showing intraclass correlation coefficients of 0.65-0.91 [53]. Instrumental reproducibility using two eNose devices of the same brand showed a Cohen's kappa ranging from 0.75-0.91 for discrimination between patient groups [53]. The intra-day (breath samples taken 5-10 minutes apart) and between-day repeatability (1-48 days apart) turned out to be good for all sensors ($p>0.05$) in controls and asthmatics [48,53,57]. Others have reported similar results using GC-MS analysis [8,9,28,61,62]. This indicates that the robustness of exhaled breath measurements in patients with airway disease is adequate.

Nevertheless, the measurements are far from being rigorously standardized. Technical calibration of eNoses is highly complex, requiring studies with selected and purified VOCs. This has recently been accomplished, comparing the limits of detection and dose-response curves within- and between two brands of eNoses [63]. Such experiments are essential for mapping between devices [64,65], which currently is the major roadblock in broad application of eNoses in biomedical research and clinical practice.

Airway inflammation

In mild to moderate COPD, the exhaled molecular profile appears to be associated with eosinophilic and neutrophilic inflammation [50]. Interestingly, molecular profiles measured by GC-MS were not only related to the presence of inflammatory cells, but also to the activation of these cells as measured by eosinophilic cationic protein (ECP) and myeloperoxidase (MPO) in sputum. The latter could be confirmed by eNose [50], suggesting that eNose assessments may be more suitable for the determination of activation of inflammatory cells (ECP and MPO) rather than their presence. Using GC-MS, Ibrahim *et al* also identified several molecular compounds that were associated with sputum cell counts in asthmatics [22]. Therefore, VOC profiles might prove to be useful in phenotyping asthma

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and COPD, based on the underlying inflammatory process in the airways. These developments are very promising in view of simplifying future office-based monitoring procedures in selecting patients eligible for anti-inflammatory therapy [66,67].

Methodological issues

When choosing a technique to collect breath samples it is essential to balance between feasibility, patient friendliness and methodological strictness. Unfortunately, as of yet no international guidelines exist to guide investigators towards optimal methodology. Table 4 lists the (potential) confounders and provides recommendations to minimize their influence on breath analysis. The most important step is standardization of breath collection, storage, breath analysis and data analysis procedures in order to make studies comparable between various laboratories [19,20,30,68-78].

Data analysis of electronic noses

The sensor array of an electronic nose provides a pattern of responses when exposed to a gas mixture (Figure 2). In general these patterns will be recombined into a smaller set of variables by techniques such as principle component reduction. New variables, called principle components or factors, are created that describe the variance within the dataset and are not correlated to each other. These factors are subsequently used as predictors to create diagnostic classification algorithms with associated ROC-curves [42].

Exhaled breath testing using eNoses may especially be suitable for exclusion of disease, with optimization of sensitivity. A trade-off is made against a relatively high percentage of false-positives, which will have to undergo further diagnostic testing. The number of false-positives should be kept as low as possible, by choosing the most appropriate algorithm and

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breathprint cut-off value. In addition, one of the main causes of false discoveries in general (i.e. finding a discrimination between classes when there is in fact no difference) is inadequate sample size [60]. The risks of false discoveries by eNose assessments are shared amongst many omics technologies and have to be minimized by using adequate recommendations [60] including an adequate sample size, correction for multiple testing and cross-validated internal and external validation.

The discriminatory potential of the model should always be checked for its accuracy in a subset of the data that was not used in the generation of the model; the validation set [59,79,80]. This can be done in three ways [60]. Internal validation/cross-validation is usually performed using the leave-one-out (jackknife) method. Similarly, this can be performed in a 2:1 or 1:1 ratio for training and validation sets from the same population. Second, bootstrapping can be performed, in which a large number of 'virtual' datasets is created and validated. A third method is a full external validation procedure which involves the inclusion of a completely new and independent validation set of patients meeting the same disease criteria. The latter closely resembles application in clinical practice and is therefore regarded as the gold standard of validation.

Factors influencing the exhaled molecular profile

Demographics and lifestyle

Exhaled molecular compounds assessed by GC-MS have been shown to be influenced by age, diet, use of cosmetics and exercise [8,81,82]. On the other hand, the discrimination between healthy controls, asthmatics and COPD patients by an electronic nose was not influenced by a difference in age [19,50,53] and old and young controls also could not be discriminated [19]. This suggests that although the individual components of exhaled breath

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change with increasing age, this does not affect the disease related VOC profiles as measured by electronic nose. Bikov *et al* showed a change in eNose breathprints in healthy controls after exercise [56]. The effect of diet and use of cosmetics on eNose breathprints has not been formally addressed.

