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REVIEW

Non-invasive methods to assess biomarkers of exposure and early stage of pulmonary disease in smoking subjects

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ABSTRACT: ***Non-invasive methods to assess biomarkers of exposure and early stage of pulmonary disease in smoking subjects. M. Malerba, B. Ragnoli, M. Corradi.***

Cigarette smoking is the major factor implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD), despite the fact that only susceptible smoking subjects develop this respiratory disease. In the last few years non–invasive techniques such as induced sputum (IS), exhaled nitric oxide (eNO) measurement and exhaled breath condensate (EBC) collection have been successfully established revealing an inflammatory status and oxidative stress indicators in the airways involved in the pathogenesis of several pulmonary diseases. Using these new non-invasive experimental tools recently, several efforts have been made to find new biomarkers in order to assess and monitor early lung damage induced by smoking. Tobacco smoke can acutely reduce eNO levels in healthy smokers and non-smoker subjects so it can play a role in anti-smoking programmes; its increase can be a positive parameter for subjects who are going to stop cigarette smoking and at the same time be used as an anti-smoking indicator. It can be useful to investigate the mechanism of cigarette-induced lung damage in an experimental setting and may potentially be useful for assessing of Environmental Tobacco Smoke (ETS) effects.

Markers of oxidative stress have been detected in induced sputum of COPD subjects even though only few studies investigated the use of induced sputum to study smoke effects on the lungs of healthy subjects. Exhaled breath condensate (EBC) obtained by cooling exhaled air under conditions of spontaneous breathing is a promising biological fluid that could provide a real-time assessment of pulmonary pathobiology. The analysis of induced sputum and of exhaled air is feasible and non-invasive, can be useful to identify new biomarkers of exposure or susceptibility in COPD patients to enhance the understanding of airways changes due to current smoking and may be useful to find new biomarkers in order to assess and monitor early lung damage induced by smoke in order to prevent the progression of obstructive disease.
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Keywords: Exhaled breath condensate (EBC), Induced sputum (IS), Exhaled nitric oxide (eNO), Chronic obstructive pulmonary disease (COPD), Early lung damage, Cigarette smoke.

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Introduction

Cigarette smoking is the major factor implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD) [1], however only some smoking subjects develop this respiratory disease. Therefore, it is important to find sensible biomarkers to assess airway inflammation and reveal early lung alterations after chronic exposure to tobacco smoke. This approach could allow the assessment and monitoring of early lung damage induced by smoke and to address secondary prevention strategies in susceptible subjects. In the last few years non-invasive techniques as induced sputum (IS), exhaled nitric oxide (eNO) measurement and exhaled breath condensate (EBC) collection have been successfully established to reveal an inflammatory status and to find oxidative stress indicators in the airways involved in the pathogenesis of several pulmonary diseases [2-5]. These approaches provide longitudinal sampling of various lung biomarkers of inflammation in the same individual, thereby facilitating the monitoring of the lung damage process. Using these new non-invasive experimental tools recently several efforts have been made to find new biomarkers in order to assess and monitor early lung damage induced by smoke.

Exhaled Nitric Oxide

Determination of eNO is a simple, rapid, non-invasive measure of airway NO [6], which is particularly sensitive to environmental pollution [7] or to pro-inflammatory airway challenges [8, 9]. Tobacco smoke can acutely reduce eNO levels in healthy smokers and non-smoker subjects [10]. Experimental evidence suggests that NO may play a role in non-specific defence mechanisms against pathogens, and may be involved in the signalling between macrophages and T cells (T helper) that are important in hosting a defence and have been implicated in chronic inflammatory diseases [11].

