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A European Respiratory Society technical standard: exhaled biomarkers in lung disease

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ABSTRACT Breath tests cover the fraction of nitric oxide in expired gas (FeNO), volatile organic compounds (VOCs), variables in exhaled breath condensate (EBC) and other measurements. For EBC and for FeNO, official recommendations for standardised procedures are more than 10 years old and there is none for exhaled VOCs and particles. The aim of this document is to provide technical standards and recommendations for sample collection and analytic approaches and to highlight future research priorities in the field. For EBC and FeNO, new developments and advances in technology have been evaluated in the current document. This report is not intended to provide clinical guidance on disease diagnosis and management.

Clinicians and researchers with expertise in exhaled biomarkers were invited to participate. Published studies regarding methodology of breath tests were selected, discussed and evaluated in a consensus-based manner by the Task Force members.

Recommendations for standardisation of sampling, analysing and reporting of data and suggestions for research to cover gaps in the evidence have been created and summarised.

Application of breath biomarker measurement in a standardised manner will provide comparable results, thereby facilitating the potential use of these biomarkers in clinical practice.

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Introduction

Volatile organic compounds (VOCs) and other different constituents were identified in exhaled breath decades ago and, from them, the fraction of nitric oxide in expired gas ($F_\mathrm{e}\mathrm{NO}$) paved the way as a potential biomarker. Many volatile and non-volatile components are present in the breath in trace amounts, making their detection a challenging task. Applying highly sensitive technology to analyse exhaled breath enables scientists to evaluate the wide scale of different molecules in these samples. Owing to the variety of sampling methods and analytics, the field developed in a largely unrelated manner in three main domains: exhaled breath condensate (EBC), exhaled VOCs and $F_\mathrm{e}\mathrm{NO}$. A fourth area, that of exhaled particles, arrived later. Official recommendations for standardised procedures are available for EBC and $F_\mathrm{e}\mathrm{NO}$ but they are >10 years old, and there are currently no official recommendations for exhaled VOCs and particles [1, 2]. There have been new developments and technological advances in the field of EBC and $F_\mathrm{e}\mathrm{NO}$, and broad experience has been gathered over the last decade; therefore, the current document focuses on these areas. For $F_\mathrm{e}\mathrm{NO}$, the specific aim of the current document is to clarify the terminology, update nasal NO measurement and introduce recommendations for NO dynamic models. Leaders in this scientific area thought it important to summarise current evidence related to sampling methods, measurement standardisation and data interpretation; to highlight gaps in knowledge; and to recommend the technical standards to be used and directions for future research. Although the field of exhaled biomarkers is large, there are common issues in sampling and also in mediator analysis. Therefore, it was felt that a joint effort from experts would be the best way to tackle methodology-related issues by using a wide platform to discuss shared problems and unsolved issues in standardisation, and, at the same time, to provide a framework for more detailed discussion in method-specific smaller groups. Thus, the aim of this document prepared by the Task Force (TF) of the European Respiratory Society (ERS) was to provide recommendations for standardisation of sample collection and, evaluation of different analytic approaches, and to highlight future research priorities in the field of exhaled biomarkers. Where previous guidelines for EBC and $F_\mathrm{e}\mathrm{NO}$ exist, areas that were not covered were evaluated and have been dealt with in the current document. The group did not aim to provide guidelines for clinical practice.

Committee composition

Two co-chairs applied for support for an ERS TF. The co-chairs invited clinicians and researchers to participate in the project on the basis of their expertise in research on one or more areas of exhaled biomarkers. The task was split into four parts and co-chairs were asked from members to take the lead for each part, *i.e.* EBC, VOCs, $F_\mathrm{e}\mathrm{NO}$ and exhaled particles. The participants were asked to join the group most relevant to their expertise. Forming groups on different topics enabled TF members to have more in-depth discussions on the specific areas of different samples.

Recommendations for technology standards and further research
Each group appraised and summarised the existing evidence and then discussed its relevance to improving standards of sampling and measurement of exhaled breath mediators. They also identified any knowledge gaps. Recommendations for technical standards and research priorities were formulated *via* discussion and consensus generated, first in each individual group then by the presentation of suggested recommendations at a TF meeting during which different aspects were brought together.

Document development
To identify relevant literature, the Medline database (assessed through PubMed) was searched using key terms such as "exhaled breath condensate", "exhaled biomarkers", "exhaled volatile organic compounds", "electronic nose", "breath print", "exhaled nitric oxide", "nasal nitric oxide" and "exhaled particles". The database search was concluded in July, 2016. Publications providing evidence for methodological questions were reviewed and used to form methodological recommendations. Only scientific publications in the English language were included.

Drafts sent to the co-chair (IH) by group leaders were merged, edited and circulated among the members for review, feedback and approval. Based on the received comments and criticisms, corrections were made to the manuscript, and then the document was submitted for pre-approval by the ERS as required by its TF operation. This document summarises the current evidence related to exhaled biomarker research and the requirements for standardised sampling and analysis where possible. Of importance, it is not intended to provide clinical guidance on disease diagnosis and management. In areas where evidence-based recommendations could not be provided, a consensus-based opinion of the TF members is expressed and the need for further research on the area is highlighted. The document structure below follows the structure of the four working groups.

Exhaled breath condensate
The definition of EBC by the previous ERS/American Thoracic Society (ATS) TF is used [1]. Briefly, EBC is obtained by cooling exhaled breath through contact with a cold surface or condenser. Samples are collected as fluid or frozen material and analysed immediately or later for volatile and non-volatile macromolecules.

General aspects of exhaled breath condensate collection
Condensing equipment
Multiple tools exist to cool exhaled air and collect EBC, ranging from homemade systems to a variety of commercial products [1].

Effect of condenser material: Collection systems have different coating materials, *e.g.* Teflon, polypropylene, glass, silicone or aluminium. Surface or coating materials have a significant influence on different biomarkers [1, 3–6]. Thus, the material of the entire collection system including sample vials should be inert or must be standardised for each EBC component of interest.

Efficacy of condensation: EBC collection devices work at different cooling temperatures ranging from zero to below −20°C. Pre-cooled devices are sensitive to higher ambient temperatures. The efficacy of condensation mainly depends on: 1) the breath volume passed through the system over time; 2) the condensing surface area; and 3) the temperature gradient between exhaled breath and sampling system. Increasing the condensing surface has been shown to increase EBC volume and the number of biomarkers detected [5]. Different components in EBC are differentially sensitive to cold temperatures, and the concentration of some constituents depends on the condensing temperature [4, 7, 8]. Efficacy is improved by the use of a closed condenser design with breath recirculation, especially in young children [9], or by fractionated sampling to separate EBC originating from proximal and more distal airways [10, 11].

Recommendations for future research: For each EBC component, the optimal condensing material and method should be defined. Comparative methodological studies on collection system and their efficacy are needed.

Exhaled breath condensate collection procedure
Safety: The EBC collection procedure is noninvasive and only requires tidal breathing. Collection does not modify airway surface conditions, and is safe and without adverse effects, even in young children and adults with severe lung disease [1].

Time of collection versus volume: Previously, subjects were asked to breathe tidally over a defined period of time. This recommendation needs to be revised because this mode of sampling results in a widely variable volume of exhaled breath. Presuming constant condenser conditions, the volume exhaled per time

(*i.e.* minute volume) has been identified as the most important factor for EBC volume collected per time [12, 13]. Consequently, the volume of exhaled breath, the volume of condensate collected from the exhaled volume and the time of collection have to be reported in order to assess efficacy of EBC collection.

Breathing pattern and lung function: The tidal breathing sampling does not affect lung function [13], but variables in the spontaneous breathing pattern may significantly influence EBC collection and composition [14]. Low airflows are advantageous because the collection becomes increasingly inefficient with increasing expiratory flow rates [13]. Hence, it is advised that subjects refrain from exercise for at least 1 hour preceding EBC collection [15]. Slow breathing cycles, *i.e.* quiet tidal breathing, are recommended because low tidal volumes and high dead-space ventilation in relation to alveolar ventilation lead to EBC samples that mainly derive from conducting airways rather than from peripheral ones. Different origins may remarkably affect EBC composition, and a larger proportion of dead-space ventilation contributes to EBC dilution (by condensed water) and to a greater influence of ambient (inspired) air [16].