Smoking

Cigarette smoke contains toxic and carcinogenic compounds, such as 2,5-dimethylfuran, acetonitrile, benzene, toluene and styrene [83]. Breathprints may be influenced by smoking in two ways [84]. First, volatile organic compounds in an exhaled breath sample may not be derived from the body but rather from the cigarette itself. Such direct influence on the breathprint can be avoided by asking subjects to refrain from smoking for a certain period before an exhaled breath sample is taken. In different studies, a two-hour period appeared to minimize the influence of smoking [50,53,58]. Second, it has been shown that cigarette smoke affects host-metabolism *e.g*. by increasing oxidative stress in the human body secondary to increasing the amount of free radicals [85,86]. This may lead to the production of oxidative stress-related molecules associated with activation of airway inflammatory pathways [87]. Notably, in COPD patients exhaled breathprints showed no difference between current and ex-smokers [50,52,53]. However, in healthy controls, differences between current and non-smokers were present [53,58]. Therefore, smoking patients in diagnostic studies should always be matched with their non-smoking counterparts to be able to eliminate the influence of smoking on the test results.

Medication

The prevailing treatment of both asthma and COPD consists of inhaled corticosteroids and inhaled short- and long-acting bronchodilators. These drugs may exert influence on the

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exhaled profile in three ways. First, they may impact the molecular composition of breath by their local and systemic effects on inflammation and metabolic pathways. Second, the administration by inhaled aerosols may directly influence the exhaled air sample by containing (parts) of the drug. And finally, drug-induced changes in airway caliber may affect exhaled breath VOC profiles. In a study by Lazar *et al*, it was shown that similar alterations in eNose exhaled breathprints were found after challenges with methacholine and isotonic saline, and that bronchoconstriction and bronchodilation *per se* did not influence the breathprints in asthma [48]. This indicates that airway caliber is not a confounder of eNose assessments in asthma. However, patients should be instructed not to use their inhaler minutes before exhaled breath measurements. In most studies patients were asked to refrain from taking inhalation medication 3 hours prior to the test [50,52,53]. This does not rule out any prolonged indirect influence of the drug on the breathprint, as it is estimated that the effect of steroids wears off only after several weeks. However, diagnostic accuracies for asthma and COPD in these studies were not influenced by the use of inhaled corticosteroids (ICS) or long-acting bronchodilators [52,53].

Comorbidity

The presence of comorbidities may influence the exhaled breathprint in patients with airway diseases. Indeed, several studies have been conducted that show the presence of fingerprints of other diseases in exhaled breath [30]. This includes diabetes [88-94], many forms of cancer [40,41,49,95-99], renal failure [100-102], heart disease [103], bacterial infections of the upper [104-106] and lower [107-109] airways, and tuberculosis [110-115]. This potential interference should be kept in mind when designing studies for determination of diagnostic accuracy for exhaled breath testing. To minimize influence of comorbidity on

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the diagnostic model and thereby the validity, studies may be designed in two ways. The first option is to construct a training set on which the diagnostic model is based, containing only 'pure' i.e. non-comorbid subjects. The second option is to construct a training set of patients with the disease under investigation with mixed comorbidities. When the latter training set is considerably large, the signal of the disease under investigation will be the common factor in all breathprints, potentially still allowing detection. This will however also worsen bias caused by confounding. Comorbid factors that happen to correlate with the class discrimination, such as risk factors associated with the disease of interest, will also be discriminatory [68]. Hence, similar to any other diagnostic procedure, comorbid conditions can limit the diagnostic accuracy of exhaled breath assessments, which requires specific studies in comorbid populations.

Future developments

Validation of results

The required next steps are:

1. To assess the diagnostic accuracy by eNose for patients with suspected airways disease, i.e. with an intention-to diagnose [80], in first and second line care.
2. To assess the influence of different comorbidities on exhaled breathprints. This should be divided into comorbidities that are related to the disease under investigation, such as nasal polyposis and gastro-esophageal reflux disease for asthma, and cardiovascular disease and diabetes in COPD, and comorbidities that are unrelated to the disease.
3. To assess the value of exhaled breath profiling in longitudinal monitoring of airway disease and early detection of exacerbations. When this is shown to be feasible, a

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clinical management study should be performed to assess whether clinical outcomes are improved by early detection of exacerbations and thereby early start of treatment.

