

# Exhaled Breath Analysis: from Occupational to Respiratory Medicine

*Massimo Corradi, Antonio Mutti*

Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, Italy

**Abstract.** Breath analysis is a technique rapidly gaining ground as a non-invasive tool to diagnose and monitor various aspects of lung diseases. Measurement of exhaled breath is safe, rapid, simple to perform, and effort independent. Given that human breath contains upwards of 250 chemicals, the potential for developing new applications is high. Much of the current knowledge on breath analysis in respiratory medicine derives from years of experience gained in occupational settings, where breath analysis has been used mainly to assess exposure to volatile chemicals. Laboratory based analysis of exhaled air is a complex, expensive and time consuming process and thus is not in wide spread use in occupational medicine. However, recent knowledge of exhaled breath analysis in pulmonology, in particular in bronchial asthma and lung cancer, and the development of fast, and easy to perform non-invasive procedures for breath analysis, re-opened possible application of exhaled breath as a novel approach for biological monitoring of inhaled pneumotoxic substances. The simultaneous quantification of biomarkers of dose and effect in exhaled air may provide new insights into lung damage occurring in workers exposed to inhaled toxicants, thus representing a new and fascinating application in risk assessment strategies.

**Key words:** exhaled breath, biomonitoring, biomarkers

## *Introduction*

In Europe, respiratory diseases rank second (after cardiovascular diseases) in terms of mortality, incidence, prevalence and costs, and a further increase in mortality is expected in the future (1). Whereas there is a substantial reduction in infectivious lung diseases, such as tuberculosis and pneumonia, there has been a progressive increase in chronic inflammatory disorders such as asthma and chronic obstructive pulmonary disease (COPD). Several tools have been developed for diagnosing, monitoring and evaluating the lung status. Lung function tests provide insights into changes in airway caliber, flow, lung volumes and gas exchange (2). Imagine techniques, especially high resolution computed tomographic scanning, provide insight into changes in lung tissue composition (3). Both these methods are indirect measurements of ad-

verse effects occurring in a biological system as a long-term consequence of exposure to toxic or noxious agents.

The concept that inflammation and oxidative stress lead to bronchial hyper-responsiveness, airflow limitation and mucus hypersecretion in lung diseases has led to a widening search for the types of inflammatory cells and mediators that are responsible for the cascade of events linking the initial stimulus to the final abnormality in airway function. Bronchial biopsies and bronchoalveolar lavage have become the "gold standard" for measuring inflammation in the lung, but the invasive nature of bronchoscopy has led to a constant search for less intrusive methods that are easier to implement (4).

Blood and urine can be collected to identify biomarkers of lung diseases, by the studying of inflammatory products spilling over into the blood or measuring

mediators or their metabolites excreted into urine (5). However, through systemic samplings, it is not always possible to achieve a sufficient specificity and sensitivity to get insights into disease processes occurring in the lung. A more direct evaluation of lung inflammation can be used by means of sputum induction. The induction of sputum is relatively reproducible and allows the quantification of inflammatory cells and mediators. Induced sputum collection and analysis is generally well tolerated by patients (6). Collection of sputum induced by inhalation of hypertonic saline can give direct information on the kind and degree of bronchial inflammation and brought to a better understanding of the role of inflammatory mediators found in the airways of asthmatic patients. However, this technique is somehow invasive, as it involves inhalation of hypertonic saline, which may induce coughing and bronchoconstriction, and cannot be used for repeated measurements, as it can cause inflammation itself.

In the last decade, there has been an increased application of exhaled breath analysis, either considering exhaled gases and exhaled condensates, in pulmonology research (7). Exhaled breath analysis has enormous potential as an easy, non invasive means of monitoring inflammation and oxidative stress in the airways, particularly in non diseased subjects. Exhaled air can be collected without the need of unpleasant stimulation of the airways as either in sputum induction or lavage sampling.