Nitric Oxide (NO) can be measured directly in the exhaled air (eNO) with a non-invasive measurement. International guidelines guarantee reproducible measurements [6, 12, 13]. Although in some inflammatory pulmonary diseases the eNO levels resulted in an increase, substantial evidence is available to show that eNO levels are reduced in smokers [10]. Horvath *et al* observed a significant reduction of eNO levels 15 minutes after smoking one cigarette in asthmatic patients. Acute cigarette smoking caused a small decrease in exhaled NO concentration, which was associated with elevation in exhaled H_2O_2 level in asthmatic patients. This suggested that smoking causes an acute release of reactive oxide molecules in the airways, which may lead to increased peroxynitrite formation and may be a factor causing a decrease in exhaled NO concentration after smoking a cigarette (figure 1) [14]. Kharitonov and co-workers showed that exhaled NO levels were over 50% lower in smokers compared with non-smokers [15]. Passive smoking may also reduce exhaled NO levels. Maniscalco *et al*. reported that exhaled NO levels in healthy individuals is reduced after short-term exposure to environmental cigarette smoke [16]. However, the decrease is transient, recovering within 30 minutes. Yates and colleagues also found a temporary decrease in NO levels after exposure to environmental cigarette smoke, which was significant compared with a decrease following simulated exposure. [17] Notably, active cigarette smoking was associated with a decrease in exhaled NO that remained low. On the basis of this data it has been hypothesized that cigarette smoking can induce chronic damage to airway epithelial cells producing NO. Decreased eNO has been reported in habitual smokers and reverts after smoking cessation suggesting an interference of NO-rich cigarette smoke with local antioxidant defences. The mechanism affecting NO levels in smokers appears to be reversible: Högman observed that subjects who stopped smoking for four weeks showed that airway NO flux increased to levels similar from non-smokers [18]. Another study showed that exhaled NO levels increased after just one week of not smoking and had increased again by eight weeks (figure 2) [19]. Moreover in smokers it is possible to find a negative correlation between eNO levels and tobacco habits expressed as number packets of cigarettes/year. The reduced eNO levels found in current and past smokers suggest the utilisation of eNO as an objective marker of tobacco consumption and exposition. These findings suggest that eNO measurement can have a role in anti-smoking programmes; its increase can be a positive parameter for subjects who are going to stop with cigarette smoke and at the same time a compliance anti-smoking indicator. It can be useful for the investigation of the mechanism of cigarette-induced lung damage in the experimental setting and may potentially be useful for assessment of Environmental Tobacco Smoke (ETS) effects.

Induced sputum

Various studies have demonstrated that exposure to tobacco smoke causes cellular oxidative stress and the release of inflammatory mediators in the airways of healthy subjects. These effects can be both acute and chronic [20, 21]. In susceptible individuals, the increased oxidative and nitrosative stress process would lead to structural changes in the airways and to the development of COPD [22, 24]. Markers of oxidative stress have been detected in induced sputum of COPD subjects even though only few studies investigated the use of induced sputum to study smoke effects on the lungs of healthy subjects. A number of studies showed modifications of inflammatory cells and mediators in induced sputum and in the

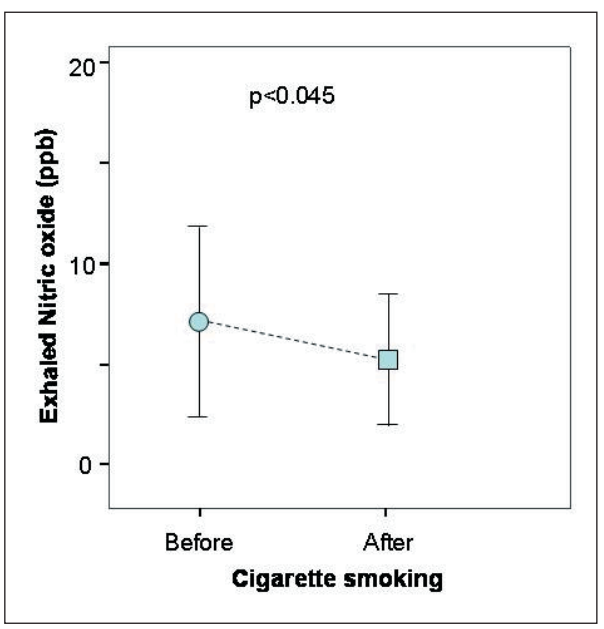

Fig. 1. - Exhaled NO concentrations in asthmatic smokers before and 15 min after smoking one cigarette.
Adapted from Horvath *et al* 2004 [14].

