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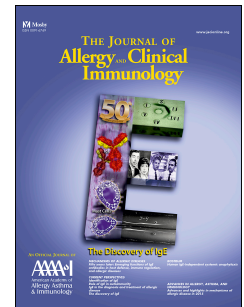
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Breathomics in asthma and COPD

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Abstract

Exhaled breath contains thousands of volatile organic compounds (VOCs) that reflect the metabolic process occurring in the host both locally in the airways and systemically. They also arise from the environment and the airway microbiome. Comprehensive analysis of breath VOCs (breathomics) provides opportunities for non-invasive biomarker discovery and novel mechanistic insights. Applications in obstructive lung diseases, such as asthma and COPD, include not only diagnostics (especially in children and other challenging diagnostic areas) but also the identification of clinical treatable traits such as airway eosinophilia and risk of infection/exacerbation that are not specific to diagnostic labels. Whilst many aspects of breath sampling and analysis are challenging, proof of concept studies with mass-spectrometry and electronic noses technologies have provided independent studies with moderate to good diagnostic- and phenotypic accuracies. The present review evaluates the data obtained by breathomics in: a) predicting the inception of asthma or COPD, b) inflammatory phenotyping, c) predicting exacerbations and d) treatment stratification. The current findings merit the current efforts step of large multi-centre studies, using standardized sampling, shared analytical methods and databases, including external validation cohorts. This will position this non-invasive technology in the clinical assessment and monitoring of chronic airways diseases.

Rationale

Asthma and chronic obstructive pulmonary disease (COPD) are medical diagnoses based on clinical criteria, derived from history taking and pulmonary function testing as outlined by the international GINA (www.ginasthma.org) and GOLD (www.goldcopd.org) guidelines, respectively^{1,2}. The evidence supporting these guidelines has been built on more than half a century of clinical and epidemiological studies, aimed to improve the identification and management of patients with these chronic airways diseases. This has led to unprecedented steps forward in the clinical control and quality of life of individual patients^{1,2}. Nevertheless, the personal and societal burden of asthma and COPD has remained unacceptably high, which has led the WHO to prioritize research in this area³.

One of the major hurdles in improving the prevention and clinical course of asthma and COPD is their complex pathogenesis and pathophysiology. This includes multiple molecular and cellular networks associated with innate and adaptive immunity, inflammation, damage and repair, which can not only vary amongst-, but also within-patients^{4,5}. It is increasingly recognized that capturing this biological complexity in asthma and COPD is required for a real step-change in improving the management and control of these diseases⁶. Rather than using singular biomarkers, such complexity is likely to be captured most comprehensively by modern high-throughput 'omics' platforms⁷, which have been made applicable for various samples (blood, endobronchial biopsies, sputum, urine, exhaled air) obtained from patients with chronic airways diseases⁸. The key issue when using these 'omics' technologies in characterizing the biological networks in medicine is to obey stringent quality control and validation criteria, which have recently been published^{9,10}.

Amongst the various 'omics' technologies, the ones that can be measured at point of care are likely to prevail in clinical practice. That is why non-invasive metabolomics of exhaled air (breathomics) has become such an appealing option, particularly for chronic airways diseases¹¹. Apart from pending technical and statistical challenges in breathomics¹², such as exchangeable sampling techniques, sensor systems, signal pre-processing, multivariate data analysis and shared databases, it is vital to formulate the clinical dilemmas that are likely to be successfully addressed by application of breathomics in patients with such diseases. This provides this technology the best opportunities of contributing to improvement of clinical outcome.

The aim of this review is to examine the current performance of breathomics in its ability to contribute to solving the major clinical dilemmas in chronic airways diseases. Only then breathomics will become relevant for clinicians or epidemiologists on the ground. These major dilemmas include (figure 1):

- a) Prediction of risk for the early inception of asthma in children¹³ or the development of COPD¹⁴;
- b) Phenotyping of patients along the whole spectrum of asthma and COPD, including inflammatory profiling^{15,16};
- c) Prediction of risk for exacerbations^{17,18};
- d) Prediction of treatment responses in order to identify the treatable traits of chronic airways disease¹⁹.