Mouth versus nose breathing: Significant differences in EBC composition have been demonstrated between mouth and nose breathing, *e.g.* with respect to exhaled biomarkers [17, 18]. When mouth breathing is performed, the use of a nose clip is advised because it: 1) prevents inhalation of air through the nose and, therefore, contamination with possible biomarkers from the nasal epithelium; 2) prevents leakage from lower airways *via* the nose; and 3) prevents mixing of nasal and bronchial air. Salivary contamination should be limited by periodic swallowing. Monitoring by amylase measurements is still controversial. Validation by sufficiently sensitive amylase assays is needed. Microbial activity in the oropharyngeal tract significantly contributes to the concentration of nitrogen oxides in EBC, and may be prevented by mouth rinsing, *e.g.* with chlorhexidine [19].

Environmental contamination: Important confounding factors may derive from outdoor or indoor ambient air. Possible mechanisms are as follows: 1) ambient air can directly contribute to EBC composition; 2) inhaled mediators can react with substances in EBC; and 3) inhaled mediators can give rise to inflammatory or immunological responses in the airways, which in turn alter EBC composition. This has been shown exemplarily for keratins, proteins and H_2O_2 [20, 21]. To avoid any contamination of EBC from undefined environmental conditions, it is highly recommended that a suitable filter be adapted on the inspiratory valve. Cleaning procedures may also influence biomarker concentrations [1]. Therefore, a careful assessment of any potential confounding arising from cleaning material should be carried out. The cleaning routine used in lung function testing and nebulisers when used in hospitals are described in detail in the relevant guidelines, but they cannot be used as direct evidence for condenser cleaning [22, 23].

Ambient conditions: Ambient temperature and relative humidity may contribute to the variability of EBC results, as shown for pH [24]. When EBC collection is implemented in field studies, it should be taken into account that breath temperature can significantly change between winter and summer, which will influence the temperature gradient between exhaled breath and the collecting system [16].

Recommendations for future research: Define exhaled volume for EBC collection and report time of collection and volume of EBC obtained, or define time for EBC collection and report the other two variables in parallel. Report: collection temperature and condenser material, characteristics of the breathing pattern, prevention of salivary and environmental contamination, cleaning procedures, and ambient conditions. The influence of these factors needs to be evaluated in further studies.

Storage and processing of exhaled breath condensate samples

EBC also contains unstable volatiles: during and immediately after collection, volatile substances can be released (evaporation), and EBC composition can change owing to ongoing biochemical processes. For example, storage for only 1 hour at room temperature causes a significant decrease in the partial pressure of CO_2 and increases EBC pH [13]. Also, data on the stability of H_2O_2 in frozen EBC samples are conflicting and range from 2 days to 2 months. Thus, measurements of at least pH and H_2O_2 have to be performed in real time or immediately after collection without freezing or storing EBC [14].

Standardisation of exhaled breath condensate pH measurement: Different lines of data suggest that the most important confounder of pH measurement in EBC is the presence of CO_2 in the samples. Standardisation of pH measurements in EBC requires the elimination of the confounding effect of CO_2. In one approach, EBC pH is measured after removing CO_2 from the sample by de-gassing (*syn.* deaeration or degasification), using an inert gas such as argon. Importantly, de-aeration cannot completely eliminate CO_2 from EBC samples. In an alternative approach, rather than attempting to remove CO_2 from EBC, samples are instead sequentially loaded with CO_2 gas. At regular time points during the CO_2 loading procedure, aliquots are taken for simultaneous pH and carbon dioxide tension (P_{CO_2}) measurements by means of a blood gas analyser. By plotting several pH/P_{CO_2} value pairs for each sample, EBC pH can be easily determined for any given P_{CO_2} value using regression analysis. This method yields highly reproducible EBC pH values at the

normal alveolar P_{CO_2} of 5.33 kPa [25–27]. Some researchers argue against artificial manipulation of EBC; however, they address only deaeration, not standardisation by CO_2 loading [16, 25–27]. Regarding deaeration, it is still unknown how many volatiles besides CO_2 are removed while bubbling gas through an EBC specimen, and how the complexity of EBC is changed by this procedure [28]. There is also no standardised protocol for de-gassing so far. It has been demonstrated that different de-gassing procedures (bubbling *versus* surface delivery) with changing durations significantly influence both losses of EBC volume and concentrations of EBC components [29]. On the contrary, CO_2 loading has been shown to provide the least variability observed so far in EBC pH measurement [26]. There is an approximate two order difference in the logarithmic scale of pH depending on the method used for standardisation, *i.e.* pH readings of de-gassed samples with CO_2 concentration close to zero *versus* pH readings at a standardised CO_2 concentration at a CO_2 level of 5.33 kPa (physiological alveolar CO_2 partial pressure) as determined by regression analysis in CO_2-loaded samples.

Storage (temperature, material, duration): If EBC samples need to be stored, conservation should be performed immediately after collection (in order to avoid any errors by evaporation of volatile components or by ongoing biochemical processes). Storage material should be inert, as recommended for EBC collection materials [6]. Recent suggestions focus on immediate freezing using dry ice and storage at −80°C until analysed [4]. Freeze-drying (lyophilisation) of EBC has been proposed as a promising method to concentrate the sample [29]. However, at present, very little is known about the reliability and reproducibility of results if freeze-drying is applied to EBC samples [30]. For most EBC components there is still a lack of knowledge of how long they remain stable during storage. No relationship was found between the concentration of EBC cytokines and storage times of up to 1 year in samples stored at −80°C [31]. No significant loss of isoprostanes was found in a 2-week stability study at −80°C [32]. In contrast, leukotrienes undergo significant degradation within a few weeks or months [33].

Recommendations for future research: The time between collection and analysis should be as short as possible, and the stability of each biomarker should be checked during the storage period. If sample additives such as assay reagents and protease inhibitors are used, this should be clearly described. Routine deaeration of EBC before pH measurement can no longer be recommended. Because of the large difference in observed values between the different methods for measuring pH, the same methodology should be applied in longitudinal and multicentre studies. Future research is needed to address the optimal choice for EBC pH measurement. In the meantime, pH measurements should be performed twice, *i.e.* before and after processing EBC, and both results reported together with the processing technique, or pH readings at a standardised CO_2 concentration at a CO_2 level of 5.33 kPa should be provided. The effects of storage time and storage conditions on the stability of different components need to be carefully evaluated for each substance. Further studies providing standardised processing protocols are warranted.

Interpretation of exhaled breath condensate data

One of the key problems is that most of the concentrations measured in EBC have been published in the units they were measured in as raw data in the liquid sample, *e.g.* $pg \cdot mL^{-1}$, $nmol \cdot mL^{-1}$ or $\mu mol \cdot L^{-1}$. In fact, 1 mL EBC cannot be considered a standardised biological specimen at all, because the percentage of condensed liquid of the exhaled volume is not constant for each collection process. Instead, one has to be aware that different collection systems and procedures will generate differently diluted condensates with variable characteristics, despite similar concentrations in exhaled breath.

How to standardise the level of a biomarker assessed in exhaled breath condensate

Dilution by water: The degree of dilution of EBC by condensed water mainly depends on: 1) the efficacy of the collection system; and 2) the individual breathing characteristics. The use of different dilution factors (*e.g.* urea, conductivity or total cations) [34] or calculating the analysed mediator in relation to the conductivity of the given EBC sample [35] have been proposed for better standardisation with a very wide range of reported physiological dilution rates (between 1000 and 48 000).

