

# Acute and Chronic Effects of Smoking on Inflammation Markers in Exhaled Breath Condensate in Current Smokers

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## Key Words

Cigarette smoke · Chronic obstructive pulmonary disease · Electrical conductivity · Exhaled breath condensate · Inflammation

## Abstract

**Background:** Long-term cigarette smoking is associated with pulmonary inflammation, but the acute effects of smoking have been less well studied. Analysis of the exhaled breath condensate (EBC) can provide noninvasive markers that might be indicative of inflammation. **Objectives:** The aim of the study was to determine whether the pH, electrical conductivity and the levels of ammonium and interleukin 8 (IL-8) of EBC were altered in smokers and whether they changed after smoking a single cigarette. **Methods:** We included 19 healthy nonsmokers (controls), 29 asymptomatic smokers, 10 patients with stable chronic obstructive pulmonary disease (COPD) [Global Initiative for Chronic Obstructive Lung Disease stages (GOLD) stages II–III], and 10 patients with exacerbated COPD. In 13 smokers, EBC was also analyzed before and after smoking. EBC was obtained during 10 min tidal breathing with a cooled RTube™. pH was determined after deaeration with argon. **Results:** Acute smoking did not alter the pH or ammonium and IL-8 levels, but raised

conductivity. As in COPD patients, the pH was significantly decreased in chronic smokers with a history of at least 10 pack-years compared to controls. **Conclusions:** EBC can be used to detect the acute and chronic effects of smoking. The increased conductivity of EBC after smoking suggests acute inflammatory effects. The reduced pH in chronic smokers shows cigarette-induced inflammation.

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## Background

Long-term tobacco smoking constitutes a major risk factor for chronic obstructive pulmonary disease (COPD), inducing inflammatory cell recruitment and activation as demonstrated by a variety of invasive and noninvasive techniques. In addition to long-term alterations, short-term, acute responses to smoking could also provide insight into the response of the respiratory tract. The noninvasive techniques now available for analyzing airway inflammation are quite attractive and are increasingly used clinically.

Such techniques include sputum induction, the assessment of exhaled nitric oxide (FE<sub>NO</sub>) and exhaled breath condensate (EBC). FE<sub>NO</sub> is a useful marker, especially in

**Table 1.** Patients' characteristics

	Male	Female	FEV <sub>1</sub> , % predicted	FEV <sub>1</sub> /VC, % predicted	Current smoking	Age, years
Healthy controls	16	13	105.2 ± 11.1	98.8 ± 9.1	(n = 0)	32.1 ± 9.5
COPD	9	1	56.4 ± 13.5	64.6 ± 18.2	(n = 3) PYs: 31.1 ± 16.2	67.9 ± 8.1
Exacerbated COPD	8	2	34.4 ± 11.4	54.7 ± 11.3	(n = 1) PYs: 47.4 ± 28.9	69.4 ± 11.6
Smokers	10	9	99.7 ± 10.6	98.1 ± 7.9	(n = 29) PYs: 21.9 ± 18.5	38.2 ± 12.2

asthma, but its interpretation is difficult in smokers [1]. Sputum induction yields information on inflammatory cells and mediators [2, 3] but has methodological limitations as well: the procedure itself affects the airways and elicits both a neutrophilic response [4] and a decrease in EBC pH [5].

A low pH in EBC is assumed to reflect airway inflammation as reported for several disorders: asthma [6], COPD [7], cystic fibrosis [8], lung transplant rejection [9], bronchiectasis [10]. pH is of great interest for clinical applications as it can be measured shortly after EBC collection using simple equipment. The same applies to electrical conductivity, which has been proposed as a correlate of the aerosol fraction of EBC, and which might also be a marker of airway inflammation [11].

The analysis of EBC has revealed chronic inflammatory alterations in smokers as compared to nonsmokers such as increased levels of interleukin (IL)-6 and IL-8 and leukotriene B<sub>4</sub> [12, 13]. Only few studies have focused on the acute effects of smoking, and only a small number of investigators used EBC to analyze local inflammatory reactions [14–16]. Smoking can elicit acute effects even in smokers as demonstrated, for example, by a raised IL-8 release from stimulated leukocytes [17]. The aim of the present study was to investigate whether smoking exerts an acute effect on pH in smokers and whether the pH of the EBC of asymptomatic smokers, as a marker of inflammation, differs from that of nonsmokers. For comparison, we included patients with COPD. Along with the pH we also assessed electrical conductivity, ammonium and IL-8 in EBC samples from participants.