Metabolomics and Breathomics

Definitions

Metabolomics is the study of the metabolic content of a given system, whether that be a cell, organ system or organism, In exhaled breath gas analysis we detect VOCs that may arise from or be affected by metabolism, and the term breathomics is currently used to describe this²⁰. However, it must be emphasized that exhaled breath VOCs do not exclusively represent lung metabolism. We will describe here the sources of breath VOCs, and the implications for sampling.

Origins of exhaled breath VOCs

The VOC content of the exhaled breath is a product of the VOCs that are inhaled, those diffusing from the tissue and circulation into the alveoli and airways at the time of sampling, and reactions occurring between these. The terms “exogenous” and “endogenous” VOCs are sometimes used to describe the origin of VOCs, although this terminology can lead to confusion (for example, are VOCs arising from the airway microbiome exogenous or endogenous?). For the purposes of further discussion here we define exogenous VOCs as those taken in from the environment (typically through inhalation, ingestion or skin absorption), and endogenous as those biologically generated inside the body *i.e.* products of host and microbe metabolism. It should be acknowledged, however, that in many cases this is a moot point as it is not possible to tell whether a given VOC arises from any particular source, unless for example the source is radio-labelled²¹. This is an intrinsic complexity of breathomics.

Exogenous VOCs arise from the environment (largely inhaled), food and drink (ingested) and drugs. Environmental VOCs are ubiquitous and arise from all but the most inert sources. Taking an outpatient clinic room as an example VOCs arise from plastics (including gloves, instrumentation and computer equipment), cleaning fluids

(for both humans and surfaces), and indeed from the breath, skin, clothing and scents of any person in the room. The study of environmental VOCs in itself is a growing area of research relevant to clinicians²². We also need to consider not only immediate but also recent environmental exposure as a source of VOCs²³.

Endogenous VOCs reflect (fragments of) metabolites, arising from processes within the body, reflect host and microbiome metabolism, including of course metabolism of the food and drugs that form part of the “exogenous” source. Volatiles will therefore also vary according the airway compartment under study: systemic VOCs passively diffuse across the capillary/alveolar interface; lower airway VOCs add those arising from bronchial diffusion, and host / microbiome airway metabolism; and whole airway VOCs add those from the mouth and nose. Attempting to isolate the origin of VOCs of interest therefore requires sampling procedures to be tailored to the specific clinical or research question.

Considerations for breath sampling and analysis

In light of the complexity of the breathome as outlined above, a number of technical, analytical and methodological issues should be considered when performing or reviewing breath research (figure 2).

- Targeted *versus* untargeted analysis. There are two broad approaches to studying volatiles in the breath: targeted, where the biomarkers of interest are known and sought prospectively; and untargeted, where an unsupervised multivariate ‘omics approach is taken. The approaches are complementary but need to be specified *a priori*, as the statistical methodologies required differ (see below). Regarding the targeted approach, it should be noted that the biochemical

origin of most VOCs is unknown¹². If the same clinical question has already been addressed in a previous study though, it is recommendable for the analytical methodology of the new study to include a search for previously identified VOCs of interest.

- Sampling methodology. Many different sampling methodologies are being employed for off-line breath analysis²⁴, making comparison between studies difficult. Furthermore, varying methods affect reproducibility differently. For methods that utilise an inspiratory VOC-filtered air supply, the length of time breathing filtered air has an effect on VOC levels, which can take varying lengths of time to wash-out or equilibrate²⁵. Where the equilibration time is standardised, the within day repeatability for breath VOC detection by electronic nose has been shown to be good²⁶. Airway caliber, as indirectly reflected by the presence or absence of airways obstruction, does not seem to have a direct effect on the breathprint²⁷. Phillips and colleagues demonstrated coefficients of variability of more than 20% for a number of VOCs measured serially in the breath of healthy volunteers and people with COPD, although it was not possible to conclude whether the source of variability lay in the individuals' breath or in the method of sampling or sample-transfer²⁸.
- Analytical methodology. Broadly speaking there are two approaches to VOC analysis (summarized in^{11,12,24}). Methods based on mass spectrometry aim to detect individual VOCs, and in some cases identify them, which is particularly useful for pathophysiological research where the VOCs of interest are not yet known. Electronic noses on the other hand are based on cross-reactive, non-specific sensor arrays and usually (but not always) are less specific, but rather respond to patterns of VOC mixtures by altering sensor properties (such as

electrical resistance) and hence producing a quantitative signal change based on pattern recognition algorithms.