4. To assess the possibility of prediction of treatment response. In asthma and COPD, not all patients respond to first or second line treatment. This will facilitate personalized treatment in asthma and COPD. Preliminary data show the feasibility of eNose measurements to predict systemic steroid responsiveness in patients with asthma [116].

Towards medical application.

When testing the diagnostic accuracy of eNoses for airway diseases, efforts should be directed towards optimization of the sensitivity of the test with high negative predictive values. This would position the test as a rapid first line diagnostic, to rule out disease. Anyone with a positive test may be referred for further evaluation as is common practice at present. Thereafter, specificity and positive predictive values can be targeted. This will require tailor-made eNoses after establishing disease-specific VOCs by GC-MS together with diagnostic algorithms for specific diseases and relevant disease subphenotypes. Identifying asthma phenotypes, including those that initially present with linked upper airway disease, will help to predict the response to anti-inflammatory therapy. Besides this, repeated analysis in well-characterized patients may be used for early detection of exacerbations.

For diagnostic and monitoring purposes there is in fact no need to identify the individual compounds present in exhaled breath, as long as the complete VOC profile measured by eNose provides adequate accuracies based on probabilistic evidence. Even response to treatment might be predicted by a composite biomarker such as a breathprint, without knowing the underlying pharmacokinetics or pathophysiological pathway.

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A technical target for future development is the translation or comparison between devices, eliminating the necessity of training each individual device for each disease [117,118]. To overcome this problem, efforts should be made in the statistical translation of one device into another [64,65], or to the design of sensors that provide identical profiles [119].

Ultimately, an umbrella database of disease breathprints or VOC profiles ('breathcloud') should be created to which newly uploaded profiles could be compared, and as soon as the diagnosis has been confirmed, can be added to expand the database [120].

Conclusion

In conclusion, achievements during the past 20 years paved the way for application of advanced electronic noses in medical practice. Several proof of concept studies have shown promising results for diagnosing different (airway) diseases. Currently the biggest limitation to progress in the field of exhaled breath diagnostics is the lack of sensors that can be produced identically in large quantities. Therefore, joined medical and technical task forces should be established to develop electronic noses exclusively for medical use, and airway diseases in particular. Furthermore guidelines on the standardization of breath collection, sampling, analysis and interpretation of test results should be formulated.

The authors declare that they have no conflict of interest.

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Legends to figures

Figure 1 The origin of exhaled volatile organic compounds (VOCs) in airways diseases.

Figure 2 Typical electronic nose sensor array response curve.

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Tables

Table 1 Pros and cons of medical application of eNoses

Pro	**Con**
• Non-invasive • Little coordination, understanding and cooperation from patient needed • Fast • Possibility to store breath samples on sorbent traps • Portable: suitable for primary care, screening settings, resource limited areas etc. • Potential application in many diseases [4,31] • Low costs per measurement • Exhaled breath matrix less complex than urine or blood [8,10,11] • No need for highly skilled personnel and equipped labs • Reversibility of the binding of VOCs to gas sensors [32] • Adequate short-term repeatability of clinical signals [48,53,57]	• Inability to identify disease-related VOCs • Influence of gender, food, environment, smoking, medication, comorbidity etc on breathprints [3,4,31,84] • Inability to distinguish between endogenous and exogenous VOCs in breath • Cannot readily replace blood biomarkers [4] • Device training necessary to recognize disease or disease state breathprints before use [1] • Limited mapping or signal exchange between devices [64,65] • Limited stability of sensors [1,118,119]

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Table 2 Pros and cons of eNoses *vs* GC-MS

	eNose	**GC-MS**
Pro	• Cheap • Portable • Fast • Possibility of direct measurement • Able to detect VOCs in low concentrations • Suitable for clinical application based on probabilistic evidence	• Detection of disease specific VOCs: target for therapy • No device training necessary • Standardized technique • Required for pathophysiological research
Con	• No detection of disease specific VOCs • Device training necessary • Standardization needed • Breathprints between different eNose types not comparable	• Expensive • Requires a lab and skilled personnel • Takes more time • Trapping on sorbent tube necessary • Difficulties detecting VOCs in low concentrations

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Table 3 eNose studies in obstructive airway diseases and healthy controls.