Exhaled breath volume per time: Owing to their solubility or reactivity, gaseous components can be assessed in the liquid EBC sample. For volatile substances, it is the quantity that has been exhaled in relation to the exhaled volume per time that is of interest [36]. This recalculation is possible when taking into account the exhaled volume, the time of EBC collection and the volume of collected EBC. When this approach was applied to volatiles and even non-volatiles in EBC, the quantities of lactate exhaled per minute and quantities of H_2O_2 or leukotriene B4 exhaled per 100 L of exhaled breath were less variable compared to concentrations assessed per millilitre of EBC [16, 37].

Particle-associated EBC components: The presence of non-volatile molecules in EBC (proteins, cytokines, *etc.*) is most likely linked to the exhalation of micro-droplets, *i.e.* aerosols or particles. Aerosol formation can be simply explained by the bronchiole fluid film burst model [38]. This hypothesis states that aerosols

are formed by a process of respiratory fluid film or bubble bursting during the reopening of respiratory bronchioles by inhalation and the subsequent fragmentation of droplet aerosols that are drawn into the alveoli until emission during the next exhalation. An increasing number of publications support this model [39, 40]. Emission of particles by exhalation is mainly dependent on individual lung physiology and respiratory pattern [40]. Consequently, the quantity of droplet-associated EBC components differs significantly between subjects, is not normally distributed and presents significant inter-subject variability that exceeds variations caused by airway diseases [16, 40]. To overcome this problem, normalisation of non-volatile EBC components in relation to the emission rate of exhaled particles has been proposed. This approach requires on-line monitoring of exhaled particles in future EBC studies [41].

Recommendations for future research: Any analysed concentration in the liquid condensate underlies multiple methodological sources of variability given by the collection process. To reduce the confounding influence of dilution, the concentration of EBC components exhaled per 100 L of exhaled breath and/or per minute should be recalculated. Further concepts of interpretation are urgently needed that take into account the exhaled particles as well.

Reproducibility, dilution factor and concentration of samples

Three important points drive the reproducibility issue: 1) the lack of standardisation of EBC collection methods and validation of biomarker measurement across different laboratories; 2) the fact that many biomarkers have been detected near the lower limits of the assay; and 3) the absence of a valid dilution factor and/or concentration method [5, 42, 43]. Regarding the dilution factor, it has not yet been convincingly demonstrated that better reproducibility can be achieved by normalising EBC data with a dilution factor.

Recommendations for future research: Give details of intra-assay and inter-assay reproducibility measurements carried out using EBC samples by using appropriate means of evaluation. Specify the lower limit of detection. Difficulties related to the sensitivity of detection methods need to be solved, appropriate statistical approaches (*i.e.* non-parametric tests) should be used and the proportion of samples below the detection limit must be reported.

Age and sex, food and drink

Some differences depending on sex and age have been detected in various EBC biomarkers [26, 44], although data are conflicting. Children adequately perform EBC collection with the same technique used in adults [11].

Food and drink might affect the levels of directly related mediators. For example, food and beverages containing oxidants, NO-related products or affecting acidity might influence any markers relating to NO, pH or oxidative stress [45, 46]. The time between food intake and EBC collection can significantly influence EBC composition [20].

Recommendations for future research: Age and sex should be recorded and taken into account as confounding factors in any EBC assessment. When measuring mediators known to be affected by certain drinks or foods, it is advisable that subjects avoid these for at least 8 hours prior to measurement. EBC collection systems suitable for infants and preschool children need to be developed.

Diurnal variability

Diurnal variability has been demonstrated for EBC H_2O_2 level in healthy subjects and patients with chronic obstructive pulmonary disease (COPD) [47, 48].

Recommendations for future research: In order to consider the potential role of diurnal variability, sampling should be planned for the same time of day. The diurnal variability for mediators in EBC should be investigated.

Ventilated patients

Numerous studies have been performed using EBC in association with mechanical ventilation. However, EBC collection in ventilated subjects is influenced by multiple factors, such as artificial humidification and ventilator mode [49].

Recommendations for future research: EBC analysis may be used to monitor lung inflammation and injury in ventilated patients. Effects of underlying mechanisms should be assessed.

Systemic diseases

The number of studies investigating EBC mediators in systemic diseases has recently increased. Possible new markers have been proposed for systemic diseases involving the lungs, such as 8-isoprostane,

prostaglandin E_2 and nitrates for cystic fibrosis; interleukin (IL)-8 for systemic lupus erythematosus; IL-4, cysteinyl leukotrienes and 8-isoprostane for systemic sclerosis; and H_2O_2 in uraemia [50, 51].

Recommendations for future research: Further investigate the role of EBC components in systemic diseases and compare with already established parameters of disease assessment.

Smoking

Different inflammatory and oxidative stress EBC biomarkers including H_2O_2 are sensitive to tobacco smoking. New "omic" markers have recently been investigated in smokers using genomic, proteomic, metabolomic and epigenomic approaches [52, 53]. A few EBC studies on IL-4, IL-5, IL-10, IL-17, γ-interferon and 8-isoprostane in water-pipe smokers are available [54].

Recommendations for future research: Consider studies of smoking cessation and the use of electronic nicotine delivery systems and their effects on different mediators.

Medications

Several studies have investigated the effect of medication on EBC constituents in a systematic manner [55–59]; however, a number of these studies lack a long-term controlled design.

Recommendations for future research: Consider the potential effect of medication. Plan to perform longitudinal controlled studies to evaluate the possible effects and/or the use of mediators as a tool of guided treatment strategy.

Reference values

Standardising EBC evaluation requires the establishment of reference values in healthy subjects. Some of the most widely studied biomarkers that have data published by independent research groups for the establishment of reference values are H_2O_2, 8-isoprostane, adenosine, pH and leukotrienes [1, 60, 61]. However, further standardisation of collection and measurement methods is required.

Exhaled breath condensate mediators in different diseases

There are a large number of studies on EBC mediators in different respiratory and other diseases [1, 9, 48, 62–82]. More details on this area are provided in supplement 1.

Recommendations for future research: Further clinical studies are needed in EBC, including the establishment of reference values.

Conclusions for exhaled breath condensates

It is unlikely that "one standardisation" will fulfil the requirements of the different substances measurable as potential biomarkers in EBC. Likely, different steps of standardisation need to be adjusted for EBC components of interest. The relevance of future EBC publications depends not only on a scientific pro/con description of specific biomarker findings in a certain group of subjects, but also on the inclusion of a systematic and meticulous description of the methods and techniques used to collect, preserve and analyse EBC. Enabling technologies facilitates the use of this human sample that is known not only for its potential, but also for the difficulty in handling it [83, 84].

Exhaled volatile organic compounds

Molecular analysis of exhaled breath volatiles (breathomics) provides a noninvasive tool for exploring the human body, including the lung and other organs [85–89]. High-throughput omics technologies based on unbiased systems biology approaches, including transcriptomics, proteomics, lipidomics and metabolomics, are increasingly used for phenotype discovery and have potential as "point-of-care" diagnostic tools [90–96].

VOCs arise in exhaled breath, urine, blood, saliva and faeces, and are also emitted by skin [97]. VOCs can have an endogenous metabolic origin either related or not to previous exogenous exposures (smoking, medication, food, *etc.*) [98] or can originate from bacteria in the gut or airways. Exhaled VOCs can also include any VOCs inhaled from the environment, including the sampling device. The biochemical background is only known for a few endogenous VOCs, such as isoprene and acetone. The concentration of exhaled VOCs is influenced by the compound-specific blood:gas partition coefficient [99], cardiac output and alveolar minute volume [100]. Furthermore, ingestion of precursors (*e.g.* valproate or ^{13}C-dextramethorphan) can result in exhalation of their volatile metabolites (*e.g.* 3-heptanone or $^{13}CO_2$). This is important in enzyme-specific breath tests [101].

Sampling

The choice of the methodology depends on the application. Issues related to exhaled breath sampling include correction for ambient inspired volatiles, type of sampling (total *versus* alveolar breath), sampling duration (single-breath *versus* fixed-time or fixed-volume breathing), effect of expiratory flow and breath hold, type of collecting materials, VOC recovery, sample pretreatment, effect of humidity, food/medications, exercise, smoking and co-morbidities [85, 102, 103].