- Data analysis. Depending on the aim, this can be performed by supervised and unsupervised statistical algorithms. Where the VOCs of interest are known and sought prospectively, data analysis is relatively straightforward. The dimensions of datasets can be reduced by using e.g. principle component analysis (PCA), selection by Akaike Information Criterion (AIC) with PCA, or partial least square analysis-discriminant analysis (SPS-DA)²⁹. Importantly, in unbiased discovery studies where multiple comparisons are made (a typical breath sample may contain 100's to 1000's of VOCs) there is a high chance of overfitting and an unacceptable false discovery rate (FDR), which means that specialist multivariable methods must be used²⁹. This includes classification analysis based on neural networks (NN), logistic regression (LR), linear discriminant analysis (LDA), support vector machine (SVM), or random forest (RF), which have recently been compared in published eNose datasets²⁹. Once proof of concept has been obtained, the findings must of course be validated in a prospective and independent cohort of patients (external validation). In all circumstances the TRIPOD recommendations on reporting multivariable prediction models should be followed (www.tripod-statement.org).

Specific applications in asthma and COPD

Diagnosis: classifying asthma/COPD versus control

All work to date has been performed in patients with an established diagnosis of asthma or COPD and in controls, in order to generate proof of concept that breath

VOCs are different in health and disease. In general there are therefore often potentially relevant clinical parameters that can differ between the case and control groups that could confound the results, e.g. medication use, comorbidities, smoking etc. If breath signals are to prove useful as (composite) biomarkers for diagnosis, they must be tested at the point of diagnosis, and in the population in which the test should clinically be used.

Asthma versus healthy controls

Electronic noses have been employed to detect VOC changes in asthma in several studies^{30–32}. Dragonieri and colleagues³⁰ initially supported this application by demonstrating that the eNose signal from the Cyranose 320 (which has 32 organic polymer sensors that change resistance on exposure to VOCs) was able to classify the breath of people with both mild asthma (*versus* young age [27±6 yrs] matched controls) and severe asthma (*versus* older [57±7 yrs] controls) with high accuracy. Interestingly, the eNose was not able to classify the breath of mild *versus* severe asthma, which might support the hypothesis that the VOCs detected were indeed related to the disease state itself rather than its severity or phenotype. The Tor Vergata eNose has also been applied to this clinical question in a study in which late expiratory breath samples were collected; eNose profiles classified 27 people with asthma from 24 healthy controls with an accuracy of 87.5%³¹. More recently a prototype of eNose technology has been integrated with spirometry (being positioned directly downstream to a pneumotachograph) allowing collection and analysis of triplicate parallel breath samples during slow expiratory vital capacity manoeuvres³², again demonstrating classification accuracy between the breath of people with asthma and healthy controls of 87% using only four sensors. In the same study the

classification accuracy between COPD and asthma was 81%. Taken together, the diagnostic eNose data in asthma provided by independent research groups are meeting proof of concept.

Gas chromatography mass spectrometry has been used as the analytical methodology in multiple studies with some success. In children, Dallinga and colleagues³³ reported a very high classification accuracy (100% using 25 VOCs in the training model) from a large cohort of children with asthma (n = 63) and age-matched healthy children (n = 57) and validated using 20-fold internal cross validation with 5% of the cohort used as a test set each time. In adults Ibrahim and colleagues³⁴ reported leave-one-out cross validation accuracy of 83% of asthmatics *versus* controls, again using GCMS as the analytical methodology. Intriguingly the same classes of discriminatory VOCs have been reported across multiple studies in asthma; exhaled methylated alkanes have now been reported to be raised in three studies from different research groups using different sampling, analytical and statistical methodologies^{30,33,34}, and have also been proposed outside asthma as potential markers of oxidative stress³⁵. In children with asthma breath sampling is feasible and acceptable and there are data to suggest that breath GCMS profiles also distinguish them from the breath of healthy non-asthmatic children³⁶.

