INTRODUCTION

Long-term cigarette smoking is the major etiological factor for the development of chronic obstructive pulmonary disease (COPD). Airway inflammation and oxidative stress are implicated in the pathogenesis. Biomarkers reflecting these responses could be analyzed in exhaled breath condensate (EBC). Recently, it became obvious that mediator concentrations in EBC could be influenced by age of the subjects, equipment, sampling properties, and the analytical assays applied. In the present study we evaluated 8 smokers (20-56 yr) and 16 non-smoking adults (18-60 yr) with normal spirometry and no episode of airway infection during 6 weeks prior to the study. EBC samples were obtained with the commercial device ECoScreen® at a controlled temperature of -20°C. Leukotriene B$_4$ (LTB$_4$, marker of inflammation), 8-iso-prostaglandin F$_2\alpha$ (8-iso-PGF$_2\alpha$, 8-isoprostane, oxidative stress) concentrations, and pH were measured. With 10 min of tidal breathing, a lower EBC volume was collected in smokers (median 1.22 ml; interquartile range 1.06-1.74 ml) than in non-smokers (1.6 ml; 1.16-2.21 ml; P=0.06). Significant differences were found in pH in smokers compared with non-smokers (7.14 (5.70-7.43) vs. 7.59 (7.28-7.73); P<0.01). No significant differences were observed in EBC concentrations of LTB$_4$ or 8-iso-PGF$_2\alpha$. The study demonstrates that acidopnea is detectable in otherwise asymptomatic smokers and might precede changes in the level of arachidonic acid metabolites. For pH is considered to be the most validated marker determined in EBC samples, it may be useful for screening asymptomatic individuals for smoking-induced early airway damage.

Key words: exhaled breath condensate, chronic obstructive pulmonary disease, inflammation, mediators, leukotriene B$_4$
According to the American Thoracic Society (ATS) criteria, forced vital capacity (FVC) and forced expiratory volume in 1 sec (FEV₁) were obtained from three acceptable lung function tests (11). Normal lung function was defined as FEV₁ ≥80% predicted and an FEV₁/FVC ratio ≥70%.

Collection of EBC

The commercial available device ECoScreen²® (FILT, Berlin, Germany) was used for EBC collection. The subjects were asked to rinse their mouths with water, otherwise no food, drinks, or smoking were allowed 2 h prior to EBC collection. The oral cavity was inspected for foci of inflammation before sampling. EBC was collected during tidal breathing for exactly 10 min while sitting comfortably. Expiratory flow, tidal volume, breathing frequency, and minute ventilation were measured by an integrated pneumotachograph. The subjects breathed through a mouthpiece and a two-way non-rebreather valve. They were instructed to swallow excess of saliva after coming off the mouthpiece. Moreover, saliva contamination was also prevented by an integrated saliva trap. The condensate was sampled in one chamber and a two-way non-rebreather valve. They were instructed to swallow excess of saliva after coming off the mouthpiece. Moreover, saliva contamination was also prevented by an integrated saliva trap. The condensate was sampled in one disposable bag on a special polyethylene surface. Parameters were defined and controlled in a way that the complete volume was condensed and collected in one ECoScreen’s chamber. The ECoScreen²® feature of exclusion a volume representing the dead-space was not used. During the collection period a temperature of -15 to -20°C was maintained by the condenser. After EBC collection, samples were immediately divided in aliquots and stored at -70°C. All EBC samples were analyzed in a blinded way expressed as median with interquartile range. Spearman rank correlation test was used to determine correlations between the EBC compounds studied. Data were analyzed by using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

EBC volume and pH

All patients were able to produce EBC. After 10 min of tidal breathing, a lower EBC volume was collected from smokers (median 1.22 ml; interquartile range 1.06-1.74 ml) than from the non-smoking control group (1.60 (1.16-2.21) ml), although the difference did not reach statistical significance (P=0.06; Fig 1A). The pH values after deaeration were significantly lower among smokers compared with the non-smokers (7.14 (5.70-7.43) vs. 7.59 (7.28-7.73), P<0.01; Fig 1B).