In a sample of breath, more than 250 volatile substances can be identified by gas chromatography, but interest in using exhaled gases in the investigation of pulmonary disease processes has occurred relatively lately (8). Nitric oxide (NO), carbon monoxide (CO) and volatile organic compounds (VOCs) are now being studied on a larger scale. Whereas air containing exhaled volatile compounds has been examined for long time, exhaled breath condensate (EBC), obtained by cooling exhaled air under condition of spontaneous breathing (9), is a relatively unexplored biological medium that could provide an assessment of lung pathobiology. EBC is a biological fluid mainly constituted by water, but also containing small droplets of airway lining fluid. The interest in EBC is justified by the fact that its collection is totally non invasive and does not cause any discomfort or risk to examined subjects.

Exposure assessment calls for noninvasive procedures to screen presumably healthy subjects, be they solvent-exposed workers or selected groups of the general population exposed to environmental pollutants (8). Exposure-dose relationships have been established and for several substances biomonitoring values can easily be converted into their ambient air monitoring equivalent (10). This is why much of current knowledge on breath analysis in respiratory medicine derives from years of experience gained in occupational settings and, more recently, from studies addressing the health effects of chemicals polluting the general environment.

Breath analysis may rely on both direct (on line) and indirect (off line) reading methods: in the on line method, breath analysis is immediately available, whereas the use of indirect methods generally involves collecting and trapping the breath sample and subsequently transferring it to an analytical instrument for analysis.

Various kinds of breath samples have been used in biological monitoring, including mixed expired air and end expired air: end-exhaled-air represents the alveolar air concentration and mixed-exhaled-air represents the gas mixture coming from the dead space of the bronchial tree and the alveolar gas-exchange space. In this present review, we will summarize the current knowledge on breath analysis in occupational and respiratory medicine.

### Exhaled permanent gases

NO measurement is now a well-known method to assess airway inflammation and increased levels of NO are detectable in exhaled air of asthmatics. NO is a common biological and neural transmitter, very important in normal airways and blood vessel tone regulation; in the airways, NO is produced by many cells, both resident or recruited during the inflammatory process. Exhaled NO is generally measured on line, having the subject to blow directly into the analyser with immediate results. However, it is also possible to have a remote breath collection into inert bags, with subsequent analysis (off line). Its patho-physiological meaning is still unclear (11), but it has been demon-

strated that NO levels in exhaled air are higher in asthmatic in comparison with healthy subjects, that its levels raise during spontaneous or induced asthma exacerbation and decrease after anti-inflammatory treatment (12). There is a positive correlation between exhaled NO and eosinophil count in induced sputum (13). Exhaled NO can also give additional information for the interpretation of occupational challenge tests (14).

Measurement of carbon monoxide (CO) has been used as a test of tobacco smoke intake (15). However, endogenous CO can arise from the metabolism of haeme by haeme oxygenase (HO) 1, an enzyme whose expression and activity is enhanced in airway macrophages by either increased oxidative stress or stimulation by pro-inflammatory cytokines (16). The increased production of CO can be detected in exhaled air. However, to date its use as a non-invasive inflammatory marker is limited, mainly because of the strong confounding by tobacco smoke and polluted ambient air (15, 17).

### **Volatile organic compounds**

The term volatile organic compounds (VOCs) refers to a group of chemicals which can vaporize easily at room temperature (18). Most of VOCs are chemicals used to manufacture and prepare many building materials, interior furnishings, textiles, office equipment, cleaners, personal care supplies, and pesticides. That is why they are a source of pollution and concern for indoor air. Some VOCs can even be found in small amounts in the air we breathe out of our lungs, and therefore exhaled breath analysis has been used to assess VOC exposure (8).

There has been a substantial progress in sampling techniques over the last decades. In 1980, an apparatus composed by the Rahn-Otis valve connected to a gasometer and to a pump equipped with activated charcoal tubes was used to investigate respiratory absorption and excretion of hexane and cyclohexane in shoe factory workers (19, 20). Such an apparatus represented a substantial progress over techniques relying on poorly standardized methods based on the use of glass tubes and manual sampling procedures, but was

difficult to assemble, cumbersome and not applicable to routine measurements in occupational settings. Moreover, air collection required at least a few minutes (the flow rate of the pump could not exceed 200 ml/min), thus confining its use to research based on carefully standardized protocols. It is worth mentioning that the rapid phase of excretion of most tested solvents range from 5 to 10 minutes, which makes sampling procedures critical.