All sampling methods require standardisation and external validation in multicentre studies. The International Association of Breath Research and a consortium of breath-researchers are pursuing this and aim to develop an open-source standardised methodology for breath sampling that currently awaits validation in clinical trials (details are available at www.breathe-free.org). Because the measurement of exhaled volatiles is in its discovery phase, the sharing of knowledge and experience is very valuable. Therefore, as a breath community, we are expressing our disagreement with patenting biomarker patterns (or signal patterns) and sampling methods of exhaled breath because it slows down the innovation cycle.

Sampling methods

Sampling devices vary between young children, adults and severely ill patients.

Two complimentary approaches are advocated to reduce the effect of environmental VOCs. First, ambient VOC concentration can be subtracted from exhaled VOC concentrations (alveolar gradient) [102], although this does not account for interactions with the patient [104]. Second, inhalation/exhalation filters can be used for breathing (relatively) VOC-free air before sampling (wash-in phase) [105]. However, alveolar gradients can be affected by several pulmonary kinetic factors [106]. In addition, set-ups designed to completely reduce background contamination have been unsuccessful [102].

Exhaled breath can be collected by mixed expiratory sampling (total breath including anatomical dead-space air) [105] or alveolar sampling (obtained as enriched alveolar breath by expired CO_2 triggers or by discarding the first part of exhalation corresponding to dead-space) [107]. The choice of focusing on VOCs derived from alveoli and/or conducting airways depends on the disease of interest. However, when sampling through the nose or mouth, the interaction of VOCs from alveolar air with the intra- and extrapulmonary airway wall cannot be avoided. Nevertheless, comparison of both methods might help to identify the VOC source within the respiratory compartments. Selective sampling of the alveolar compartment can reduce oral contaminant concentrations [106], but is technically more demanding [108].

Breath sampling during a fixed time can reduce variations in breath-to-breath volatile concentrations reported in single-breath analysis [106, 109]. Alternatively, breath sampling from a predefined fixed volume is also used. Recently, a new method of integrating real-time breath sampling with spirometry was technically and medically validated. Physico-chemical properties of individual volatiles and collecting materials affect relative VOC recoveries [110]. Ideally, collection devices should be inert and/or disposable, given that many materials and cleaning agents emit VOCs with a high risk of carry-over effects even under stringent conditions [108]. As an alternative to single use, stringent cleaning regimes for collection devices (*e.g.* Tedlar or Mylar bags) by repeated washings with ultra-pure nitrogen have been shown to provide acceptable repeatability [111, 112]. It should be noted, however, that remaining or variable VOC contributions by Tedlar bags are difficult to recognise using cross-reactive sensors in electronic noses (e-noses).

Conditioning volatile organic compound samples

VOCs can either be analysed directly (real time) or undergo several pretreatment steps. VOC concentrations are generally in the parts per billion to parts per trillion (nM to pM) range [113]. For this reason, pre-concentrations of breath samples by adsorption onto sorbent traps or coated fibres may be required to provide a sufficient signal on the used analyser. VOC adsorption onto sorbent traps, the most common sample pretreatment procedure, also increases sample stability [114]. However, different sorbents absorb VOCs selectively. This enables focus to be directed to target volatiles, but reduces molecular information. The detection limits of different analysers vary widely, but new developments are bringing high-sensitivity analytical technologies into marker detection that are capable of detecting volatiles in the low parts per billion range, bringing the analysis of exhaled VOCs closer.

The response of some types of sensor is affected by water vapour; this is a critical issue for e-nose research on exhaled samples [103, 115]. Different approaches have been used to reduce the effect of ambient/sample humidity on breath VOC analysis, including the use of silica gel [105] and potassium chloride traps [107]. However, these methods can also affect VOC levels and completely removing water vapour has thus far not proved possible. Adsorbent materials (*e.g.* Tenax®), which have a very low water-binding capacity, can reduce the water vapour effect [116]. Another approach is to leave out known water vapour signals from the

analysis [117], but by doing this one may lose signals generated by biomarkers on the same sensor; therefore, this potential shortcoming needs to be taken into account.

Breathing pattern

Exhalation flow-dependency has been reported for acetone [118], ethanol [119], isoprene [120, 121] and pentane [120], suggesting that these compounds derive, at least partially, from the airways. In line with this, exhalation flow alterations significantly affect exhaled breath patterns assessed with e-nose, contributing to the variable ability of these devices to discriminate breathprints [117]. Similarly, exhaled VOC profiles as determined by e-noses integrated with spirometry are dependent on expiratory flow [109]. It is as yet unknown if changes in airway calibre affect VOC concentrations, but e-nose breathprints have been shown to be independent of acute changes in airway calibre in asthma [122].

During single-breath sampling, a single expiratory vital capacity manoeuvre is generally used [104, 107]. During this procedure subjects are instructed to exhale immediately after a deep inhalation to total lung capacity because VOCs, including acetone [118, 119], ethane [120], isoprene [120, 121], methanol and dimethyl sulfide [118], may accumulate in the alveoli and airways during prolonged breath hold. Infants may require tidal breathing sampling with minimal respiratory resistance [123, 124], while VOCs can be sampled from the ventilator circuit in patients ventilated in the intensive care unit [125, 126].

Subject/patient

Potential confounding factors in breath analysis include diet [119, 127, 128], inter-subject variability in drug elimination [129], pharmacological treatment [87, 130], exercise [15, 131–133], smoking [134, 135] and co-morbidities [136]. Common efforts to reduce the impact of these factors on breath analysis include prohibition of exercise, smoking, food and drink intake 2–3 h prior to testing. Whether these factors improve reliability is, however, still to be determined.

Volatile organic compound measurements

Exhaled breath analysis can roughly be split into two main streams [137]: 1) an analytical molecule identification-based stream; and 2) a sensor technology, pattern recognition-based stream. The analytical mass spectrometry (MS) track, often coupled to a separation technique like gas chromatography (GC), is focused on identifying biomarker compounds related to particular disease conditions and the accompanying pathophysiology. The second is a cross-reactive sensor technology that is purely based on pattern recognition of complex mixtures. This is represented by e-nose technologies [103, 115] that are used for probabilistic predictive values in relation to health and disease.

Identification of volatile organic compounds and their concentrations

During MS, ions are created from the VOCs present in a gas mixture. On the basis of the mass-to-charge ratio (m/z) of their product ions, compounds can be detected and identified. Ionisation is achieved using electron or chemical ionisation. GC-MS and two-dimensional GC-MS (GCxGC-MS) mainly use electron ionisation, causing fragmentation of compounds and giving library-searchable spectra, whereas chemical ionisation and atmospheric pressure chemical ionisation are often used to elucidate structure. Other analytical identification techniques include proton transfer reaction MS (PTR-MS) and selective ion flow tube MS (SIFT-MS), which are driven by soft chemical ionisation. GC-MS is seen as the leading technology for VOC analysis, with very high sensitivity and selectivity. However, it cannot be used for real-time exhaled breath measurements. PTR-MS and SIFT-MS can both be applied in real time and have the option (and limitation) to ionise compounds selectively [138].

The use of MS technology is valuable for gaining insights into pathophysiological pathways in the field of pulmonology. Compounds that can be linked to the presence of pathogenic fungi and bacteria can be found [139–142], along with data on VOCs related to inflammation, obstructive lung diseases and lung cancer [86, 119, 143–148].

Pattern recognition of volatile organic compounds

Whenever biomarkers are not known, many non-selective sensors are needed to obtain a fingerprint of the measured VOC mixture [149, 150]. The registered fingerprints are treated with pattern-recognition techniques (within the machine-learning field) to be recognised as pertinent to certain classes (diseases). This probabilistic classification workflow allows diagnosis, phenotyping and monitoring based on VOC fingerprints:

1) Starting points:
   - sensors technology for VOC mixture content capturing and assessment [151–153]
   - clinical parameters to be correlated with sensor outputs [154]
   - suitable and reproducible exhaled breath sampling protocols [114, 155].