The potential for exhaled VOCs for the diagnosis of asthma has been highlighted in a recent meta-analysis³⁷, which summarized studies using various methods of breath sampling and analysis. They conclude that VOC profiling may be used as an add-on tool for asthma diagnosis in conjunction with other diagnostic tests. This is not unexpected and clinically realistic. Any 'omics' technology in diagnosis and

monitoring will eventually be used in conjunction with available clinical information. Eventually, the complementary information provided by breathomics, which cannot sufficiently be captured by merely clinical assessment, will be decisive for its clinical implementation at point of care.

COPD versus healthy

Several groups have investigated the VOC profile of the exhaled breath of people with COPD over recent years using various methods of breath collection and analysis. Breath signals analysed by the Cyranose 320 were able to discriminate the breath of patients with lung cancer from those with COPD with an internally cross-validated correct classification rate of 85%³⁸. Using the integrated eNose / spirometer mentioned above, the same team was also able to classify people with COPD *versus* lung cancer (cross validation accuracy 80%) and healthy controls (accuracy 78%)³², although there were important demographic differences between groups in particular in terms of age and smoking status.

Van Berkel and colleagues used GCMS derived breath VOCs to generate a predictive model in a cohort of patients with COPD (n = 50) and healthy controls (n = 29). When the model was tested in a new test set of volunteers (16 COPD, 16 healthy), 29 of 32 were classified correctly, and a further 14 of 15 steroid-naïve COPD patients thus implying that the effect was not steroid-related³⁹. The model again comprised VOCs including methylated alkanes, as for asthma. Basanta and colleagues reported GCMS profiles that could discriminate breath from healthy individuals *versus* COPD with cross-validated accuracy of 70%, aldehydes representing the most frequently occurring discriminatory compounds⁴⁰. Another

large study again showed that GCMS could classify COPD versus controls with good accuracy, and emphasized the importance of controlling for current smoking status in such studies⁴¹. An important confounder that needs to be considered and tested in these and other breath VOC studies is the effect of study site; Gaida and colleagues recently published data showing the discrimination between COPD and healthy controls was significantly influenced by this factor⁴². Even though a significant effect of study site could not be confirmed in a recent eNose study³², these data underline that measures should be taken to explore and to correct for site-bias.

Ion mobility spectrometry, a form of MS that has potential for point-of-care use, has also been used to analyze the breath of patients with COPD^{43,44}. Proton transfer reaction-MS can analyze VOCs in near real-time, and intriguingly has suggested breath VOC profiles differ between the COPD phenotypes of emphysema, airflow obstruction and impaired gas transfer⁴⁵.

Asthma versus COPD

Several studies from the group of Sterk have shown the eNoses can classify the breath of people with asthma versus COPD with moderate to good accuracy^{32,46,47}. In the largest of these the classification model was tested in a true external validation set and was still able to differentiate between the diagnostic categories with an accuracy (in the prospective external validation set) of 90% in current non-smokers⁴⁷. This accuracy may approach optimal values given the inevitable clinical overlap between asthma and COPD¹⁹.

At risk populations

Prediction of disease development is one of the most important premises of any biomarker in chronic airway diseases. Breath analysis is especially suitable for this purpose as measurements can be repeated frequently, which allows for monitoring the likelihood of disease development. Additionally, breath collection is one of the few acceptable sampling methods to obtain material for molecular analysis in pre-school aged children due to its non-invasive nature. The predictive value of exhaled breath analysis for the development of asthma in children was investigated in the “Asthma Detection and Monitoring” study, a prospective follow-up study of wheezy children at risk for the development of asthma⁴⁸. Multiple breath samples were taken in 246 children from two to four years of age. About one third of the children were diagnosed with asthma at the age of 6. A model comprising 12 VOCs were predictive of asthma several years before the diagnosis. The accuracy of the prediction was good, with an area under the receiver operating characteristics curve (AUROC) of 0.78⁴⁸. In a further analysis, the additive value of this breath test on top of the asthma predictive index (a clinical prediction score for the development of asthma) was reported⁴⁹. The AUROC increased from 0.61 for the asthma predictive index alone to 0.89 when combined with the 12 VOCs. This increase in predictive accuracy was better than for exhaled breath condensate, gene expression in peripheral blood and lung function. These results illustrate that breath analysis is not only easier to perform in children than exhaled breath condensate sampling or blood draws, but also that a strong biological signal is present in this matrix.

