

## CLINICAL STUDY

## New techniques for assessment of occupational respiratory diseases

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**Abstract:** Recently, as several studies have demonstrated, these non-invasive techniques, such as induced sputum (IS) or analysis of the exhaled air; exhaled nitric oxide (eNO) or exhaled breath condensate (EBC) provide fairly reliable results that correlate with those of „gold standard“ methods (bronchoscopy, bronchoalveolar lavage) which are more invasive and uncomfortable for patients. Although novel approaches have attracted the attention of scientists, they have not been examined in relation to occupational settings and professional diseases. The IS is a useful biological medium for the diagnosis of occupational asthma and for the assessment of exposures to harmful dust at workplaces. The eNO analysis can serve as an easy and comfortable diagnostic tool for the professional asthma after exposure to various allergens. The examination of EBC biomarkers evaluates local doses of hard metals in the lung, as well as detection of oxidative stress markers (malondialdehyde, H<sub>2</sub>O<sub>2</sub>). Due to the need to standardize the procedures for EBC collection, further studies on EBC validation and the subsequent application in the clinical and epidemiological fields are required. The techniques listed in this article may serve as optimal tools for diagnosis of occupational respiratory diseases and for screening/monitoring programs following inhalation exposures in future (*Ref. 64*). Full Text (Free, PDF) [www.bmj.sk](http://www.bmj.sk).

Key words: non-invasive methods, occupational respiratory diseases, biomarkers.

**List of abbreviations:** SWORD – Surveillance of work-related and occupational respiratory diseases, BAL – bronchoalveolar lavage, EBC – exhaled breath condensate, NO – nitric oxide, IS – induced sputum, DNA – deoxyribonucleic acid, IL – interleukin, TNF- $\alpha$  – tumor necrosis factor- $\alpha$ , COPD – chronic obstructive pulmonary disease, OA – occupational asthma, MMP – matrix metalloproteinase, LT – leukotriene, WTC – World Trade Centre, NOS – nitric oxide synthase, ATS – American Thoracic Society, ERS – European Respiratory Society, ETAAS – electrothermal atomic absorption spectroscopy, ICP-MS – inductively coupled plasma-mass spectrometry, MDA – malondialdehyde.

Occupational respiratory diseases represent a specific group of illnesses caused by materials inhaled at the workplace. The most common are rhinitis, laryngitis, tracheitis, bronchitis and

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bronchiolitis, bronchial asthma, chronic obstructive pulmonary disease, lung cancer, interstitial lung disease, and inhalation fever. The incidence of diseases caused by mineral dust has recently declined in post-industrial countries, and asthma has emerged as the principal occupational lung disease (1): according to the SWORD (Surveillance of work-related and occupational respiratory diseases) program, occupational asthma was responsible for about 25 % of all cases of the occupational diseases in recent years. The highest reported rates of asthma were in craft and related occupations, followed by plant and machine operatives, and associated professional and technical workers. The analyses of asthma by industry show rates generally higher in primary and manufacturing industries, but much lower in utilities, construction, and the health and social services. Causing agents are classified into four main groups, the largest of which is represented by organic or chemical elements, followed by metallic and miscellaneous elements and few are unknown/unspecified. Isocyanates and metals are prominent agents in craft related workers and plant and machine operators with the addition of flour/grain, wood dust, solder/colophony, and welding fume in the craft related occupations. Also prominent are the specific associations between glutaraldehyde and latex, and health and social services, and technical and associate professional occupations (2).