EBC LTB₄ and 8-iso-PGF₂α

LTB₄ was undetectable in 3 smoking and 4 non-smoking subjects; 8-isoprostane was detectable and could be quantified in every sample. Significant differences were not found in EBC concentrations (pg/ml) of both biomarkers between the study groups. Because the EBC volume was different for the groups, the total amount of the markers (pg) in the EBC samples was additionally calculated. Again, significant differences were not found (Table 2).

There was a strong positive correlation between LTB₄ and 8-iso-PGF₂α concentrations (as well as in the total amount, data not shown) in the EBC collected from both groups respectively. However, only in the smokers, there was also an inverse correlation between these biomarkers and the EBC pH. High concentrations of LTB₄ or 8-iso-PGF₂α correlated with low EBC pH (Table 3).

Specific enzyme immunoassay kits were used to detect LTB₄ or 8-iso-PGF₂α (Assay Designs, Ann Arbor, USA). The kits use a polyclonal (LTB₄) or monoclonal (8-iso-PGF₂α) antibody to bind the relevant marker. The optical density was used to calculate the concentration of the biomarker by using the Softmax Pro 4.7.1 software utilizing a 4-parameter logistic curve fitting program. In each assay, the lowest standard was set as the limit of quantification (LOQ) of the assay. The LOQ of LTB₄ was 11.7 pg/ml and the maximum cross-reactivity was 5.5% for 6-trans-12-epi-LTB₄. The LOQ of 8-iso-PGF₂α was 6.1 pg/ml and the maximum cross-reactivity was 4.6% for PGF₂α. The intra-assay %CV varied from 5.8 to 6.8% and the inter-assay %CV from 5.0 to 16.5% (for low to high concentrations of LTB₄) (12).

Statistical analysis

Value distribution was assessed using the D’Agostino & Pearson omnibus normality test. Comparisons of data were performed with unpaired t-test and Welch’s correction, where appropriate. Values below the LOQ were set 2/3 of LOQ. A two-sided significance level of 0.05 was chosen for all tests. Data are expressed as median with interquartile range. Spearman rank correlation test was used to determine correlations between the EBC compounds studied. Data were analyzed by using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA).

![Fig 1](image)

**Fig. 1.** Volume (Panel A) and pH (Panel B) of exhaled breath condensates in asymptomatic smokers and healthy non-smokers.
Table 1. Characteristics of the study subjects.

<table>
<thead>
<tr>
<th>Category</th>
<th>Smokers</th>
<th>Non-smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.5 (20.0-56.0)</td>
<td>50.5 (18.0-60.0)</td>
</tr>
<tr>
<td>FVC % predicted</td>
<td>107.8 (93.5-133.2)</td>
<td>105.8 (91.1-136.1)</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>98.3 (80.1-118.9)</td>
<td>100.3 (80.3-128.4)</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>80.2 (70.1-94.6)</td>
<td>80.9 (70.3-96.2)</td>
</tr>
</tbody>
</table>

Data are expressed as median (range). FVC - forced vital capacity; FEV1 - forced expiratory volume in 1 s.

Table 2. EBC leukotriene B4 (LTB4) and 8-iso-prostaglandin F2α (8-iso-PGF2α) values in the groups studied.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Smokers</th>
<th>Non-smokers</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>464.4 (223.0-661.1)</td>
<td>264.2 (82.7-713.1)</td>
<td>0.73</td>
</tr>
<tr>
<td>LTB4 pg/ml</td>
<td>22.8 (8.9-36.7)</td>
<td>14.1 (7.8-40.3)</td>
<td>0.67</td>
</tr>
<tr>
<td>PG pg/ml</td>
<td>52.1 (35.5-67.7)</td>
<td>56.3 (19.0-66.5)</td>
<td>0.70</td>
</tr>
<tr>
<td>8-iso-PGF2α pg/ml</td>
<td>727.2 (334.5-1021.0)</td>
<td>488.8 (95.1-998.3)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

There were no significant differences found in either the concentrations or the absolute amounts of the biomarker, respectively.