Just a few years ago, a simple device (BioVoc®) was developed to address the same biomonitoring techniques at the UK Health and Safety Executive (HSE), thus making collection of alveolar air at the workplace feasible and meaningful. An improvement to the HSE technique was proposed in our laboratory by Poli et al. (21), who used solid phase microextraction (SPME) instead of the cartridges proposed by HSE, which are potentially contaminated by pollutants contained in ambient air. Beside their application in biological monitoring, VOC analysis could also serve as non-invasive biomarkers of various disease states such as hyperlipidemia (22) and lung cancer (23). In fact, the process of free radical formation and subsequent peroxidation of polyunsaturated fatty acids, i.e. of linoleic and linolenic acid, induced the pulmonary formation of volatile hydrocarbons, which could be identified and quantified in breath samples.

The observation that patients with lung cancer exhale different amounts of VOCs in comparison with healthy controls raised interest, due to the possibility of identifying biomarkers of lung cancer in exhaled air for use as a screening tool. The qualitative and quantitative evaluation of breath compounds is of interest because it is quick and safe, and is well acceptable by the patient.

### **Volatile organic compounds (VOCs) and lung cancer**

In 1999 Phillips et al. (24) described a simple apparatus for VOC collection and analysis, where subjects were requested to breathe tidally in a sorbent trap, which was then thermally desorbed. VOCs were then separated by gas chromatography and identified by mass spectrometry. Using this method he was able to pick out 22 VOCs, mainly alkanes and benzene de-

rivates, whose levels discriminated between patients with and without lung cancer. Phillips et al. (25) further extended their observations reporting a set of 9 VOCs (most of them different from those previously identified) as having a strong discriminator power between healthy volunteers and patients with lung cancer. However, much of the raised interest was then tapered mainly due to technical problems related to VOC measurements; in fact, most of VOCs are present in very low concentrations, thus requiring sophisticated techniques for sample concentration and analysis. In addition, most of the reported studies included a mixed group of lung cancer patients, with primary (non small and small cell cancer) and secondary lung cancer; furthermore, there are no studies, which compared the levels of VOCs in exhaled air of patient with lung cancer with those having clinical conditions which often precede the development of cancer, mainly COPD or the condition of asymptomatic smoker. Finally, in most of the studies that dealt with VOC analysis, the chemical identification was simply based on the similarity of the respective computer-based library mass spectra but not with standard identification and a precise quantification of each VOC was missing.

In order to overcome these limitations, we recently performed a study aimed at identifying and quantifying VOCs in exhaled air of patients with primary non small cell operable lung cancer and to compare the levels with those of subjects with COPD or normal smokers (21). Exhaled breath was collected with the commercially available device (Bio-Voc®) described above. Briefly, subjects are requested to perform a single slow vital capacity into a one-way valve connected to a Teflon®-bulb, which traps the last portion of exhaled air (150 ml). The breath collecting method we used allows the sampling of a fixed volume of alveolar air, limiting environmental and upper airway contaminations; in addition, it does not have carryover effects and it allows to add internal standards to the samples, thus improving the reproducibility of the data. To summarize the pathophysiological meaning on the observed differences in VOC concentrations among groups, we must underlie that none of the selected VOC levels was specific for the different clinical conditions. However, the combination of exhaled VOCs derived either from lipid peroxidation process-

es and from ambient pollution, may be useful to properly classify patients, therefore it may improve the non-invasive approach to early lung cancer detection (21).

A further interesting analysis of exhaled VOCs may be performed using a relatively new technology known as electronic nose. Electronic noses rely on arrays of chemical vapor sensors that respond to specific chemical pattern characteristics of an odorant molecule, particularly VOCs. Published data confirm that exhaled breath of patients with lung cancer have distinct characteristics that can be identified with an electronic nose (26). Combining information from quantitative measurements relying on reference techniques (gas-chromatography-mass spectrometry) and those obtained so far by using electronic noses could lead to produce more selective and sensitive sensor for routine use.