Most of the initial assessments of particular burden and involvement of inflammatory and structural cells in occupational respiratory diseases have been done in studies using fiberoptic bronchoscopy in conjunction with bronchoalveolar lavage (BAL)

analysis (3), or based on experimental studies on BAL alone (4). The relative invasiveness of these techniques has restricted the use of bronchoscopy to a limited number of specialized centers, and hampered its development into a practical and suitable tool for screening programs, exposure evaluation or repeated follow-up of workers exposed to hazardous dust in large populations (5). However, these sampling methods have allowed the characterization of some biological mechanisms in pulmonary diseases, and still represent the reference techniques (6). BAL is regarded as the most reliable method for sampling the lining fluid of the lower respiratory tract (6, 7). More importantly, the associated inflammatory reaction seems to limit the comparability between subsequent sampling times, thus preventing their use for monitoring purposes, which remains the most relevant approach to the use of biomarkers both in epidemiological and clinical settings (6). Apart from these techniques, there are imaging and pulmonary function exams available for diagnosis of occupational respiratory diseases, but they also represent a burden on the patient's organism. For these reasons, there is a need to develop new techniques characterized by low invasiveness, which would improve the knowledge of pathogenic phenomena, but would be simple to apply in the study of groups of subjects at risk and in the occupational setting of workers exposed to hazardous pollutants.

Recently, there has been an increasing interest in the investigation of the lungs using non-invasive techniques including sputum induction and measurement of biomarkers in exhaled breath including nitric oxide (NO), which have been extensively investigated and are now accepted as reflecting airway inflammation (7, 8, 9, 10), and those found in exhaled breath condensate (EBC), obtained by condensing exhaled breath at low temperatures (10). Exhaled breath analysis has an enormous potential as an easy, non-invasive means of monitoring inflammation and oxidative stress in the airways (6) and to identify new biomarkers of lung processes (10, 11).

### Induced sputum

Sputum can be obtained either spontaneously or using the hypertonic saline solution, depending on whether or not the patient has a productive cough (12). Induction with the hypertonic saline is so far the most common, with the possible analysis of spontaneously expectorated sputum only in patients who have a productive cough as a part of their disease (e.g., chronic bronchitis or acute asthma exacerbation).

Induced sputum (IS) provides a non-invasive alternative method to evaluate the respired particulate matter and the lung inflammatory response (5, 13, 14). However, it was only in the early 1990s that the first attempt to standardize the method of induction and processing was made. Since then, numerous studies have been published attesting to the safety and relative feasibility of this method, and eventually leading to the establishment of the first task force on induced sputum methods sponsored by the European Respiratory Society (ERS) (15).

Hypertonic saline is delivered in the form of an aerosol produced by an ultrasonic nebulizer. There is a consensus that ultra-

sonic nebulizers should be used, with an output of 1 mL/min being sufficient for a successful induction. The duration of inhalation of saline should be kept constant whenever possible. Current recommendations state that the optimal time is 15 min; however, induction can be continued for another 5 min if an adequate sample has not been obtained. Problems may arise when hypertonic saline causes bronchoconstriction. Contamination of the sputum sample with saliva is a major concern and a common problem. Because of their size, squamous cells cover the inflammatory cells in cytopins, making the counting of these imprecise. Various methods have been recommended to reduce squamous cells: blowing the nose prior to induction and wearing a nose clip during induction, washing the mouth with water before each expectoration, and wiping the mouth with paper tissue (15).

There are two valid and widely used methods for processing induced sputum: the first consists of selecting the visibly viscous (mucoid) parts of sputum using an inverted microscope, and the second consists of processing the entire expectorated sample (12). The sputum analysis provides the evidence of total and differential cell counts and soluble markers. The differential cell count is determined by counting a minimum of 400 non-squamous cells and is reported as the relative numbers of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells, expressed as a percentage of the total non-squamous cells. The percentage of squamous cells should always be reported separately (5). Moreover, the supernatant of sputum can be used for the measurement of a variety of soluble mediators, including eosinophil-derived proteins, tryptase, myeloperoxidase, deoxyribonucleic acid (DNA), albumin, fibrinogen, nitric oxide derivatives and cytokines, such as interleukin (IL)-5, IL-8 or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (16). Enzyme assays have also been used successfully to analyze sputum. Sputum is rich in enzymes such as neutrophil-derived elastase and cathepsin G, which can be detected using specific chromogenic substrates that produce a colored product that can be quantified by spectrophotometry. The activity of some proteases, such as matrix metalloproteinases, can be quantified using the substrate gel zymography (15).