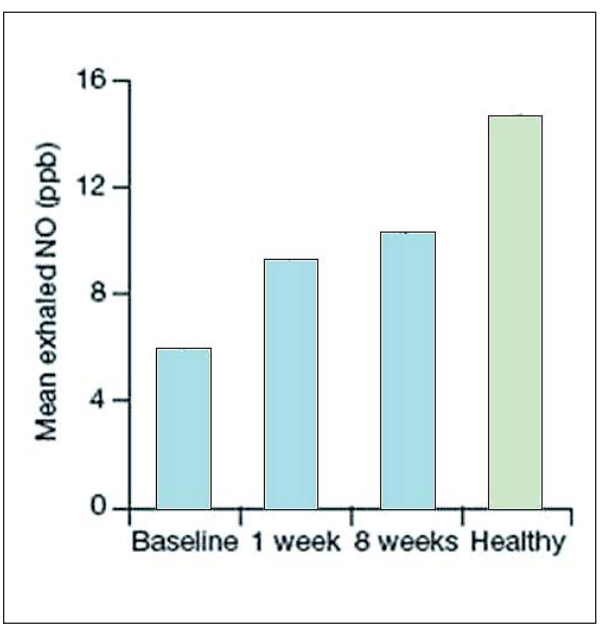

Fig. 2. - Mean exhaled NO levels (10 mL/s) in smokers following cessation of smoking and in healthy controls.
Adapted from Robbins *et al* 1997 [19].

BAL of smoking subjects. An increased number of neutrophils was found in the BAL of smokers: this was related to the number of smoked cigarettes and the degree of airflow limitation [25]. It was demonstrated that smokers had significantly higher values of LTB4 than normal subjects and this is consistent with the increase in neutrophils number in BAL and induced sputum observed by Keatings *et al*. [26]. In agreement with this hypothesis, LTB4 has been found previously to be elevated in sputum [27] and in the BAL of smokers [28]. Other studies showed modifications of inflammatory cells and mediators in induced sputum of COPD patients. A significant reduction in IL-10 levels (an anti-inflammatory cytokine) and a small number of IL-10-expressing cells was found in the sputum of patients with asthma, COPD and smokers compared with healthy non-smokers [29] suggesting an important role of the anti-inflammatory cytokines in the development of chronic obstructive diseases. Another study investigated the effect of smoking cessation on cellular inflammation of the lungs in patients with COPD. The authors compared the cellular composition of induced sputum from smokers and ex-smokers with COPD patients and they found no significant differences in the cellular profile of the two groups. This result may indicate that smoking causes persistent inflammation changes in the airways in patients predisposed to COPD [30]. More recently, different studies investigated the effect of acute and chronic cigarette smoking on induced sputum inflammation in healthy smokers. Van der Vaart *et al*. [20] studied the acute response to cigarette smoke in intermittent smokers, since repetitive acute smoke effects may cumulate and lead to irreversible changes. They found that smoking rapidly increases the number of sputum neutrophils between 3 and 12 hours after smoking and they observed a biphasic response in sputum lymphocytes, that, after an initial smoke-related suppression increases more with smoking. They hypothesised that the fast increase in neutrophils might result from detachment of neutrophils from the pulmonary vascular endothelium or by recruitment from the bone marrow, while the initial reduction in lymphocytes might result from increased adherence in lung tissue or to a fast up-regulation of adhesion molecules after smoking. Several markers of oxidative-nitrosative stress were found to be increased in sputum of healthy smokers and symptomatic smokers without airway obstruction (GOLD stage 0 COPD) such as: neutrophil numbers, elevated expression of inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO, a marker of neutrophils activation), 4-HNE (a marker of lipid activation) [31]. Moreover markers of airway remodeling were found in healthy smokers: levels of total MMP-9 (a matrix metalloproteinase) are present in high concentrations in smokers and its expression is correlated with tobacco exposure [32]. The maintenance of the active MMP-9/TIMP-1 ratio in healthy smokers may explain the absence of progressive airway obstruction, for this reason the authors propose the measurement of active MMP-9 concentration in induced sputum to assess airway remodeling. Another metalloproteinase, MMP-12 (an elastolytic proteinase firstly found in alveolar macrophages of cigarette smokers) seems to be involved in cigarette-smoke-induced emphysema. A recent study [33] showed an increased expression of MMP-12 in induced sputum of healthy smokers compared to non-smokers, supporting the suggestion that smoking may increase the expression of this enzyme. Another recent study [34] found that vascular endothelial growth factor (VEGF) levels are increased in induced sputum of both asymptomatic and COPD smokers, suggesting VEGF as a marker reflecting the inflammatory process occurring in smoking subjects. The findings of these studies underline the fact that the analysis of the induced sputum may enhance the understanding of airways changes due to current smoking and may be useful in finding new biomarkers in order to assess and monitor early lung damage induced by smoke in order to prevent the progression of obstructive disease.