Table 3. Correlations between pH, leukotriene B4 (LTB4), and 8-iso-prostaglandin F2α (8-iso-PGF2α) in exhaled breath condensate derived from smokers (■) and non-smokers (□).

<table>
<thead>
<tr>
<th>pH</th>
<th>LTB4</th>
<th>8-iso-PGF2α</th>
</tr>
</thead>
<tbody>
<tr>
<td>r=0.81 P=0.015</td>
<td>-0.81</td>
<td>r=0.76 P=0.033</td>
</tr>
<tr>
<td>LTB4</td>
<td>r=0.18 P=0.522</td>
<td>r=0.94 P=0.017</td>
</tr>
<tr>
<td>8-iso-PGF2α</td>
<td>r=0.15 P=0.615</td>
<td>r=0.87 P&lt;0.0001</td>
</tr>
</tbody>
</table>

DISCUSSION

Long-term cigarette smoking is the major etiological factor for the development of chronic obstructive pulmonary disease (COPD) and airway inflammation and oxidative stress are implicated in its pathogenesis (7, 13, 14). Biomarkers reflecting these responses could be analyzed in exhaled breath condensate (EBC) (6) and were shown to temporally increase during exacerbations of COPD (15). EBC is an attractive non-invasive method but methodical limitations hampered its clinical use (5). There is accumulating evidence that mediator concentrations in EBC could be influenced by the physical surface properties of the collecting devices, the temperature and the analytical assays applied (9). Moreover, for some markers the age of the subjects, was identified as a confounder (10).

The objective of the present study was to evaluate chronic subclinical changes in otherwise healthy current smokers. Subjects refrained from smoking prior to EBC collection to avoid possible acute effects by exposure to cigarette smoke (16). Non-invasive markers of acidopenia (pH), neutrophil inflammation (LTB4), and oxidative stress (8-iso-PGF2α) were assessed in EBC. EBC was collected with the new commercial available temperature-controlled device ECoScreen2® at -20°C according to general methodological recommendations on the collection and analysis of EBC (5). ECoScreen2® is a single-use disposable condensing and collection system. In preliminary experiments, ECoScreen2® was demonstrated to be superior in comparison with the commonly used collecting system ECoScreen concerning EBC volume and biomarker concentrations (leukotriene B4, prostaglandin E2, 8-iso-prostone) measured with specific enzyme immunoassay kits (data not shown). For the present study, a control group consisting of healthy non-smokers was recruited; thereby smokers and non-smokers demonstrated equal normal lung function assessment. Confounding was further reduced by comparable age of the groups and by applying sampling of EBC for both groups ante meridiem.

We found that collecting EBC from smokers resulted in a lower sample volume than with non-smokers. Since the duration of collection for both groups was exactly 10 min and breathing patterns (measured by pneumotachograph) were similar, the observed differences might reflect a decrease in aerosolization of the airway lining fluid as a result of cigarette smoke-induced epithelial injury (17). As there was a difference observed in the EBC volume of the groups, biomarker values were calculated for their absolute amount in the EBC sample in addition to the concentration presented.

One main finding was a significant difference in EBC pH revealing lower values in smokers. Published data demonstrate airway acidification in several respiratory diseases and reveal the important pathophysiological role of airway acidic stress (2). There are different recommendations concerning the general conditions of EBC pH measurements. In the present study, we assessed pH values after deaeration with argon. This method was previously shown to be robust and reliable (18) and normative data were characterized in healthy subjects (19). In our study, measured EBC pH values are in accordance to published data on pH measurements in healthy non-smokers referring to deaeration with argon gas (9).