Sputum has been most widely used in the asthma evaluation (15, 17, 18, 19) and several studies have demonstrated that the number of eosinophils in the induced sputum is associated with asthma severity (20). The presence of elevated eosinophil counts above the upper limit of 3 % of non-squamous cells can help to diagnose this condition in the absence of typical findings on examination and lung function testing. One would hope that effects of occupational and environmental hazards might be amenable to evaluate using the induced sputum (21). The evaluation of sputum has been also very useful in chronic obstructive pulmonary disease (COPD), but it should not be used for diagnosis (15).

### Induced sputum and occupational respiratory diseases

IS is now being widely used in medicine. In the period 1992–2004, more than 650 papers were published on the application of IS in the diagnosis and management of asthma, and ~200 on

its use in COPD and chronic bronchitis. However, much less attention has been paid to the application of this technique in occupational and environmental exposures (5).

### **Induced sputum and occupational asthma**

Occupational asthma (OA) is defined as a disease characterized by variable airflow limitation and/or airway hyperresponsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace (23). The features of OA are similar to those of more common atopic forms of the disease (15). Exposure to occupational agents induces marked changes in sputum in subjects with OA. Eosinophilic airway inflammations have been observed in subjects with OA after an exposure to occupational agents in the laboratory (19). When at work, subjects with OA show a predominant eosinophilic airway inflammation, which decreases or resolves after a period away from work. Since then, evidence has shown that the prevalence of eosinophils is a useful marker for monitoring OA. The percentage of sputum eosinophils increases after the exposure to occupational agents in the laboratory when compared to baseline data, and significant changes are seen in sputum eosinophils when workers are exposed to a sensitizer at their workplace in comparison to the levels counted after a period away from the workplace. Authors of related studies conclude that the proportion of eosinophils and levels of eosinophil cationic protein in sputum is particularly high at work in patients with OA, suggesting that the measurement of these factors can supplement other physiological outcomes in establishing the diagnosis of OA (17, 18, 24).

The study of PARK et al (25) looked at soluble mediators in workers exposed to isocyanates, and demonstrated that exposure to toluene di-isocyanate leads to the overproduction of matrix metalloproteinase (MMP)-9, which may induce airway inflammation and remodelling, and then contribute to persistent asthmatic symptoms. Lemiere et al (26) recently reported the results of a study dedicated to the airway inflammation induced by exposure to isocyanates that showed that the neutrophilia observed after exposure to isocyanates is likely to be related to the release of leukotriene (LT)B<sub>4</sub>, probably enhanced by the increased expression of LTB<sub>4</sub> receptor on neutrophils, as well as by the release of IL-8. In several other studies, investigators demonstrated the utility of sputum cell analysis in the investigation of the pathogenesis, pathophysiology, and treatment of both asthma (12, 22) and OA (18, 19, 20). All the authors agree that IS can be used as an additional, non-invasive investigation to validate the diagnosis of OA.

### **Induced sputum and hazardous dust**

The hypothesis that the quantitative and qualitative analysis of particles recovered by IS can be used as a biological monitoring method in the periodic health examinations of healthy workers exposed to hazardous dusts, in addition to the traditional occupational parameters of past history and environmental mea-