	Study population	**Result**(accuracy unless otherwise stated)	**eNose**	**Type eNose**	**Ref**
Asthma	10 mild asthma 10 severe asthma 20 healthy non-smoking controls	Mild asthma - controls 100% Severe asthma - controls 90%	Cyranose	32 polymer sensors	[19]
	27 mild asthma 24 healthy controls	Mild asthma – controls 86% (combined with FeNo 93%)	Prototype eNose Tor Vergata	8 quartz microbalance gas sensors coated by Metallo-porphyrins	[20]
	10 mild to moderate asthma	Similar change in breathprints after methacholine challenge and after saline challenge. Breathprints reproducible between days.	Cyranose	32 polymer sensors	[48]
COPD	10 COPD GOLD I-III 10 NSCLC 10 non-smoking controls	COPD - Lung cancer 85%	Cyranose	32 polymer sensors	[49]
	12 COPD GOLD I 16 COPD GOLD II	Breathprints associated with sputum ECP (r=0.84) and MPO (r=0.72)	Cyranose	32 polymer sensors	[50]
	10 COPD AAT 23 COPD GOLD II-III 10 non/ex-smoking healthy	COPD – AAT COPD 58% COPD - controls 68% AAT COPD - controls 62%	Cyranose	32 polymer sensors	[51]

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	controls				
Asthma and COPD	30 COPD GOLD II-III 20 mild-severe asthma 20 smoking controls 20 non-smoking controls	COPD - Asthma 96% COPD - Smoking controls 66% Asthma – Non-smoking controls 95% Breathprints controls reproducible within-day and between-day.	Cyranose	32 polymer sensors	[53]
	40 COPD GOLD II-III 39 asthma, reversible 21 asthma, fixed	External validation of [53]: COPD - rev. asthma 83% COPD - fixed asthma 88% Rev.asthma - fixed asthma 58% (not significant)	Cyranose	32 polymer sensors	[52]
	20 Asthma (9 GORD) 17 COPD (9 GORD) 7 Controls	Asthma - Controls 70.2% Asthma - COPD 70.4% Asthma - Asthma GORD 85% COPD - COPD GORD 64.7% Obstructive lung disease – Obstructive lung disease with GORD 67.6%	Cyranose	32 polymer sensors	[54]
Healthy	14 ozone responsive subjects	No relation to ozone response	Cyranose	32 polymer sensors	[55]
	10 healthy non-smoking subjects	Physical exercise changes breathprint. Breathprint correlated to EBC pH (r=-0.34)	Cyranose	32 polymer sensors	[56]
	78 pregnant	External validation 80% (3rd	Cyranose	32 polymer sensors	[57]

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	52 non-pregnant women	trimester p=0.02; 2nd trimester p>0.05). Breathprints non-pregnant controls reproducible within day and between days.			
	Smokers and non-smokers	External validation 95%	Cyranose	32 polymer sensors	[58]

FeNo: fractional exhaled nitric oxide. COPD: chronic obstructive pulmonary disease. GOLD: Global Initiative for Obstructive Lung Disease. NSCLC: non small cell lung cancer. ECP: eosinophil cationic protein. MPO: myeloperoxidase. AAT: α1-antitrypsin deficiency. GORD: gastro oesophagal reflux disease. EBC: exhaled breath condensate.

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Table 4 (Potential) breath analysis collection confounders

(potential) Influence on breath analysis	**Action to minimize influence**
Exogenous VOCs (investigator, environmental) [84]	Inspiratory VOC filter [19]
Exogenous VOCs (breath collection equipment, disinfectants, reminiscent VOCs from previous users)	Baseline sample by passing ambient air through breath set up that the patient will use
Humidity of breath sample [30,32,73,74]	1. Silica filters at expiratory port 2. Heating of breath sample 3. Heating of sensors
Diffusion of VOCs from or into sampling bag [68,121]	1. Disposable bag 2. Thorough cleaning of bag 3. Direct sampling without bag
Ageing of sample during storage or transport	Use of sorbent tubes for prolonged stabilization of VOCs [75-78]* *this changes the breath sample, but the discriminative signal between asthma-controls is preserved [78]
Limited sensitivity of certain eNose sensor types for disease specific VOCs	Use of an eNose platform combining various eNose sensor types [117]
Incompatibility of individual eNose sensor responses; necessity to train each device	Use of an eNose platform and sorbent tubes for centralized measurements in multicentre trials [117]