    2) Explorative analysis:
- to study sensor correlation with specific (target) parameters
- sensor selection for the target disease.

    3) Supervised analysis devoted to:
- disease discrimination (diagnosis and monitoring) [99, 150, 156]
- disease stage identification (diagnosis and monitoring) [157, 158]
- disease characterisation (phenotyping) [143, 159].

Organic and inorganic films and polymers are used as sensing materials and they are coupled with many different working principles: conductometric, acoustic, optical and electrochemical [115]. Each of them needs specific temperature and humidity operating conditions [150, 160]. Sensing material, working principle and operating conditions strongly influence the selection of specific VOC spectra [160].

Calibration

The quantitative analysis of breath samples by GC-MS [161, 162], e-nose [151, 163], ion mobility spectrometry (IMS) [164, 165], field asymmetric IMS [165, 166], SIFT-MS [167, 168] *etc.* requires quality control by means of an appropriate calibration procedure [169–171]. Further details on the required calibration procedures are given in supplement 2.

Analysis
Processing

The signal that results from the VOC measurement (ion count in GC-MS or cross-reactive sensor response) has to be processed into a matrix of features that is suitable for statistical analyses [89]. One important preprocessing step is normalisation, which aims to reduce the effect of systematic variation (*e.g.* due to variability in sample volumes) between samples by adjusting signal intensity by, *e.g.* 1) total intensity; 2) highest value; or 3) intensity of a standard [171]. However, it remains unclear if the basic assumptions to allow normalisation are always met in breath analysis [89]. Nevertheless, careful preprocessing, normalisation and environmental correction are required for optimal results [109] and therefore should be extensively reported in all studies.

Statistical analysis

Appropriate statistical analysis is of major importance [89, 95, 136, 172–175], see supplement 3 for further details.

Clinical accuracy

The potential clinical usage of exhaled breath biomarkers has traditionally been explored by examining associations of the presence of a disease with one or more individual VOCs or particular composite patterns of VOCs [148, 149]. For correct interpretation of findings (similar to assessing other potential biomarkers), authors should follow international standards to accurately report findings described by different expert panels (*e.g.* STARD, CONSORT and TRIPOD) [176–179].

Internal validation and external validation

Internal and external validation of omics data is crucial [94–96, 103, 148, 149, 180–187], see supplement 4 for further details.

Priorities for technical research on exhaled volatile organic compounds

There is a great need for a wide-scale multicentre study to compare different sampling methods, equipment and determination of exhaled volatiles by different techniques at different sites to address issues of standardisation and to assess the current clinical utility of the available technology. Further research is needed both in the technical areas of breath sampling and testing and on the clinical relevance of breathomics [109, 175, 188]. A detailed list of important areas for further research is provided in supplement 5.

Recommendations for future research: There is a great need for further standardisation of sampling and analysis procedures of exhaled breath VOCs to substantiate the promise of volatiles in clinical medicine [103, 137, 189]. Such standardisation requires a sensible balance between imposing restrictions and allowing ample room for innovation [103, 190]. Further details of research priorities are provided in supplement 5. We are still relatively at the beginning of VOC research in medicine and have yet to further discover and define VOCs and VOC patterns with established clinical add-on value with regard to diagnosis, phenotyping or prediction of clinical course in medical practice [191–207]. This will require

stringent procedures for stepwise validation of composite biomarkers [94–96, 176, 178]. Only then will VOCs find their way into daily clinical management.

In this rapidly growing scientific area we are in the exploratory phase of relevant research. Improved understanding of the source, the dynamics of VOCs present in the breath in health and disease, their influencing factors, and the complex interplay between different compartments and between the microbiome and metabolism [208–213], together with critical evaluation of currently used methods and active development of data handling, analysis and interpretation have changed the landscape in this field at an unexpectedly fast speed [103, 214–217]. Shortening the innovation cycle brings new technology to medical research in the area of VOCs [217, 218]. The process is further facilitated by studies in other species that help in our understanding of the factors in physiological variability [219–221], which are also aided by active research in the field of infective agents [222, 223].

Exhaled nitric oxide

The last ATS/ERS recommendations for exhaled NO (F_{eNO}) measurements described several aspects of standardisation [2] and are used worldwide. The ATS/ERS 2005 document is still valid and is mostly still applicable in 2016; therefore, these parts are briefly summarised. Since the development of these recommendations, the field has grown further, and new devices have been developed and used. Furthermore, mathematical modelling of pulmonary NO dynamics, sometimes called extended NO analysis, has been developed [224]. The aim of the current document is to add to the existing recommendations by clarifying the terminology further (see Box 1), by evaluating nasal NO measurement in light of recent publications and to introduce recommendations for NO dynamic models in order to compare and pool data for further analysis.

F_{eNO} emerged in the 1990s as a noninvasive marker of airway inflammation in asthma, but since then has been studied in many other disease entities, including COPD, scleroderma, obstructive sleep apnoea syndrome, nasal epithelia disorders, cystic fibrosis and hepatopulmonary syndrome [2]. F_{eNO} has also been widely used in studies of respiratory effects of environmental exposures [225–227].

Measurement of F_{eNO}

Recommendations for the determination of F_{eNO} have been published [2, 228, 229] and the ATS/ERS guidelines [2] that cover most areas are still valid, but there are technical issues that need updating. This was felt to be important even if, regarding clinical utility, the discriminating power (specificity and sensitivity) in diagnosing or tailoring the treatment of asthma has not allowed this method to be part of the World Health Organization (WHO) recommendation for asthma diagnosis or monitoring [230]. $F_{eNO_{50}}$ may be elevated in healthy subjects [231] and may be normal in subjects with asthma [232, 233]. Thus the cause and even the origin of exhaled NO seem to be complex. However, more studies have recently indicated the usefulness of F_{eNO} as a biomarker for asthma phenotyping and management [234]. Most specifically, it is now considered that elevated $F_{eNO_{50}}$ values *per se* are not sufficient to ascertain the diagnosis of asthma. Rather, high $F_{eNO_{50}}$ values help to identify T-helper 2 cell-type inflammation, a trait commonly seen in patients with asthma [235]. $F_{eNO_{50}}$ in asthmatic patients is associated with the risk of exacerbations and the likelihood of a positive response to inhaled corticosteroids [236, 237]; therefore, its measurement bears clinical significance. For $F_{eNO_{50}}$ measurement, inhalation to total lung capacity is recommended by the ATS/ERS guideline [2]. However, arguments supporting a more comfortable procedure for the patients without affecting the results [238] have

Box 1	Terminology
F_{eNO}	fractional concentration of exhaled NO in the gas phase (ppb). Exhalation flow rate is given as a subscript in mL·s^{-1}. A flow rate of 50 mL·s^{-1} is written $F_{eNO_{50}}$.
F_{nNO}	fractional concentration of nasally aspirated/exhaled NO. The flow rate of the aspirated NO, usually 5 mL·s^{-1}, is given as a subscript in mL·s^{-1} (*e.g.* $F_{nNO_{5}}$). A nasally exhaled NO value at 50 mL·s^{-1} is given as $F_{nNO_{50}}$.
C_{ANO}	concentration of NO in the gas phase of the alveolar or acinar region (ppb).
C_{awNO}	tissue concentration of NO of the airway wall (ppb).
D_{awNO}	airway compartment diffusing capacity of NO from the airway wall to the gas stream (mL·s^{-1}).
J_{awNO}	total flux of NO in the conducting airway compartment (nL·s^{-1}) that takes into account the value of C_{ANO} ($J_{awNO}=(C_{awNO}-C_{ANO})\times D_{awNO}$). If C_{ANO} is zero, the flux is equal to the theoretical total maximum flux, which is $J'_{awNO}=C_{awNO}\times D_{awNO}$.
V'_{NO}	elimination rate of NO (pL·s^{-1} or nL·s^{-1}).
V'_{E}	exhalation flow rate (mL·s^{-1}).

been put forward [239]. Such methods have been adopted both by clinicians and by manufacturers. In this method, FeNO measurement is performed with a deep inhalation through the mouth and slow exhalation, with feedback of the flow rate for the subject. Velum closure is mandatory and achieved by using a positive pressure of 5–20 cmH$_2$O against exhalation. An approved measure is one in which the flow rate is within 10% of the target value, *i.e.* 45–55 mL·s^{-1}. When a chemiluminescence detector is used for NO determination, a minimum of two NO measurements is recommended by the ATS/ERS guidelines [2]. The technical requirement for acceptable measurements is that the two plateau values should be within 10% of each other; if not, then another measurement is necessary [2]. In the last 10 years, great developments have been achieved in various methods of NO analysis [240, 241] and comparisons made between them [242]. When using electrochemical sensors, no plateau is visible, and there are limited data demonstrating that performing one exhalation manoeuvre is appropriate for some electrochemical devices, with good repeatability [243, 244]. The ATS/ERS guidelines recommend two measurements and this is still a valid recommendation, although it is appreciated that if only one measurement can be performed owing to financial or other constraints, use of the mentioned handheld devices could provide valuable data.