With the ECoScreen2® condensing equipment and the specific enzyme immunoassays applied in our study, the concentrations of LTB4 were in most samples within the limit of quantification and 8-iso-PGF2α could be validly quantified in any sample. There was an overlap of EBC LTB4 and 8-iso-PGF2α values in healthy smokers showing no significant difference compared with control subjects.

8-iso-PGF2α belongs to the group of F2-isoprostanes and is formed by free radical-catalyzed peroxidation of arachidonic acid, reflecting oxidative stress and lipid peroxidation (20). Compared with a previous study on healthy smokers using ECoScreen, which reported detectability in about half of the subjects (8), we could detect 8-iso-PGF2α in all samples with high immunoassay activity. The levels of 8-iso-PGF2α in EBC have been shown to be elevated in healthy smokers compared with normal subjects. However, there seems to be a difference in pulmonary function (FEV1 pred 93.0±4.3 in healthy non-smokers vs. 87.3±4.1 in healthy smokers) (16). This discrepancy might be explained by younger age of our subjects. Our results are in line with a recently published study demonstrating no significant difference of EBC 8-iso-PGF2α in healthy smokers compared with non-smokers (3). Smokers were aged 43±12, which is close to the smoking subjects (aged 41±14) evaluated in our study.

LTB4 is enzymatically formed from arachidonic acid by 5-lipoxygenase and is a potent chemotactant of neutrophils. Significant elevation in EBC LTB4 concentrations were reported in patients with COPD compared with matched healthy smokers (21). There is one study comparing healthy smokers with non-
smokers demonstrating increased LTB4 concentration in EBC of smokers (7). LTB4 was assessed only in a subgroup of the indicated group (10 out of 21) and detailed characteristics of these subjects were not presented.

In the present study, concentrations (and also the total amounts) of LTB4 correlated with 8-iso-PGF2α in EBC derived from smokers as well as non-smokers. The interrelationship of the indicated biomarkers is in agreement with a recently published study (4). Smoking caused a significant reduction in EBC pH obtained from smokers, while LTB4 and 8-iso-PGF2α were not different compared with control subjects. However, in smokers the decreased EBC pH significantly correlated both with LTB4 and 8-iso-PGF2α concentrations, indicating activation of neutrophils and resulting oxidative stress. LTB4 plays an important role in recruitment and activation of neutrophils. In this context, it is intriguing that neutrophils also migrate down pH gradients, seeking out areas of lower pH (22). On the other hand, neutrophils can contribute to lowering of pH in fluids. Our results are in line with data suggesting that endogenous acidification is attributed to neutrophilic inflammation (2). Low airway pH has been implicated in COPD pathophysiology, as it could cause bronchoconstriction, impair ciliary motility, or damage to epithelium (23).

In conclusion, our results demonstrate ECoScreen2® to be a suitable device for detecting biomarkers in EBC widely applied for assessing oxidative stress (8-iso-PGF2α) and inflammation (LTB4). In otherwise asymptomatic smokers acidopnea is detectable whereas no changes in the level of arachidonic acid metabolites could be detected in comparison with healthy non-smoking subjects. Collection of EBC is safe and does not interfere with the underlying disease; thereby it can be performed repeatedly. For pH is considered to be the most validated marker with the underlying disease; thereby it can be performed for epidemiological field studies. J Physiol Pharmacol 2007; 58(Suppl 5): 289-298.

Conflict of interests: None declared.

REFERENCES


Received: July 27, 2009
Accepted: October 15, 2009

Author’s address: Dr. Frank Hoffmeyer, BGFA, Forschungsinstut für Arbeitsmedizin der Deutschen Gesetzliche Unfallversicherung, Ruhr-Universität Bochum, Bukele-de-la-Camp Platz 1, 44789 Bochum, Germany; Phone: +49 234 302-4549. Fax: +49 234 302-4542; E-mail: hoffmeyer@bgfa.de