However, several factors should be taken into account before drawing possibly overoptimistic conclusions. Firstly, the 12 VOCs test panel was not definitively identified and confirmed: although the mass spectra were matched to a library, this tentative identification was not validated with the injection of pure chemical

standards. Secondly, external validation was not performed and this should be the focus of follow-up studies. Finally, a study in the EUROPA birth cohort suggests that rhinovirus infection moderates the discrimination by exhaled breath analysis with an eNose between wheezing and non-wheezing children⁵⁰. Therefore, stratification for viral infection status may be required to increase the diagnostic accuracy of exhaled breath analysis in wheezing children. More interestingly, the authors found that rhinovirus infected wheezy children could be separated from the other children consistently, even after resolution of the infection. Given the potentially pathogenetic role of early-life rhinovirus infection in the inception of asthma⁵¹, they speculate that the underlying genotype-environment interaction that may result in asthma also contributes to the persistent changes in exhaled breath profiles.

Identification of treatable traits

Asthma or COPD are complex and heterogeneous, and it has been argued that further improvements in care for these chronic airways diseases require precision medicine, aiming to identify treatable traits that can be targeted with specific therapies¹⁹. There are several reports suggesting that breath analysis can be used to distinguish phenotypes of patients with asthma or COPD. Patients with COPD due to cigarette smoking or alpha 1-antitrypsin (AAT) deficiency could be discriminated with high accuracy using an electronic nose and treatment with recombinant AAT led to a change in the breath profile⁵². This may suggest that the composition of VOCs in exhaled breath is influenced by the etiology of COPD development. However, this study was very small with 10 patients per group and no correction for confounders was performed.

Airway inflammation

Inflammatory phenotyping is increasingly important in asthma and COPD in view of intervention with steroids or novel anti-inflammatory biologicals^{19,53}. Irrespective of the underlying cause for disease, multiple studies reported on a correlation between exhaled VOC profiles and inflammatory cells in sputum and broncho-alveolar lavage fluid. Two studies showed exhaled breath profiles obtained with an eNose are significantly associated with pulmonary eosinophilia in asthma⁵⁴ and with pulmonary neutrophilia in COPD GOLD I, but not in GOLD II patients⁵⁵. In both studies, it was the second principal component that correlated with inflammatory cell counts. In conjunction with the finding that the first principal component frequently contributes to disease discrimination this implies that information on the inflammatory subtype is enclosed in a different part of the exhaled breath signal than the factors that discriminate between diseased and non-diseased individuals. GC-MS measurements do indeed suggest that different VOCs contribute to asthma discrimination than to the establishment of the disease phenotype³⁴. In that study, several branched alkanes and ketones were found to be associated with airway eosinophilia and neutrophilia. However, these compounds were not found in an *in vitro* comparison of the VOCs produced by eosinophils and neutrophils⁵⁶. This might be due to the complex biological environment of eosinophilic and neutrophilic inflammation *in vivo*. Without additional information on the biochemical pathways that contribute to the production of the measured VOCs it remains uncertain whether the correlations between volatile compounds and inflammatory cells are a direct effect of production by the cells themselves or an epiphenomenon. However, this would not hamper the clinical application of a breath test for inflammatory phenotyping in any case.