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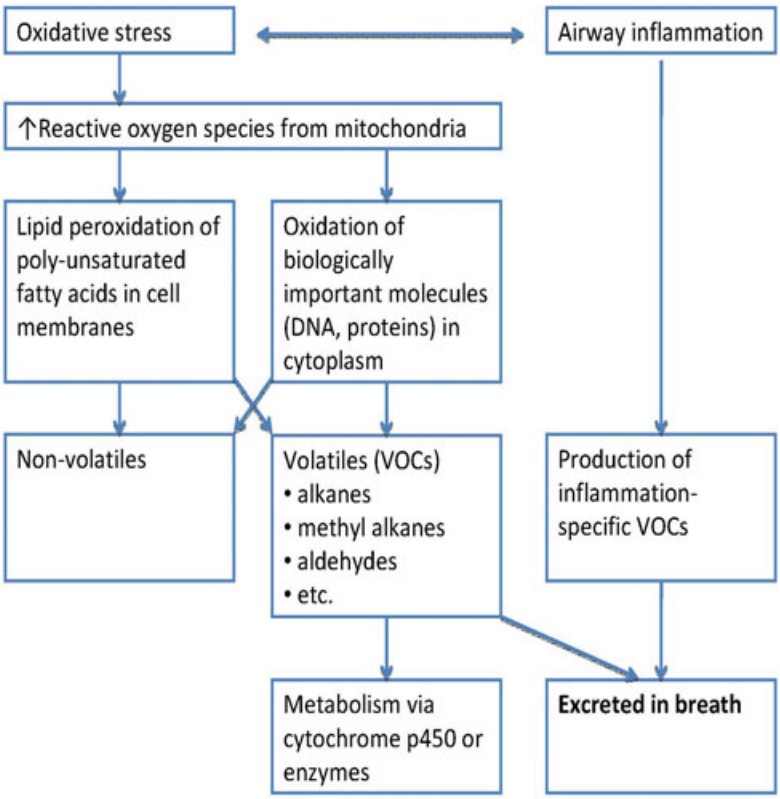

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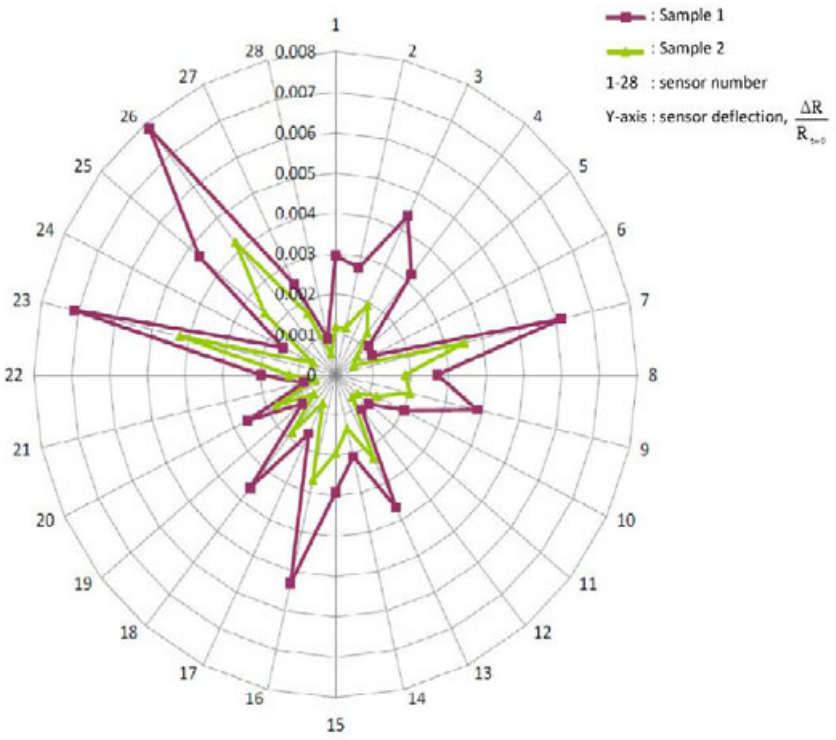

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