### Exhaled breath condensate (EBC)

Collection of exhaled breath condensate (EBC) is another non-invasive approach to study ongoing inflammatory processes in the lung. EBC is collected by cooling or freezing exhaled air and is totally non-invasive (27). The collection procedure has no influence on airway function or inflammation, and there is accumulating evidence that abnormalities in condensate composition may reflect biochemical changes of airway lining fluid. Several volatile and non-volatile chemicals, including proteins, lipids, oxidants, and nucleotides have now been detected in EBC (Tab. 1).

The use of EBC is a very promising technique in the assessment of airways inflammation (28). Nevertheless, there are still open problems, of which the lack of a standard procedures to collect samples and express results remains the most important. Collection temperature is perhaps critical, as it may have influence on the volume of EBC and thereby on the concentration of non-volatile biomarkers.

#### *Methodological issues in EBC collection*

In order to standardize EBC collection, our group recently collaborated with a producer to devel-

**Table 1.** Selected EBC biomarkers that have been proposed to assess and monitor lung injury and diseases

Markers	Meaning	Methods	Conditions	Selected references
Hydrogen peroxide	Cell released oxidant	Colorimetric or fluorimetric methods	Asthma, COPD, bronchiectasis, cystic fibrosis, acute lung injury	48, 54
Nitrotyrosine	Nitrosative stress biomarker	Enzyme immunoassay (EIA), mass spectrometry (MS) techniques	Asthma, cystic fibrosis	46
Eicosanoids	Arachidonic acid derived inflammatory mediators	EIA, radioimmunoassay, High Performance Liquid Chromatography (HPLC)	Asthma, COPD	42, 43, 52
8-isoprostane	Lipid peroxidation biomarker	EIA and gas chromatography/MS	Asthma, COPD, cystic fibrosis, ozone exposure, obstructive sleep apnea, acute lung injury	45, 53
Aldehydes	Lipid peroxidation biomarker	Liquid chromatography-tandem MS	Asthma, COPD, workers exposed to hard metals,	36, 32 44
Glutathione	Anti oxidant	HPLC	Asthma	44
pH	Airway acidity	pH electrodes and indicator dyes	Asthma, COPD, cystic fibrosis,	49
Cytokines	Inflammatory mediators	EIA	COPD, lung cancer, acute lung injury	50
Toxic metallic elements	Biomarkers of exposure	Electrothermal atomic absorption spectroscopy (ETAS) and Inductively coupled plasma –MS (ICP-MS)	Workers exposed to hard metals	32

op and validate a new type of condenser: the TURBO-DECCS, acronym for Transportable Unit for Research on Biomarkers Obtained from Disposable Exhaled Condensate Collecting Systems (purchased from Italchill, Parma, Italy). The instrument was specifically designed to control the temperature of EBC collection and to test the effect of different condensation temperatures on the recovery of selected biomarkers [ $H_2O_2$ , MDA (as markers of inflammatory and oxidative stress processes, respectively) and total condensed volume and conductivity, reflecting overall subject ventilation and both concentration and charge of non-volatile electrolytes, respectively].

In a recent study, 24 healthy subjects collected EBC at four different temperatures: -10, -5, 0 and +5°C (29). Our results suggest that: (i) the tempera-

ture of EBC collection should be controlled and reported. Collection temperature should be chosen on the basis of analytical needs (stability of the analytes, minimum required EBC volume, sensitivity of the method, etc.); (ii) water is the main variable dilution factor, thus total condensed volume should be recorded; (iii) cooling temperature related to EBC collection influence in different ways selected biomarkers and is less critical for volatile or semi-volatile substances; (iv) potential normalizing factors should be homogeneous with the analytes to of interest (e.g., in terms of relative volatility and solubility).

TURBO-DECCS is particularly suitable for occupational monitoring purposes. In fact, the condenser was specifically designed to collect EBC in working settings, being transportable. In addition, the

respiratory circuit is disposable, which allows the performance of multiple collections from different subjects in a short period of time, avoiding the need for cumbersome sterilization procedures that can even contaminate EBC samples. Moreover, it offers the possibility to control breathing temperature, a variable which can interfere with the recovery of all non-volatile substances.