Treatment response

Corticosteroids are the cornerstone of treatment of inflammation and potentially airway remodeling in chronic airway diseases⁵⁷. However, steroids have several side-effects and should not be administered if a patient is not steroid responsive, at least in COPD⁵³. Airway eosinophilia and fractional exhaled nitric oxide (FeNO) are important predictors of steroid responsiveness⁵⁸ and, as described above, eosinophils are associated with altered VOC profiles. In a proof of principle study van der Schee *et al.* used breath analysis with an electronic nose to directly predict oral steroid responsiveness in mild to moderate asthmatic patients that were previously withdrawn from steroids. Breath analysis was superior to sputum eosinophils and FeNO with an AUROC of 0.88. The breathprints also showed a strong correlation with eosinophils in sputum, thus strengthening the pathophysiological link between exhaled breath profiles and the inflammatory status within the lung. As pointed out in the accompanying editorial⁵⁹ the high internal validity of this result in a highly controlled experiment with a limited number of subjects (N=18) limits the generalizability. Thus far no prospective studies have been published to validate the predictive performance in patients starting inhaled corticosteroid therapy. With the development of new, targeted therapeutics that are very costly prediction of treatment response is becoming ever more important¹⁹.

Prediction of exacerbation

Exacerbations are inversely associated with quality of life in patients with chronic airway diseases and additionally seem to play an important role in the decrease of lung function over time, at least in COPD^{18,60,61}. Therefore, it would be very helpful if the development of exacerbations could be predicted⁶². As patients are chronically

at risk for exacerbations, a non-invasive test like breath analysis is ideal to allow for monitoring. There are no data on the prediction of exacerbations with exhaled breath analysis in adult patients but a recent study in 40 children with asthma reported promising results. Breath samples were collected every two months for a year, during which time approximately half of the children developed an exacerbation⁶³. Classification of exacerbations was very good with a sensitivity of 100% and a specificity of 93% using a model comprising six VOCs when baseline samples were compared to exacerbation samples within a patient. Between-patient analysis, comparing exacerbation samples and non-exacerbation samples of other patients, yielded a lower accuracy, with a sensitivity of 79% and a specificity of 100% with seven VOCs. The VOCs that were included in the statistical models did not overlap between the intra- and inter-patient comparisons. None of these markers correlated with asthma control⁶⁴.

There is evidence that the breathprint of COPD patients with an exacerbation and positive sputum cultures is different from similar patients with negative sputum cultures⁶⁵. This might be important as a marker of those exacerbations for which antibiotic treatment can be withheld. Breathprints also discriminate between colonized and non-colonized airways in patients with stable COPD⁶⁶, thus these observations are not unique to exacerbations. This is not surprising as bacteria produce a wide range of VOCs that have not been described in human metabolism^{67–69}.

Conclusions

Most studies on breathomics in obstructive airways diseases have focused on the diagnosis of asthma or COPD. The major limitation of all except one⁴⁶ of these studies is that most were not intended to newly diagnose patients. Furthermore, the disease label may not be as important as the identification of treatable traits¹⁹. There is a clear need for non-invasive biomarkers in the stratification of patients. Exhaled breath analysis as a substitute for sputum eosinophils may lead to immediate clinical usage in the selection of patients for therapies directed against eosinophilic and/or type-2 inflammation. Stratification may also become more important in non-eosinophilic disease in the near future. Exhaled breath analysis has advantages over blood and sputum sampling as it is non-invasive, cheap and potentially gives instant results. However, external validation between cohorts and research groups is pivotal for the future of breathomics.

The ideal analytical instrument is highly sensitive and identifies compounds with high precision. GC-MS meets these needs, but is not available at the bed site and does not provide a result rapidly. Sensor array systems (eNoses) on the other hand can give an instant probabilistic result, but does not selectively measure any specific VOCs. Some systems such as MCC-IMS or FAIMS seem to combine the best of both worlds, but have not yet reached the selectivity or specificity that is required in clinical practice. The coming years will reveal how and when breathomics will mature into a clinically relevant test in the diagnosis, phenotyping and monitoring of chronic airway diseases.