The above information together with data showing that handheld devices are not interchangeable needs to be considered when these devices are used either for research purposes or for clinical practice. The reasons that these different handheld devices are not interchangeable are likely that some cells have better accuracy than others and the programme applied within the devices limits repeatability. A hypothetical reason is the interval when sampling into the cell takes place. At least 30 s relaxed tidal breathing is required between measurements. When collecting off-line exhaled breath, it is necessary to be able to separate dead-space gas to be able to compare with on-line NO values. Therefore, the initial 150–200 mL should be discarded, or sampling should be started when a CO$_2$ signal is present, which can help identify the dead-space volume. Ambient NO levels should be recorded and the use of NO-free air for inhalation is preferable.

Considerations

Various factors that are not disease-related can influence NO values. One such factor is spirometry, which should therefore not be performed prior to NO measurement [2]. Bronchoconstriction may cause a decrease in FeNO$_{50}$ and bronchodilation an increase [245, 246]. FeNO$_{50}$ can vary in uncontrolled asthma and is proposed to be a biomarker of asthma control and a predictor of exacerbation [247]. Drugs that may affect airway calibre should be documented as well as anti-inflammatory drugs. A questionnaire for NO measurement is recommended (supplement 6). Mouthwash is recommended when physiological research is done, because bacteria in the mouth can influence exhaled NO concentration. Because this influence is minor and does not have clinical relevance, mouthwash is not required when the measurement is done in clinical practice [248, 249]. A too-deep inhalation, with over-distension, may affect the patient's control over exhalation flow rate. There is no specific lower age limit for FeNO$_{50}$ measurements and normal values are published for children from 4 years of age. The 2005 ATS/ERS recommendations are still valid for preschool children who are unable to perform appropriate controlled exhalation and for infants [2]. Note that FeNO has been shown to be age-dependent in three distinct phases in healthy individuals [250].

Reference values

Reference values for healthy adult subjects have been published [251–253] and recommended cut-off values have been discussed to help clinical decision-making [254, 255]. In clinical practice a FeNO$_{50}$ between 25–50 ppb in adults (20–35 ppb in children) should be judged within the clinical context and NO values >50 ppb (>35 ppb in children) may be used to predict a response to anti-inflammatory therapy [254, 255]. A personal best FeNO$_{50}$ might be estimated from the use of NO parameters and be most useful when evaluating treatment with anti-inflammatory drugs [256, 257].

Measurement of FnNO

Within the nasal region, NO output is high from the paranasal sinus epithelium, where inducible nitric oxide synthase is expressed under basal conditions [258]. Altered nasal NO levels have been described in a number of conditions, including allergic rhinitis, sinusitis, nasal polyps, cystic fibrosis and primary ciliary dyskinesia (PCD); a disease characterised by low nasal NO output [259]. Several studies demonstrate that FnNO accurately identifies individuals with PCD, supporting its usefulness as a screening tool [260].

Like FeNO, FnNO is flow dependent [239]. Usually the intrinsic flow of the NO analyser could preferably be used, *i.e.* 5 mL·s^{-1}. However, the flow rate must be tested. Data can be presented as concentrations but NO output should also be given. The choice of flow rate depends on the subject and methods. There are two recommended methods: aspiration with serial sampling and exhalation with parallel sampling [261–263]. Before measurements start the patient should blow their nose and assure free airflow in both nostrils.

Aspiration with serial sampling
Aspiration is done through one nostril with the use of a tightly fitting nasal olive with the other nostril open. The subject performs a deep inhalation and holds their breath to obtain velum closure, while air circulates from one naris to the other around the posterior nasal septum. Velum closure may also be obtained by oral expiration against a resistance of 10 cmH$_2$O, by pursed-lip breathing *via* the mouth, or by voluntary elevation of the soft palate. Velum closure can be monitored by measuring nasal CO$_2$. In infants and children who cannot hold their breath, measurement can be obtained during silent tidal breathing although values tend to vary more with this method.

Exhalation with parallel sampling
Measurement is done after a deep inhalation with NO-free gas, followed by slow exhalation through the nose (with the mouth closed) into a tightly fitted mask covering the nose [262] or *via* parallel tightly fitting nasal olives [263]. The subject should have feedback of the flow rate. An approved measurement is one in which the flow rate is between 45–55 mL·s^{-1} and a plateau is established at 10 s. If NO values vary more than 10% [264], another measurement should be added and a mean value displayed. Additional information to record is the ratio between FeNO$_{50}$ and FnNO$_{50}$.

Measurements of nasal NO during humming can be done to specifically study if there is gas transfer between paranasal sinuses and the nasal cavity [265].

Recommendations for future research: On-line FnNO measurement has been well developed but the optimal measurement technique is yet to be determined. Determination of the optimal sound frequency for humming needs further research. The available studies are flawed by a small numbers of subjects and the use of multiple methods, such as different NO analysers, sampling flow and breath manoeuvres. Low flow rates require longer to reach an NO plateau than higher flow rates and can give values outside the calibration range for the instrument. Most studies in the literature employ a chemiluminescence method, sampling nasal gas by aspiration directly from one nostril, using the intrinsic flow of the chemiluminescence analyser or an external pump. The recent introduction of portable electrochemical devices for handheld NO measurements poses additional methodological difficulties, but the use of exhalation at a flow rate of 50 mL·s^{-1} can be recommended.

A reliable and standardised method for FnNO measurement is necessary to enable comparison of results from different laboratories and to formulate appropriate reference values.

Researchers have promoted nasal NO measurements as being potentially useful in screening for disease and in monitoring the effects of treatment. However, this has been proved only for PCD and more work is needed to establish nasal NO as a potential biomarker of other diseases.

Reference values
Reference values, although desirable, are not available. Cut-off values have been proposed based on a limited number of healthy controls and diseased patients but at different flow rates [266–269].

Estimation of alveolar and airway nitric oxide parameters
Exhaled NO at low flow rates reflects NO dynamics mainly in the large central airways and is quite insensitive to the changes in NO dynamics in the periphery of the lungs (small airways and lung parenchyma). Mathematical modelling of pulmonary NO dynamics, sometimes called extended NO analysis, has been presented based on a simple and robust two-compartment model of the lung. In short, the model consists of an expansible part representing NO dynamics in respiratory bronchioles and alveoli (alveolar or acinar compartment) and a single cylindrical tube representing larger conducting airways from the trachea to respiratory bronchioles (bronchial compartment) [270, 271]. The model has been further fine-tuned by taking into account the increasing cross-sectional area of airways towards the lung periphery (trumpet-shaped airways) and the possibility of axial back diffusion of NO from conducting airways towards the alveoli [272]. Statistical methods have also been used to improve the estimation of NO dynamics [273].

General equation of the two-compartment model
The two-compartment model predicts FeNO (or NO output) as a function of flow through the non-linear equation:

$$V'_{NO} = V'_E \times F_{eNO} = V'_E \times (C_{awNO} + (C_{ANO} - C_{awNO}) \times \exp(-D_{awNO}/V'_E)) \tag{1}$$

J_{awNO} can be calculated as follows:

$$J_{awNO} = (C_{awNO} - C_{ANO}) \times D_{awNO} \qquad (2)$$

The most frequently used models in the literature are the linear and the non-linear models. When new models are developed they should preferably be compared to one of these.