Exhaled breath condensate

Exhaled breath condensate (EBC) obtained by cooling exhaled air under conditions of spontaneous breathing is a promising biological fluid that could provide an assessment of pulmonary pathobiology. It can be easily and non-invasively collected from patients of any age [35]. The analysis of exhaled air is feasible and non-invasive [36] and can be useful in identifying new biomarkers of exposure or susceptibility in patients with lung diseases. Recently it has been hypothesised that long-term exposure to tobacco smoke leads to an increased lung uptake of toxic metals (particularly cadmium) that can also be used as markers of environmental pollution [37]. Moreover some biochemical compounds could be used as biomarkers of lung damage, which can indicate the limitation of the pulmonary tissue's ability to respond to the challenge of exposure to a xenobiotic substance [38]. In particular oxidative stress and the related inflammation could be assessed through the measurement of exhaled hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and leukotrienes (LTB4) [39].

It has been hypothesised that cadmium may be responsible of the smoke related toxicity on the lungs, in fact it was observed that subjects who died for COPD or emphysema had increased levels of cadmium in their liver when compared with subjects who died for other causes [40]. Some authors also found elevated cadmium concentrations in emphysematous lungs [41]. A recent study compared the content of cadmium in alveolar macrophages in smokers and non-smoker subjects using BAL [42]. The authors found that significant amounts of cadmium accumulate in the alveolar macrophages of smokers compared to non-smokers, with a correlation between cadmium content of alveolar macrophages and smoking history expressed as number of cigarettes smoked per day [42]. More recently EBC was used to compare the

presence of toxic metals in COPD and control subjects, this paper confirmed the ability of EBC to identify cadmium pulmonary contamination and the authors observed that cadmium in EBC was higher in COPD patients than in the control subjects [37]. The authors concluded that cadmium and other toxic-heavy metals could be biomarkers of current tobacco smoking. Moreover there were differences between the COPD patients and control subjects when the former were classified into smokers and ex-smokers or non-smokers. The authors suggested that exhaled metal levels may also provide a measure of cumulative long-term exposure to tobacco smoke and environmental exposure to toxic metals. One of several potential links between cigarette smoking and the development of inflammatory lung diseases is the overburden of lower airways with oxidants and free radicals [43]. There is considerable evidence that oxidative stress is increased in patients with COPD and that reactive oxide species, such as hydrogen peroxide (H_2O_2), may contribute to the pathophysiology of COPD [44]. Measurement of H_2O_2 in EBC suggested reflecting free radical generation and tissue damage in the airways [45]. H_2O_2 in EBC is unstable; therefore, EBC should be rapidly frozen and analysed after collection the determination of its peroxide concentration. The most frequently used methods of measuring H_2O_2 in EBC are the colorimetric or fluorimetric measurements [46]. Compared with healthy non-smokers, exhaled hydrogen peroxide is increased in EBC of healthy smokers [47] and this increase is more pronounced in patients with stable COPD [44,45] reflecting the disease severity [48]. A positive correlation between H_2O_2 levels in EBC and cumulative cigarette consumption (pack-years) was observed in 17 asymptomatic cigarette smokers, EBC was collected after 12 h restraining from smoking (figure 3)

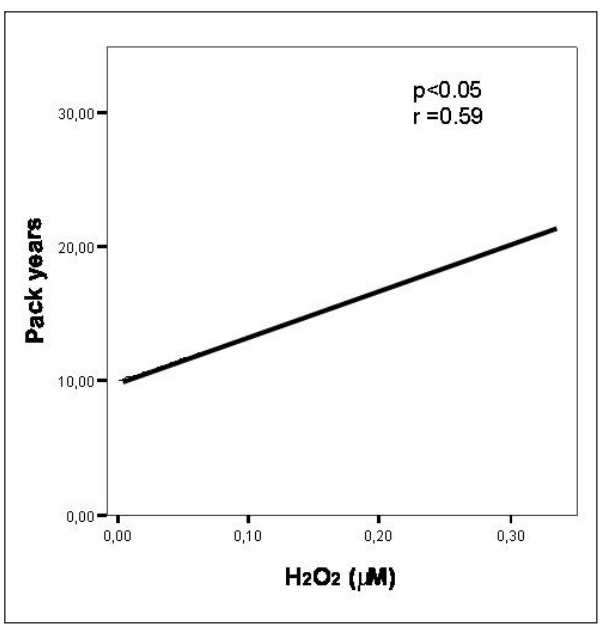

Fig. 3. - Positive correlation between H2O2 levels in EBC and cumulative cigarette consumption (pack-years) in 17 asymptomatic cigarette smokers.
Adapted from Novak *et al* 2001 [47].