### *EBC and exposure to pneumotoxic metals*

Considering that EBC is water practically free of potentially interfering solutes, it represents an ideal biological matrix for elemental determination by relatively common techniques, such as electrothermal atomic absorption spectroscopy (ETAAS) or less commonly available instruments, such as inductively coupled plasma – mass spectrometry (ICP-MS). EBC elemental analysis may be used to assess target tissue levels of pneumotoxic metals and essential trace elements, and hence the probability of local effects resulting from highly reactive or poorly soluble species retained by the lung for long time. To our knowledge, there are no previous reports considering elements excreted in exhaled air, too. Considering that almost 500 ml of water is expired per day, the amount of metals which are expired every day is not negligible. For several elements, it may represent about 20% of total daily excretion.

Blood and urine are currently used for the biological monitoring of workers exposed to pneumotoxic metals (30). In either cases, biomonitoring represents a surrogate measurement of absorbed dose, which is of paramount importance in the risk assessment of systemic effects. However, the concentration of toxic metals and trace elements in blood and urine is not always a good indicator of its concentration in the critical tissue, particularly when the former is poorly soluble and the latter is represented by the epithelium representing the organism's interface with ambient air (31). Indeed, inhaled toxic chemicals can act locally on the lung, which represents the route of entry of most environmental pollutants.

EBC analysis is a novel approach to biological monitoring, which allows the simultaneous quantification of both target tissue levels of inhaled metallic

elements and the measurement of biomarkers of effect directly sampled from the epithelial lining fluid.

Recently, our group showed that EBC is as a suitable matrix to assess lung dose and effects in workers exposed to cobalt (Co) and tungsten (W) (32). Data showed that both Co and W concentrations in EBC are correlated with respective urinary levels but, most of all, EBC Co, but not its urinary levels, are positively correlated with biomarker of lung damage, such as aldehydes derived from lipid peroxidation. This study demonstrated that Co and W can be measured in the EBC of occupationally exposed workers and that the levels of these elements in EBC correlate with a marker of oxidative stress, namely MDA (33), thus suggesting the potential use of this matrix as a novel approach to monitor target tissue dose and effects occurring in the respiratory tract upon exposure to pneumotoxic substances.

We further extended the application of EBC in biological monitoring in chrome (Cr) plating workers. The respiratory tract is the major target organ for Cr [for both valence states, trivalent chromium (Cr III) and hexavalent chromium (Cr VI)] following inhalation exposure in humans (34). Chronic inhalation exposure to chromium (VI) is much more toxic than chromium (III), for both acute (short-term) and chronic (long-term) exposures in humans (35).

We showed that Cr levels in EBC and in urine were higher in exposed subjects than in controls, and they were positively correlated with each other, being both correlated with ambient air Cr levels (Caglieri et al. submitted). Thus, both Cr in EBC (Cr-EBC) and in urine (Cr-U) can be considered valid biomarkers of exposure. However, looking at the different correlations between variables, Cr-U and Cr-EBC levels seem to provide different information: there were weak or no correlations between Cr-U and biomarkers of effect, whereas Cr-EBC concentrations closely correlated with both MDA and H<sub>2</sub>O<sub>2</sub> levels in EBC, thus suggesting that they may be more representative of the lung dose responsible for local toxic effects.

In both the above-mentioned studies, we assessed the lung effects of metal exposure relying on biomarkers of oxidative stress, which may be sensitive endpoints for evaluating early biochemical changes in the

airways. This approach will probably overcome the limitations of traditional spirometric tests, which often indicates late and frequently irreversible lung dysfunction.

EBC can be thus considered a suitable medium for quantifying both long-term and recent Cr exposure at target tissue level and, together with biomarkers of effect, can provide insights into the oxidative lung damage occurring in workers exposed to pneumotoxic metals.

To better understand inhaled Cr toxicity and kinetics, we also carried out elemental speciation of Cr (aimed at distinguishing Cr VI and Cr III) both in ambient air and in EBC (Goldoni et al. submitted). In fact, whereas it is usually assumed that only Cr III is detectable in urine, there are no data indicating whether a similar behaviour also occurs in EBC. Different individual concentrations of reducing agents (glutathione, ascorbic acid etc.) in lung lining fluid may affect Cr reduction and subsequent lung toxicity. Kinetic data showed that airborne Cr VI was reduced by 50% in epithelial lining fluid sampled at the end of exposure and that a further reduction by 50% required about 15h. The persistence of Cr VI in EBC samples after the last exposure probably explains the observed lung effects.