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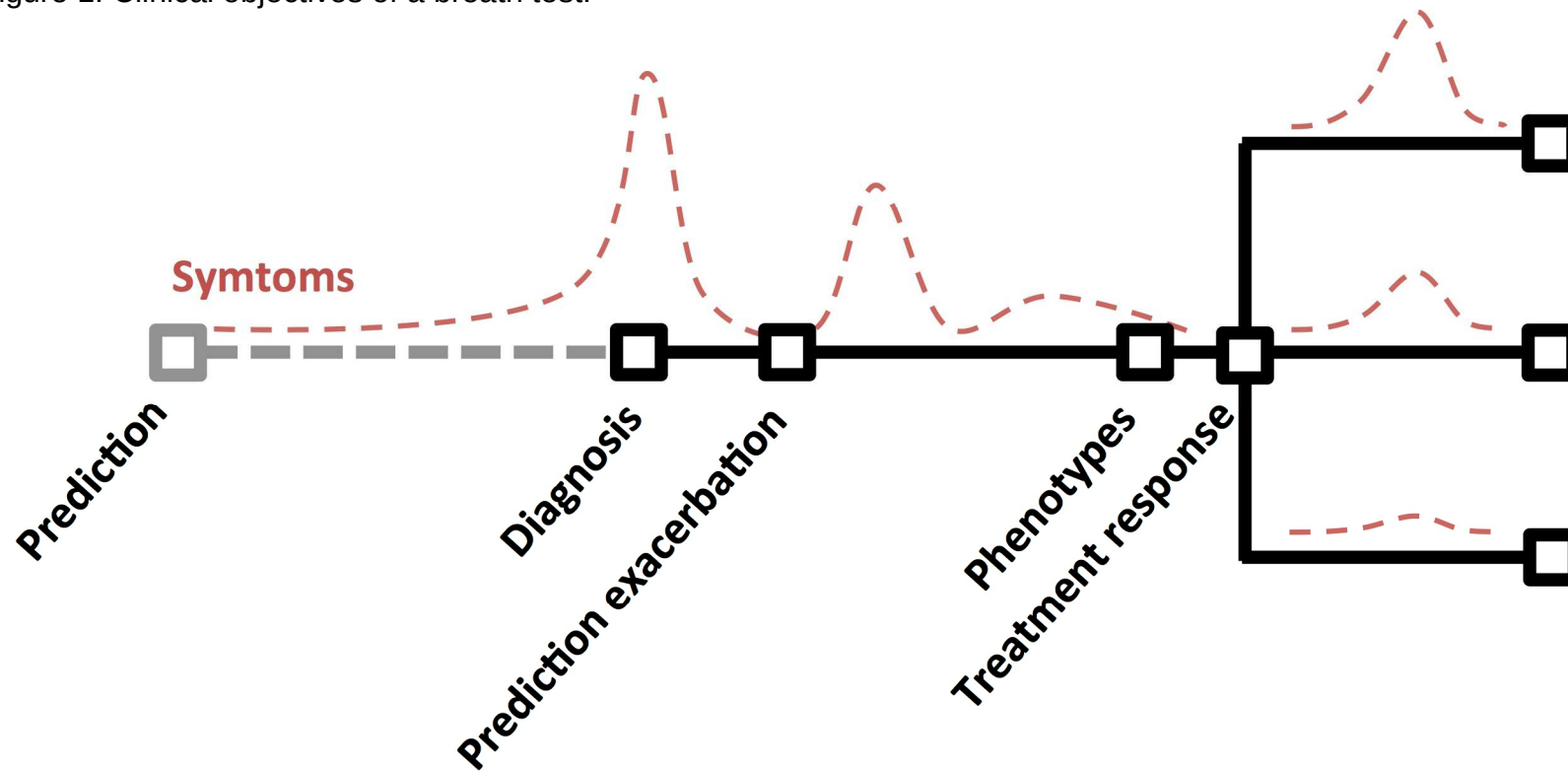
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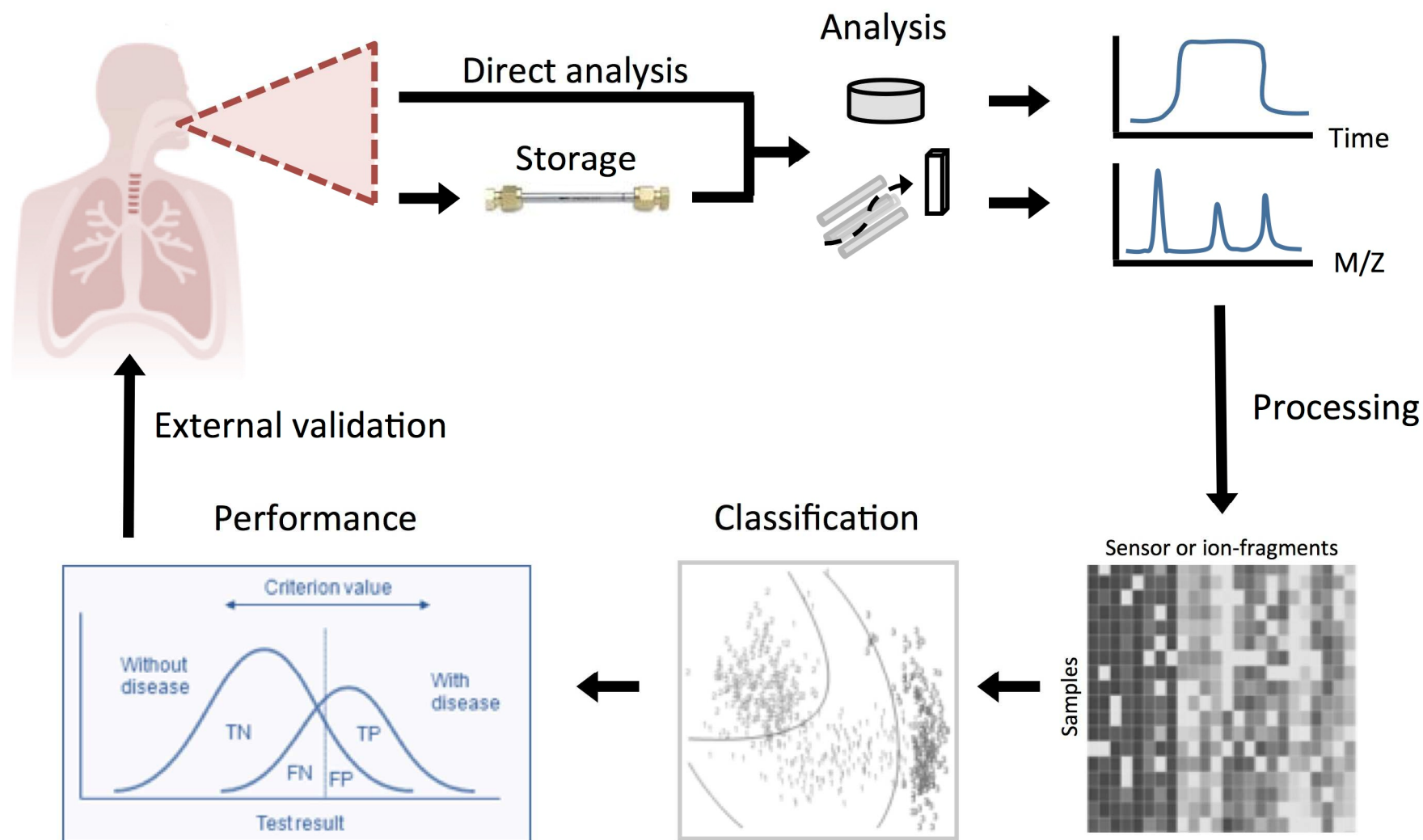
727 Figure 1: Clinical objectives of a breath test.



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731 Figure 2: Methodological steps in breath research.



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734 Box: Future research priorities for breathomics in asthma COPD. eNOSE = electronic

735 nose; PTR-MS = proton transfer reaction-mass spectrometry; SIFT-MS = selected

736 ion flow tube-MS; GC-MS = gas chromatography-MS

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Technical	Standardization of sampling methods	Development of robust and deployable methods suitable for: outpatient clinic and emergency department
	Online analytics	Testing of online methods for breath analysis e.g. eNOSE, PTR-MS, SIFT-MS and developing shared data-bases amongst centres
	Offline analytics	Refinement of analytical methods and development of VOC library for GC-MS based methods
Clinical	Diagnosis	Prospective identification of disease (e.g. asthma) in newly-presenting patients
	Stratification	Prospective identification of treatable traits with implications for treatment or prognosis e.g. airway eosinophilia; infection

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