## Participants and Methods

### Study Participants

The study population consisted of 29 asymptomatic smokers (16 males/13 females aged 38 ± 12 years) with a smoking history of 21.9 ± 18.5 pack-years (PYs); 20 subjects reported >10 PYs (26.8 ± 17.4 PYs) and 9 subjects <10 PYs (3.2 ± 2.6 PYs). As control

group, we enrolled 19 healthy nonsmokers (10 males/9 females aged 32 ± 10 years) without a history of pulmonary disease. Two out of the healthy nonsmokers were ex-smokers with 5 PYs. Airway obstruction was ruled out by spirometry in both healthy nonsmokers and asymptomatic smokers (table 1).

For comparison, we included patients with stable COPD [Global Initiative for Chronic Obstructive Lung Disease stages (GOLD) stages II–III] as defined by GOLD criteria, with no signs of exacerbation (9 males/1 female aged 67.9 ± 8.1 years). No patients had received treatment with oral steroids for at least 4 weeks before the measurements. Five were current smokers and 5 were ex-smokers. As a group with acute inflammation, we enrolled 10 patients with COPD exacerbation (8 males/2 females aged 69.4 ± 11.6 years) defined by standard criteria [18]. At the time of measurement, they had not received any systemic corticosteroids. The study was approved by the local Ethics Committee and informed consent was obtained from each subject.

All participants answered a questionnaire regarding symptoms, smoking habits, health status, medication and medical history. Moreover, blood samples were taken and differential cell counts performed. Systemic inflammation was ruled out in control subjects and smokers by low leukocyte counts and low serum levels of C-reactive protein and procalcitonin. Spirometry, following American Thoracic Society/European Respiratory Society guidelines [20], was used to exclude airway obstruction in healthy controls and smokers, and to assess the stage of COPD according to GOLD criteria [21]. Spirometry was not performed in the patients admitted due to an acute exacerbation.

### Study Design

In this observational study, EBC was collected at least once in all subjects and 3 times in the subgroup of smokers who were tested before and after smoking a cigarette. The smokers refrained from smoking at least 2 h prior to measurements. In a random subgroup of 13 asymptomatic smokers, the acute effect of smoking was assessed after smoking a single cigarette of a commercial brand (Gauloises Red) over a time period of 5–10 min. EBC was collected 5–15 min after the end of smoking, as well as 15–25 and 25–35 min after the end of smoking. All samples were analyzed for pH.

In addition to pH, electrical conductivity, IL-8 and ammonium levels were determined in the EBC samples from 10 healthy control subjects and 10 smokers. In the smokers, in addition to pH, the EBC collected 5–15 min after smoking a single cigarette was used for these analyses. Limitations in the amount of available EBC prevented us from performing the additional measurements in all subjects and at all time points.

## Methods

### EBC Collection

EBC samples were collected during 10 min of quiet breathing through a single-use disposable RTube™ collector (Respiratory Research, Inc., Charlottesville, Va., USA), while subjects were wearing a nose clip. The aluminum sleeve of the device had been cooled to an initial temperature of  $-20^{\circ}\text{C}$  prior to collection. After collection, the plunger was used to pool the condensed material within the tube into a single sample (about 1.0 ml). Samples were stored for a maximum of 4 weeks in reaction tubes at a temperature of  $-20^{\circ}\text{C}$  before IL-8, conductivity and ammonium were measured. pH measurements were performed directly after EBC sampling.

### Assessment of pH

For pH determination, 250  $\mu\text{l}$  of EBC were transferred into a polyethylene tube that had been washed with double-deionized water. Samples were de-aerated with a gentle argon flow (Linde Gas, Germany, purity 99.9%) for  $\geq 20$  min until pH readings were stable. pH was determined with a microelectrode and a pH meter (Windaus<sup>®</sup>, Clausthal-Zellersfeld, Germany), which were calibrated before each sequence of measurements, using pH values of 4, 7 and 9. Samples used for pH determinations were not utilized for other measurements. pH measurements were performed directly after EBC sampling.

### Electrical Conductivity

Conductivity measurements were performed in 100  $\mu\text{l}$  EBC using a glass microcell (LDM/S; WTW, Weilheim, Germany) at a temperature of  $25^{\circ}\text{C}$ . The microcell was flushed with deionized water and dried with argon. All measurements were done in duplicate. Readings were always stable within few seconds, far above the detection limit of the device, and reproducible within  $<2\%$ .