Non-linear model: Fitting a non-linear curve (Eq. 1) on a plot of NO output *versus* flow rate allows the derivation of C_{ANO}, D_{awNO} and C_{awNO} [271]. The Högman & Merilänen Algorithm (HMA) is such a model [239, 274, 275]. A special added feature of the approach by the HMA is to use an algorithm to test if the measured set of data points is mathematically consistent with the model. The condition required for data to guarantee a valid solution for D_{awNO} and C_{awNO} can be written in general form:

$$\frac{F_{eNO_{low}} - F_{eNO_{medium}}}{F_{eNO_{medium}} - F_{eNO_{high}}} < \frac{V'_{E \cdot high}}{V'_{E \cdot low}} \times \frac{V'_{E \cdot medium} - V'_{E \cdot low}}{V'_{E \cdot high} - V'_{E \cdot medium}} \qquad (3)$$

If this is not the case, the data set suffers from errors in measuring the flow rate or the NO concentration, or both. Then the measured data points are not mathematically consistent with the model.

The results of the NO parameters (C_{ANO}, C_{awNO} and D_{awNO}) can be fed into an algorithm in a standard Microsoft Excel environment to create a curve form with its respective flow rates and NO exhaled volumes. The $F_{eNO_{50}}$ is estimated and given for comparison to the measured $F_{eNO_{50}}$ as a quality control. Non-linear modelling has the least error, suggesting it is the best method at the moment [276], although it is more challenging for the patient.

Linear model: A Taylor's development of Eq. 1 around zero limited to the first term allows a simpler model to be proposed that linearly links NO output to flow rate [270]:

$$V'_{NO} = C_{ANO} \times V'_E + J_{awNO} \qquad (4)$$

A linear regression of NO output against flow rate gives C_{ANO} as the slope of the line and J_{awNO} as the intercept on the y-axis. The r-value of the regression line is mandatory because this gives the quality of the measurements, and should be >0.95.

Flows

The same technique as for $F_{eNO_{50}}$ should be applied, but the flow used must be chosen based on which method of extended NO analysis chosen, namely the linear or non-linear method. The exhaled NO plateau value is normally achieved after 10 s at a flow of 50 mL·s^{-1}. The 2005 ATS/ERS document recommended the plateau NO concentration be evaluated over a 3 s window [2], which has been included in the software of many analysers. With extended NO measurement, it takes longer to reach the plateau value at a low exhalation flow rate than at high flow rates. The plateau value at high flow rates may not extend for 3 s and may therefore be manually judged.

Non-linear model: NO values from at least three flows are needed: one low ($\leqslant$20 mL·s^{-1}), one medium (100 mL·s^{-1}) and one high (350 mL·s^{-1} or even 400 mL·s^{-1}) [270]. A minimum of two measurements are done on each flow and mean values of flows and NO concentrations are used to calculate the NO parameters. $F_{eNO_{50}}$ can be estimated. For children and adults with expiratory difficulties who are unable to perform the high flow rate, a lower flow rate can be accepted if the estimated $F_{eNO_{50}}$ is found to be within 5 ppb of the measured $F_{eNO_{50}}$. This is only applicable if the measured set of data points is mathematically consistent.

Linear model: In practice, the linear model starts with a measurement of $F_{eNO_{50}}$ that should always be given for comparison but is not included in the model. Then three exhalation flows of at least 100 mL·s^{-1} or higher with increasing steps are performed, with the highest flow rate of 350 mL·s^{-1}, or even 400 mL·s^{-1}. A minimum of two measurements are done for each flow rate. When children are unable to perform the highest flow rate, a lower one can be accepted, *i.e.* 250 mL·s^{-1}. NO output is calculated for each measurement by using the measured flows with the corresponding NO values. NO eliminations are plotted against exact measured flows and a regression line is drawn.

Recommendations for future research

The most important advantage of modelling NO dynamics is the gain of C_{ANO} that may be useful in assessing inflammation in small airways or lung parenchyma both in airway diseases and interstitial lung

diseases [224]. J_{awNO} is closely related to $F_{eNO_{50}}$ and does not necessarily add much clinical value over $F_{eNO_{50}}$, but dividing J_{awNO} into its components, namely C_{awNO} and D_{awNO}, can increase understanding of the physiological processes with increased NO excretion from conducting airways.

The drawback of modelling NO dynamics is the need for a more complex estimation method. Mathematically, at least two NO values at different flow rates ($\geqslant100$ mL·s^{-1}) must be used to compute C_{ANO} and J_{awNO}, reflecting NO dynamics in the peripheral compartment and central compartment, respectively [270, 277]. To divide J_{awNO} into its components C_{awNO} and D_{awNO}, a third low (<20 mL·s^{-1}) flow must be included [275]. In addition, a number of different mathematical solutions to calculate these NO parameters have been presented [271] and standardisation of both flows and mathematics is therefore needed to allow for comparison between studies.

Models of NO production and transport incorporating molecular diffusion emphasised that the latter brings back NO molecules from peripheral airways into the alveolar compartment, increasing C_{ANO} independently from *in situ* NO production [272, 278]: the so-called back-diffusion phenomenon. Therefore, C_{ANO} correction formulae have been proposed based on airway NO output. It is extremely important to understand that these corrections were established, and are therefore only applicable, in healthy subjects or in patients free of obstruction. In case of peripheral reduction of airway calibre, back diffusion may be less important and blind application of these formulae in disease states may lead to overcorrection [275, 279]. Calculations of NO parameters are also flow dependent and the use of correction factors will over-adjust the C_{ANO}, sometimes into negative values [275]. Therefore, the use of correction factors for axial back diffusion is not recommended (supplement 7).

If, despite no error detection in the data set, quality controls (r>0.95 for the linear model or Eq. 3 for the non-linear model) are not fulfilled or a negative C_{ANO} value is derived, the models may be inadequate. Physiologically, a calculated C_{ANO} should be positive. A negative C_{ANO} is more an indication that the model of NO production of the respiratory system is inadequate.

Reference values
There are only two publications that provide reference values for healthy adults [252] and children [280]. A few publications report values in children with asthma using the linear model [279–283] and the non-linear model [284–286], but further research is needed to evaluate their applicability in clinical settings.

Instrumental considerations for nitric oxide measurements
Basic equipment specifications for NO analysers have previously been described [2]. Currently there are several technologies available for clinical use that are based on chemiluminescence, electrochemical sensing and laser-based detection [240]. The chemiluminescence devices were the first to be implemented and are therefore considered as the standard technique. All later-developed detection methods for measuring NO are introduced in comparison to chemiluminescence.

Chemiluminescence instruments
This type of analyser measures NO indirectly, *via* light generation due to a chemical reaction with ozone. Calibration is generally recommended once a month with a calibration gas up to 2000 ppb and zero is set daily. In addition, a yearly check of chemical reaction converters and the ozone generator and its peripheral components is required. Chemiluminescence instruments provide a fast response time, between 0.5 and 0.7 s, and a good detection limit (0.1–0.5 ppb). Repeated measurements can be performed directly. The analysers can be equipped with a unit for controlling the exhalation flow rate. The investment and running costs are high for these instruments, limiting their use in routine clinical applications or home monitoring.

Electrochemical sensors
Electrochemical sensors have quickly gained popularity because they are handheld devices, weighing <1 kg. They measure NO concentration *via* a detectable electrical signal (*e.g.* current). Their detection range is from about 5 ppb up to 300 ppb and has an accuracy of ±5 ppb or ±10% of the measured value. The response time is <10 s and the analysing time is 60–100 s. In contrast to chemiluminescence analysers, the electrochemical technology that supports the handheld analysers does not allow for pre-test calibration. Each analyser includes a replaceable sensor that is changed after ∼1–2 years or after a number of valid measurements that is specified by the manufacturer. Because no regular calibration can be performed, the performance characteristics of such analysers might change over time or when a sensor is replaced [287]. The analysers are equipped with a pressure measurement set to perform an exhalation pressure of 10–20 cmH$_2$O and maintain a fixed flow rate of 50±5 mL·s^{-1}. The patient is either guided by the display or by a flow indicator.