surements, has been proposed. This screening could be done in tandem with the biological monitoring of workers exposed to toxic agents, such as metals (e.g. lead, cadmium) and solvents (e.g. toluene, trichloroethane), the levels of which can be estimated in an individual by measuring the chemical or its metabolite in blood, urine or exhaled air (27). Sputum analysis for asbestos bodies has been shown to be an insensitive method for assessing the lung asbestos burden. Several investigators have shown the relevance of asbestos bodies in spontaneous sputum when compared to BAL (28). IS has been found to be useful in screening of the occupational lung cancer. Semi-automated sputum cytometry appears to be sensitive and reliable in the detection of malignant changes in the tracheobronchial mucosa in a limited number of patients with occupational radon or asbestos exposure (29). Evaluation of silica- and hard metal-exposed workers has revealed that BAL and IS specimens yield similar quantitative and qualitative results in terms of the number of particles present in the samples and the chemical analysis of the particles (30). In the study conducted by Akpınar-Elci et al (31), the question of whether exposure of non-smoking workers to flavouring agents in microwave popcorn production is associated with airways inflammation examining inflammatory parameters in IS was investigated. The results confirmed that neutrophil concentrations in this group were significantly higher than those in the healthy non-smoking control group and interleukin-8 and eosinophil cationic protein levels were significantly higher in high-exposure workers than in low-exposure workers, although there were no significant differences between both types of exposure groups in spirometric values. This analysis supported the evidence that popcorn production workers are prone to a significant occupational hazard through exposure to flavouring agents. Another possible field of use of IS in occupational exposures is the exposure to diesel exhaust fumes. The study conducted by Nordenhall et al (32) concluded that short-term exposure to diesel exhaust fumes induces a time-dependent inflammatory response in the airways of healthy subjects, causing an early increase in IL-6 and methylhistamine levels and neutrophil percentages possibly followed by a late increase in the percentage of lymphocytes. The study conducted by Dragonieri et al (33) examined airway inflammation in traffic policemen daily exposed to traffic related air pollution compared to a group of healthy subjects without any occupational exposure. Traffic policemen showed a statistically significant increase in the percentage of neutrophil cell count. Traffic related air pollution can be considered as an occupational hazard for workers performing physical work close to traffic. Particularly dangerous airway tissue damage can result from high-level occupational exposures during emergency rescue action. In New York City, IS examinations were performed in fire-fighters who were exposed to particulate matter and combustion/pyrolysis products during and after the World Trade Centre (WTC) collapse on 11th September 2001, and the outcomes were compared to controls. Various cell counts and MMP-9 levels were significantly different in fire-fighters as compared to controls and the differential counts for neutrophils and eosinophils increased with cumulative WTC workday exposure intensity (13).

There are several limitations of IS as a diagnostic tool for the large-scale use. The main limitation is the fact that the sputum is inevitably diluted by saliva resulting in the potential disadvantage of a considerable contribution to the induced sputum sample. Moreover, the hypertonic solution can induce bronchoconstriction and inflammation response. The technique is not always applicable due to the occasional provocation of nausea.

However, many advantages of this method outweigh the disadvantages. The technique offers a rapid, safe, non-invasive approach in the field of research and diagnosis of occupational diseases of the lung. Probably the greatest benefit of induced sputum is its application in the evaluation of airway inflammation in children, where the use of bronchoscopy has been limited for ethical and safety reasons (22).

### Exhaled breath analysis

#### *Exhaled nitric oxide*

Nitric oxide (NO) is an endogenous, soluble gas whose highly reactive molecules are involved in many biological and pathophysiological organic processes. NO is generated from the oxidation of L-arginine to L-citrulline by the nitric oxide synthase (NOS; 34). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (35). The type II iNOS is released by many cells in the lung (macrophages, fibroblasts, neutrophils, and epithelial cells) (36), and is thought to be a pro-inflammatory mediator with immunomodulatory effects (35). Expression of iNOS is upregulated by interferon  $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ , all cytokines known to be active in airway inflammation. These cytokines are suspected to be a source of airway NO in subjects with asthma (37). The production of NO under the oxidative stress conditions generates strong oxidizing agents (reactive nitrogen species) that may modulate the development of chronic inflammatory airway diseases and/or amplify the inflammatory response (38). NO may play a role in the non-specific defensive mechanisms against pathogens and may be involved in the signalling between macrophages and T cells. CD4+ T helper (Th) cells are important in the host defence and have been implicated in chronic inflammatory diseases. NO, derived from airway epithelial cells, plays an important role in amplifying and perpetuating the Th2 cell-mediated inflammatory response, both in allergic and non-allergic asthma (35). In vitro studies have demonstrated that DNA synthesis and proliferation of human airway smooth muscle cells, that are important determinants of airway hyper-responsiveness in asthma, are reduced by exogenous administration of NO donors (39). Nitrogen oxides can promote either cell survival or cell death, depending on the chemical species, redox state, concentration, and target cell type (35). With more than 900 publications, exhaled nitric oxide (eNO) is widely accepted as a non-invasive marker of airway inflammation for research. American Thoracic Society (ATS) standardized techniques for the measuring of eNO: subjects inhale at total lung capacity, and exhale at a constant flow rate of 50 mL/s. A constant flow is essential, because eNO levels are flow-dependent. After few seconds, a steady eNO concen-