[47]. The concentrations of exhaled hydrogen peroxide are further elevated in patients with COPD exacerbations [43]. Horvath *et al* observed that smoking increases the oxidative stress in the asthmatic airways, supporting the hypothesis that smoking worsens the oxidative stress [14]. The increased exposure of smoker's lung tissue to oxidants may result from the presence of large amounts of free radicals and oxidants in cigarette smoke. Another, perhaps more remarkable, source of oxidants in the respiratory tract of cigarette smokers is activated phagocytes. Cigarette smoke causes enhanced recruitment of mononuclear phagocytes and polymorphonuclear leucocytes to the lower airways [49,50]. These cells have altered oxygen metabolism and release more H_2O_2 and other reactive oxygen species than phagocytes from non-smokers.

On the basis of the reported papers, an elevated content of H_2O_2 in expired breath may represent an indirect marker of free radical-mediated processes and reflects harmful oxidant overload in the lower airways related to cigarette smoking so exhaled hydrogen peroxide analysis may be useful to assess lung oxidative stress in exposed subjects.

Evidence suggests that exhaled leukotrienes may be involved in tobacco smoke lung toxicity [51]. Cigarette smoke can induce the production of TNF-α by macrophages and epithelial cells and can help macrophages to release other inflammatory markers like IL-6 and LTB4 [52]. The presence of LTB4 in EBC was confirmed by different study groups [52, 53]. Increased numbers of neutrophils are found in the BAL of smokers and are related to the number of cigarettes smoked and the degree of airflow limitation [25]. It was demonstrated that smokers had significantly higher values of LTB4 than normal subjects and this is consistent with the increase in neutrophil numbers in BAL and induced sputum observed by Keatings *et al*. [26]. In agreement with this hypothesis, LTB4 has been found previously to be elevated in sputum [27] and in the BAL of smokers [28]. LTB4 in EBC was found increased in ex smokers COPD patients compared to healthy non-smokers [54], and more increased during COPD exacerbations [55]. Moreover, a mild increase in LTB4 concentration was observed in the EBC of healthy smokers compared with healthy non-smokers [56], this confirming a possible role of LTB4 as markers of early lung disease in smoking subjects. Conversely, more recently it has been hypothesised that the presence of LTB4 in EBC may be due to a salivary contamination [57], the authors claimed future measures of LTB4 in EBC including sensitive alpha-amylase assay.

It has been demonstrated that smokers present an increased oxidative stress as documented by elevated levels of MDA [58]. MDA-DNA adducts are also increased in lung cancer cases with respect to controls, but only in smokers [59].

Increased values of MDA were observed in EBC of COPD patients as compared with non-smoking controls [60, 61].

Conclusions

New non-invasive techniques as eNO, induced sputum and exhaled breath condensate have been successfully introduced to study the inflammation processes involved in the pathogenesis of pulmonary diseases while other methods to directly assess airway inflammation that have been tried before are quite invasive. In the last few years these techniques have been proposed to find sensible parameters to reveal early lung alterations after exposure to tobacco smoke in healthy subjects, as it is known that cigarette smoking is implicated in the pathogenesis of chronic obstructive disease. Exhaled nitric oxide is acutely decreased after cigarette smoking, probably due to the release of oxide species while chronic exposition to cigarette smoke may induce damage to airway epithelial cells producing eNO. This effect seems to be reversible, for this reason eNO can have a role in anti-smoking programmes and may be useful for assessment of environmental tobacco smoke effects. Induced sputum may be helpful to identify early modifications in inflammatory cells and mediators in healthy smoking subjects to prevent the progression towards obstructive diseases. EBC, finally, have shown that smokers have an increased oxidative stress demonstrated by increased levels of LTB4, H_2O_2, MDA and an increased lung uptake of toxic metals that can be used as markers of environmental pollution and as biomarkers of lung damage.

These findings suggest that above mentioned parameters may be sensible markers to reveal early lung alterations caused by chronic exposure to tobacco smoke in susceptible subjects to prevent the COPD development.

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