Electrochemical analysers are exclusively used for $F_{eNO_{50}}$ measurements [243, 288]. The reproducibility of the measurements is less than 3 ppb of the measured value for values <30 ppb and <10% of the measured

value for values >30 ppb [288]. The difference in $F_{e}NO_{50}$ values when comparing with the standard chemiluminescence technique is within ±4–10 ppb. These results are considered by most users to be clinically acceptable [243]. Some analysers consider one exhalation sufficient for reliable clinical data; for others, at least three exhalations are necessary [241]. For children, multiple tests may be necessary because the analysers do not record $F_{e}NO_{50}$ from poor exhalation manoeuvres, a frequent situation when measuring exhaled NO in children. In general, electrochemical sensors appear to be suitable for routine clinical practice.

Additional techniques

Other small sensors are under development, such as the smart breath health diagnostic system [289] based on smart solid-state microsensor technology. Optical sensors are also available for the detection of NO concentrations at low levels (ppb). These measure the decrease of light intensity from a laser source due to absorption by NO. Several research groups have developed laser-based NO sensors [286, 290–294]. The instrument is designed for multicomponent analysis using lasers (NO, CO_2 with one quantum cascade laser and CO and N_2O with another), can detect <0.3 ppb of NO within 1 s, and includes a breath sampling system for multiple exhalation flows.

Technical maintenance

Chemiluminescence instruments need daily maintenance of flow rate and NO zero setting due to drift of the signals. Calibrations of NO and flow rate are done at intervals recommended by the manufacturer but any drift of the signal should be corrected before measurements are taken. Electrochemical sensors need zero setting and manufacturers present different solutions. Laser-based analysers do not require consumables, allowing for minimal maintenance. An indication of flow rate should be mandatory given that the NO signal is flow dependent. There are different methods to measure exhalation flow rate and it is critical that NO measurement is performed at the exact flow rate that it is aimed at. Compared to electrochemical cells, chemiluminescence instruments have the advantage of a fast response time and the ability to measure NO at a wide range of flow rates. The use of CO_2 is recommended with off-line measurements or with nasal NO measurements to indicate that accurate sampling is performed.

Conclusion for exhaled nitric oxide

The current statement does not aim to interpret the potential clinical uses of $F_{e}NO$ measurement or extended NO analyses. Despite the large number of studies that exist in the field of exhaled nitric oxide measurement, further studies are needed with regards the reference values of NO modelling to reach data comparability. Further exploratory work is also needed for the appropriate standardisation of nasal NO measurement.

Particles in exhaled breath

Exhaled breath contains an aerosol of small particles. All non-volatiles identified in EBC are probably derived from these particles. Condensation is, however, an inefficient method to collect non-volatiles because many particles pass the condenser without being collected. Importantly, there is large inter-individual variation in the endogenous production of exhaled particles that needs to be accounted for when aiming at quantitative analyses [295, 296].

The number of particles in exhaled breath (PEx) is very low during tidal breathing, much lower than in the ambient air, and does not seem to be influenced by obstructive airway disease [297]. By using a breathing manoeuvre allowing for airway opening following airway closure, there is a substantial increase (up to 18-fold) of the number of PEx [298], enhancing analyses of their chemical composition but also indicating the origin of these particles. A decrease in the number of PEx in subjects with airway obstruction has been observed [299]. These results indicate the majority of PEx originate from the very distal airways where airway closure and reopening take place. This conclusion is further supported by a dose-dependent increase in the number of PEx with increasing breath-holding times at residual lung volume and the decreasing number of PEx if breath holding is performed at total lung volume [300], the latter possibly causing a deposition of the largest formed particles within the airways.

PEx, when sampled in a manner that allows for airway opening, contain ~75% phospholipids and 25% proteins. The observed phospholipid composition of PEx is similar to that of the respiratory tract lining fluid. Time-of-flight secondary ion MS has shown that the phospholipid composition of PEx is altered in asthma and that subjects with asthma have fewer unsaturated phospholipids and that smokers have more protonated phospholipids than non-smokers [301]. In animal studies, the altered phospholipid composition of surfactant has recently been strongly associated with the development of e.g. COPD [302], indicating that this is an interesting area for future research.

In a shotgun proteomic study of pooled PEx samples from healthy individuals [301, 303], 124 proteins were identified, the majority of which were extracellular. Amylase was not detected, excluding the possibility of oral contamination of the PEx samples, and neither were any mucins, further supporting the peripheral origin of the samples.

An ELISA has been adapted and validated to analyse surfactant protein-A (SP-A) in PEx [304]. When examining the levels of SP-A in PEx and in EBC after sampling of 100 L of exhaled breath from nine healthy subjects, SP-A was well above the detection limit in all PEx samples, but only in five out of 18 EBC samples. SP-A in PEx has also been shown to be increased in COPD (stage II–IV) compared to in age-matched healthy controls, and correlated significantly with lung function in patients with COPD [305]. A new method to sample non-volatiles in exhaled breath, based on impaction, has been developed: Particles in Exhaled Air (PExA™). This method enables the number of exhaled particles in different size intervals to be counted, and thereby permits quantitative chemical analyses [284, 289]. It also allows repeated sampling of the respiratory tract lining fluid from the small airways. Other methods to sample exhaled particles, based on collection by filters, by liquid impingement and by using wetted wall cyclones, have so far only been evaluated in experimental settings [295, 300]; Teflon filters performed best, catching on average 20–30% of cytokines from a nebulised aerosol with spiked concentrations of cytokines [306]. The relatively low efficiency of particle collection by this method might explain why the concentration of these cytokines is below the detection limit of ELISA tests (while in EBC at least some of them are detectable). Using the PExA method, another approach has therefore been necessary, *i.e.* to determine the more common constituents of the lining fluid of small airways both in the healthy and disease states. This work is, however, in its early infancy.

In conclusion, sampling of exhaled particles provides a novel opportunity to identify, quantify and monitor pathological processes in the small airways. There have as of yet been no multicentre comparative studies, but standardisation of this method is crucial for future research, including the breathing manoeuvre, sampling technique and analysis of the samples.

Recommendations for future research: Further research is suggested to facilitate understanding of particle release from the airways and to better understand the potential clinical relevance of particles as exhaled biomarkers.

Overall conclusions and future expectations

Exhaled biomarkers form a rapidly growing field of research [307]. Previous guidelines have contributed to the fruitful networking and streamlining of research efforts in the field. The expectation of this document is the same: to provide an overview of the current knowledge and to look for new horizons. As in many other areas of medical research, the roots of exhaled biomarker research go back centuries to when olfaction (smell of the breath of the patient) was mentioned as being important in the detection of liver and renal failure. This, together with the ability of canines to discriminate between breath samples obtained from patients with different cancers and from healthy subjects [308], highlights those specific aspects that link the field of exhaled biomarkers to the ancient communication channels through volatile compounds. Honeybees can be trained to signal the presence of heroin and research into insect-based biosensors may also have an effect on the field of exhaled biomarkers [309, 310]. As the field of exhaled biomarkers continues to grow, we may rely more on further development of artificial or even biosensor-derived olfaction systems to provide an even firmer background for the recognition of biomarker patterns in the breath. Breathomics may become a cornerstone of personalised medicine [311], but this will require more attention to be paid to the human microbiome and its confounding effect on "our" breath biomarker profile [312]. The potential offered by the rapidly growing area of e-nose technology resulting in wearable e-noses (fabric-based electronic sensors) are also great for "smelling" the difference between health and disease [313]. Eventually, medically acceptable, positive and/or negative predictive values for diagnoses, phenotypes and prediction of clinical course have to be delivered, before widespread implementation can be established.

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