tration plateau is reached and this is taken as the eNO value exhaled by the subject. Repeated exhalations are performed to obtain two or three reproducible values (40). The advantage of this technique is its non-invasiveness, simplicity, and possibility to transport the equipment home.

### eNO as a diagnostic tool in the occupational respiratory diseases

eNO is generally accepted as a diagnostic tool for asthma, but there are only a few studies that have examined the role of eNO in the occupational asthma (OA). Individuals with positive specific inhalation challenges to occupational agents seem to show a greater increase in eNO than those with negative specific inhalation challenges (41). Determination of this parameter can be informative in the interpretation of occupational challenge tests in cases with borderline functional changes (42). The study conducted by Piipari et al (43) used the eNO measurements to assess asthmatic airway inflammation during specific bronchial challenge tests using the occupational agents in patients with suspected occupational asthma. In patients with normal or slightly increased basal eNO level and late bronchoconstriction, a significant increase in eNO was seen, but patients with high basal eNO level and significant bronchoconstriction did not show any significant eNO increase. Results indicate that eNO measurements give useful additional information for the interpretation of occupational challenge tests in cases with borderline functional changes, increasing the validity of the tests. The investigation by Menzies et al (44) that examined the markers of inflammation using eNO in bar workers before and after a legislative ban on smoking in public places, have demonstrated that asthmatic bar workers had less airway inflammation, with reduction in eNO after the ban. The study conducted by Olin et al (45) determined whether exposure to high peaks of ozone could increase the prevalence of asthma or respiratory symptoms among bleachery workers and whether eNO was increased in the exhaled air of these workers. They found a higher median concentration of eNO when compared to controls, and results of this study confirmed that workers who have been repeatedly exposed to ozone have an increased prevalence of adult-onset asthma, even if these authors did not find significant differences in eNO between exposed subjects and controls in a previous study (46). Lund et al (47) found increased eNO levels in exhaled air of non-smoking aluminium pot room workers when compared to controls employed in the same plant but working outside of the pot room atmosphere. The authors concluded that eNO can be used as an early marker of asthma in aluminium pot room workers. Other recently published studies have found an increase of eNO in work-related isocyanate exposure (48), in latex allergen exposure of symptomatic healthcare workers (49), and in exposure to toluene, xylene and methylethyl ketone in shoe and leather workers (50). The study conducted by Lehtonen et al (51) showed a significant increase of eNO levels in patients with asbestosis. On the other hand, a study conducted by Tan et al (52) showed a decrease of eNO levels in occupational latex exposure

in healthcare workers. eNO was measured before and after a controlled latex challenge at the beginning and at the end of the working week. eNO levels did not show a clear relationship with routine workplace exposure. Also Akpınar-Elci et al (53) have found significantly lower eNO levels in a high-exposure group of workers in a microwave popcorn production plant as compared to low-exposure workers and controls.

In conclusion, many investigators have demonstrated that eNO, as well as lung function deterioration after specific occupational challenge tests, are valuable parameters to differentiate between occupational and non-occupational airway disorders. Naturally, eNO levels are influenced by many possible confounding factors of non-occupational origin. However, it has been suggested that the eNO increase in % is a more appropriate diagnostic tool than absolute eNO values. This plays a special role in occupational medicine, because the casual relation of functional changes due to exposure plays a key role in compensation (48). Defining the exact role of eNO as a predictor of airway disease requires further investigation.