These studies demonstrate that EBC is a suitable matrix to assess target tissue dose and early effects of pneumotoxic elements. Novel insights can be obtained about kinetics and dynamics, which to the best of our knowledge have never been explored. Such novel information can contribute to open new approaches to the toxicology of metallic and transition elements.

#### *EBC and Biomarkers of disease*

EBC has been used to assess inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease, lung cancer, interstitial lung disease, and acute respiratory distress syndrome (36-40). Recently, EBC application has been extended to biological monitoring of workers exposed to cobalt and tungsten (32).

EBC contains many non-volatile substances including proteins, lipids and oxidation products that seem to reflect the composition of bronchoalveolar extracellular lining fluid (41). Several inflammatory

and/or oxidation markers have been measured in EBC of healthy subjects and patients with different diseases. High levels of cys-leukotrienes (LTE<sub>4</sub>, LTC<sub>4</sub> e LTD<sub>4</sub>) have been found in EBC of patients with moderate to severe asthma (42, 43). Levels of 8-isoprostane seem to be correlated with the severity of asthma irrespectively from the presence of corticosteroid therapy (44), while malondialdehyde levels decrease with corticosteroid therapy (45). High levels of 3-nitrotyrosine and nitrosothiols are also reported in patients with asthma (46, 47), and an increase in H<sub>2</sub>O<sub>2</sub> concentrations is reported in severe and unstable asthma (48). Airway acidification detectable in EBC has been reported in several lung disorders (49). EBC cytokines analysis successfully characterized important differences in stable COPD compared to exacerbation or smoking and non-smoking healthy individuals (50).

Despite the enthusiasm of a few research groups, much of the skepticism about the validity of EBC as a diagnostic and monitoring tool lies on analytical problems associated with the measurement of trace amounts of unstable and non specific mediators, mainly relying on immunochemical methods lacking reference methods and materials, and affected by poor sensitivity, specificity and selectivity. However, reference analytical techniques such as gas and liquid chromatography tandem mass spectrometry are being currently used to validate EBC analysis: validation studies clearly demonstrated that several mediators such as 8-isoprostane, cys-tLT, LTB<sub>4</sub>, malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are really present and detectable in EBC (51-54). Further validation studies will lead to the development of simpler and cheaper methods of analysis (such as colorimetric strips or sensors) which will be more practical for routinely EBC use.

#### *EBC and COPD*

Several biomarkers of disease in EBC have been proposed to assess and monitor COPD, mostly requiring validation in terms of collection and analysis. A novel concept being addressed in our laboratory is the use of EBC to analyze its elemental composition, to identify biomarkers of exposure and susceptibility to noxious pneumotoxic substances.

The elemental composition of EBC can be used to assess long-term exposure to tobacco smoke, which is associated with increased lung uptake and burden of toxic metals. Indeed, subjects with COPD and with a long time history of cigarette smoking do have higher levels of toxic elements known to be contained in cigarettes, such lead (Pb), cadmium (Cd) and aluminum (Al), in comparison with nonsmoking controls. Exhaled metallic elements do not represent simply a means to assess current exposure to tobacco smoke, but may also provide a quantitative estimate of the target tissue burden; in fact, considering ex smoking COPD patients only, Pb, Cd and Al EBC levels were still higher compared to controls (Mutti et al., in preparation).

EBC toxic metal analysis in COPD patients may be also of relevance to understand disease pathogenesis. In fact, an association between Cd exposure and the development of emphysema has been reported (55, 56) and *in vitro* evidence suggests that Cd may play an important role in the pathogenesis of emphysema associated with chronic inhalation of fumes by inhibiting the production of connective tissue proteins (57).

Our data suggest the possible contribution of novel toxicological knowledge to a better understanding and possibly treatment of lung pathology associated with exposure to tobacco smoke and environmental pollutants.

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