### Ammonium

Ammonium measurements relied on the classical Berthelot reaction involving phenol, sodium hypochlorite, and sodium pentacyanonitrosylferrat (III). After incubation (pH 11.8–12.4,  $50^{\circ}\text{C}$ , 1 h), the amount of  $\text{NH}_4^+$  ions in 50- $\mu\text{l}$  samples of EBC was determined photometrically at 620 nm (UV-1602; Shimadzu, Duisburg, Germany). Four standards and 1 blank value treated in the same way as the samples were used to obtain the final concentrations. The limit of detection was 5 ng  $\text{NH}_4^+$  in 50  $\mu\text{l}$ , and the standard deviation of repeated measurements was about 2.5%.

### Interleukin 8

The level of IL-8 in EBC was determined by ELISA in duplicate – using two 100- $\mu\text{l}$  samples without preconcentration – and an ultrasensitive IL-8 assay (Ultra Sensitive Kit, Biosource, Nivelles, Belgium). Measurements in blinded samples were performed by FILT GmbH, Berlin, Germany. The limit of detection was  $<100$  fg/ml. The standard curve was fitted between 0 and 25 pg/ml.

### Analysis

Depending on the distribution of each variable, mean values and standard deviations (SD) or median values and interquartile ranges (IQR) were computed for data description. The different groups of control subjects were compared using the nonparametric Kruskal-Wallis analysis of variance (ANOVA). For (post hoc) comparisons between specific groups, the Mann-Whiney U test

was employed. Friedman's nonparametric ANOVA served to evaluate the time course of pH after smoking, and the Wilcoxon matched-pairs signed-ranks test was used to compare specific time points. Spearman's rank correlation coefficients ( $r_s$ ) were computed to quantify the degree of association between variables. No correction for the multiplicity of tests was performed, but p values are given explicitly wherever reasonable. Statistical significance was assumed at 5%.

## Results

### *Acute Effects of Smoking on EBC*

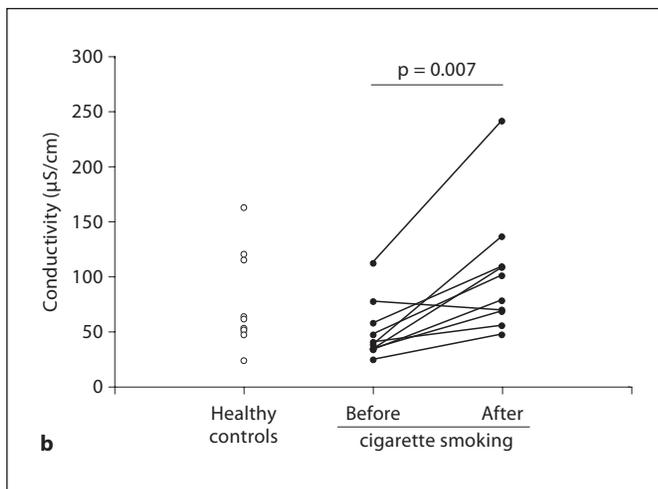
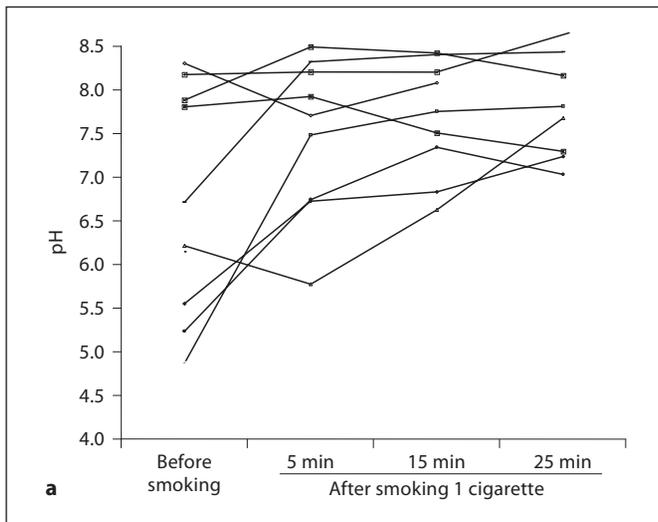
EBC was collected from 13 current smokers at different time points before and after smoking. Median (IQR in parentheses) pH before smoking (baseline) was 7.18 (2.12), and 5–15, 15–25 and 25–35 min after smoking it was 7.41 (1.39), 7.58 (1.21), and 7.78 (0.59), respectively. Despite a tendency for some subjects to show an increased pH (fig. 1a), these 4 measurements were not statistically significantly different from each other ( $p = 0.392$ , Friedman).