#### *Exhaled breath condensate (EBC)*

The EBC is a fluid obtained by cooling the exhaled air during spontaneous breathing, and has been used to study several lung pathologies (54, 55, 56). The EBC is essentially formed by condensed water and contains non-volatile compounds, including cytokines, lipids, surfactant, ions, oxidation products, adenosine, histamine, acetylcholine, and serotonin. In addition, EBC traps potentially volatile water-soluble compounds, including ammonia, hydrogen peroxide, nitrogen oxides (NO<sub>x</sub>), ethanol, and other volatile organic compounds. Moreover, EBC has readily measurable pH (10, 55, 57). All the regulation mechanisms that concur to form EBC are not fully known. However, a plausible hypothesis is that liquid droplets from bronchoalveolar lining fluid can be transported by the exhaled airflow due to convective processes and consequently collected in the condenser (55). The available condensing systems reported in the literature generally collect 1–2 mL of EBC in 10–15 minutes (57). The factors that determine the dilution of lining fluid droplets are not fully known, but superficial tension, velocity and humidity of exhaled air, and exhalation turbulence play a significant role (55). EBC collection is suitable to assess biomarkers of local inflammation and oxidative stress, which may be sensitive endpoints for identifying early biochemical changes in the airways occurring in exposed subjects (4). Studies of EBC could be useful in assessing lung redox deviation, acid-base status, and degree and type of inflammation in acute and chronic asthma, chronic obstructive pulmonary disease, adult respiratory distress syndrome, occupational diseases, and cystic fibrosis (57). Collection of EBC is a completely non-invasive method of studying the status of the respiratory tract and has the advantage that it can be repeated several times with short intervals between sampling. Collection devices can be portable and can be used in a wide range of settings including intensive care units, outpatient clinics, workplaces and home (10, 57).

The American Thoracic Society (ATS) and the European Respiratory Society (ERS) have developed guidelines for EBC

collection and measurement of exhaled biomarkers, and have collaborated on publishing recommendations that summarize current knowledge, including optimal collection techniques. The main recommendations for oral EBC collection are following:

- collection should occur during tidal breathing in the sitting position using a nose-clip and a saliva trap, with a defined cooling temperature and collection time (temperature and time can vary depending on the study objective);
- surfaces contacting the EBC should be inert to the compounds of interest;
- inclusion of expiratory flow resistance of filters is not required.

EBC samples should be immediately frozen after collection and stored at -70 °C (10). As EBC mainly consists of water that is practically free of potentially interfering solutes, it is an ideal biological fluid for elemental determinations based on relatively common techniques, such as electrothermal atomic absorption spectroscopy (ETAAS), or inductively coupled plasma-mass spectrometry (ICP-MS) (57).

The technique is now attracting broad research interest. More than 100 peer-reviewed articles in the international literature (at least 35 in 2000–2001) have described methods and reported its utility in the investigation of lung diseases and occupational illnesses (57).

To date, there is only a limited number of head-to-head comparisons of EBC assays with other methods of assessing airway biochemistry and inflammation, such as exhaled NO, BAL, IS, and biopsy (7, 58, 59). It must be kept in mind that the exact ionic composition of the human airway lining fluid in healthy and ill subjects is not known, so there is as yet no available gold standard by which to make comparisons (57).

#### **EBC in occupational settings**

Most publications related to the use of EBC as a potential diagnostic tool have been targeted on non-occupational diseases, mostly on asthma and COPD (54, 56, 60). Nevertheless, these studies give a clear indication that breath analysis may have an important role to play in clinical management of asthma and COPD.

Considering the fact that EBC is practically free of interfering solutes, it represents an ideal biological matrix for elemental characterization. Published data show that several toxic metals and trace elements are detectable in EBC, raising the possibility of using this fluid to quantify the lung tissue dose of pneumotoxic substances in occupational settings. This novel approach may represent a significant advance over the analysis of alternative media (blood, serum, urine), which reflect systemic rather than target tissue dose of toxic metals. Certain metallic elements are less soluble and are responsible for local effects (inflammation, cancer) (4). Several recently published studies demonstrate that EBC can be used for assessing the target tissue dose of pneumotoxic substances from polluted workplaces. Goldoni et al (61) have shown that cobalt (Co) and tungsten (W) can be measured

in the EBC of occupationally exposed workers and thus suggest 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. The concentration of malondialdehyde (MDA) in EBC was used as a biomarker of pulmonary oxidative stress. MDA levels were increased depending on Co concentration and were enhanced by co-exposure to W. Such a correlation between EBC MDA and both Co and W levels was not observed in urinary concentration of the same elements. Another study of Goldoni et al (62) has followed the kinetics of Cr (VI) in chrome plating workers using EBC samples. Kinetic data showed that airborne Cr (VI) was reduced by 50 % in airway lining fluid sampled at the end of exposure and that there was a further 50 % reduction after 15 h. The persistence of Cr (VI) in EBC supports the use of EBC in assessing target tissue levels of Cr (VI). Caglieri et al (63) have investigated Cr levels in EBC of workers exposed to Cr (VI) and assessed their relationship with biochemical changes in the airways by analyzing the EBC biomarkers of oxidative stress, namely hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and MDA. Cr-EBC levels increased from the beginning to the end of Friday but were considerably lower on Monday morning. A similar trend was observed for H<sub>2</sub>O<sub>2</sub>-EBC levels and MDA-EBC levels. All inscribed levels correlated with each other as well as with urinary Cr levels. The results of this study demonstrate that EBC is a suitable matrix that can be used to investigate both Cr levels and biomarkers of the free radical production by sampling the epithelial-lining fluid of workers exposed to Cr (VI). Another study used EBC as a tool for the detection of toluene concentration in healthy subjects after a short period of exposure (20 min). EBC samples were collected before and at the end of the exposure, while the environmental concentration of toluene was continuously monitored. Before the exposure, toluene concentrations in EBC were lower than the detectable limit in all subjects, while after the exposure, toluene was detectable in all EBC samples. A significant correlation was found between the environmental toluene levels and toluene in the EBC. Furthermore, a significant relationship between increased EBC toluene levels and urinary hippuric acid after the exposure was found. The authors concluded that EBC is a promising matrix for toluene assessment, although its application in humans requires further investigation (64). The study conducted by Lehtonen et al (51) investigated whether EBC could be used to assess inflammation in asbestosis. Patients with asbestosis had elevated levels of LTB<sub>4</sub> and 8-isoprostane in EBC when compared to healthy controls. On the basis of these results, the authors suggest that EBC is a promising non-invasive means of assessing inflammation in patients with asbestosis.

In conclusion, the techniques presented in this article represent a new approach in diagnostics of occupational diseases of the respiratory tract. The studies on EBC can be considered as a promising starting point to detect levels of markers of local exposure to hard metals and their relationship with specific biomarkers of effect, thus facilitating the diagnosis of professional asthma by the examination of parameters related to this disease (H<sub>2</sub>O<sub>2</sub>, nitrosin, 8-isoprostane, aldehydes, glutathion, pH). Nonetheless,

there is still a relative lack of research on how some of these biomarkers relate to clinical outcomes, such as progression of the disease, severity of disease, clinical subtypes, or response to therapy. (54). It is clear that EBC contains many potential biomarkers. It is now important to optimize their measurement and study the clinical value of monitoring biomarkers in the breath in a variety of lung diseases and to establish the reproducibility of these measurements. One of the current limitations of EBC measurement is the low concentration of many biomarkers making their measurement dependent on the sensitivity of assays (10)

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