There was also no significant ( $p = 0.508$ , Wilcoxon) difference in the level of IL-8 in EBC before [85 (31) pg/l] and 5–15 min after smoking [82 (22) pg/l]; those data were based on the EBC from 10 smokers, for whom paired samples of sufficient volume were available. In those subjects, the ammonium concentration in EBC before and 5–15 min after smoking was 6.0 (4.0) and 5.6 (2.2)  $\mu\text{g/ml}$ , respectively, which is not significantly different ( $p = 0.554$ , Wilcoxon). Electrical conductivity before and after smoking was 40 (24) and 90 (41)  $\mu\text{S/cm}$ , and these values are significantly different from each other ( $p = 0.007$ ) (fig. 1b).

Correlation analyses did not reveal statistically significant correlations between the different measurements or their changes after smoking, except for a correlation between conductivity and ammonium after smoking ( $r_s = 0.72$ ,  $p = 0.019$ ; fig. 2).

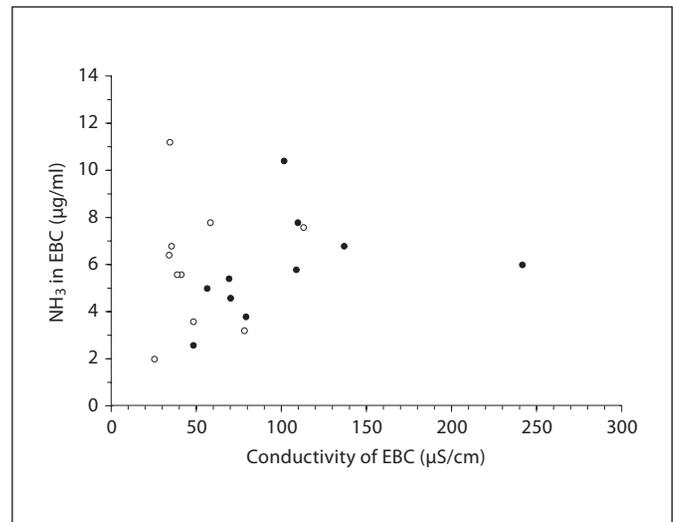
### *Chronic Effects of Smoking on EBC*

EBC was collected in subjects with a smoking history of  $>10$  PYs ( $n = 20$ ). In those subjects, the median (IQR in parentheses) pH was 7.40 (2.26), while in those with a history of  $<10$  PYs ( $n = 9$ ) it was 8.17 (0.68), and in control subjects ( $n = 19$ ) 8.20 (0.40) (fig. 3a). There was a significant difference between groups ( $p = 0.001$ , Kruskal-Wallis). Specifically, smokers with  $>10$  PYs showed lower values than control subjects ( $p < 0.001$ , Mann-Whitney) or smokers with  $<10$  PYs ( $p = 0.013$ ), whose values did not significantly differ ( $p = 0.731$ ) from those of controls.



**Fig. 1. a** Time course of pH of EBC in asymptomatic smokers over a time period of 35 min after smoking a single cigarette. Each collection of EBC took 10 min, and the time points indicate the start of the collection. There were no statistically significant changes of pH over time. **b** Individual values of electrical conductivity of EBC in healthy subjects (○) and asymptomatic smokers before and after smoking a single cigarette (●). While baseline values did not differ between groups ( $p = 0.007$ ), there was a significant increase in conductivity after smoking.

To further elucidate these differences, we compared subjects from different groups selected at random from the population of control subjects, patients with stable COPD, patients with an exacerbation of COPD, and smokers with >10 PYs. Each group consisted of 10 subjects. There was a significant difference between these four groups ( $p = 0.014$ , Kruskal-Wallis), and the Mann-Whitney U tests confirmed that the median (IQR in pa-



**Fig. 2.** Relationship between the values of conductivity and ammonium in the EBC of asymptomatic smokers before (○) and after (●) smoking a cigarette. There was a significant correlation between the values assessed after smoking ( $r_s = 0.72$ ,  $p = 0.019$ ).

renteses) pH in control subjects [8.16 (0.22)] was greater than in smokers [7.58 (1.17);  $p = 0.010$ ] and patients with stable [7.36 (1.10);  $p = 0.004$ ] or exacerbated COPD [7.05 (2.70);  $p = 0.019$ ] while the last 3 groups did not show any significant differences (fig. 3b).

In contrast to pH, IL-8 levels did not differ significantly ( $p = 0.198$ ) between the 4 groups, median (IQR in parentheses) values being 67 (80), 85 (31) 66 (9) and 61 (38) pg/ml in healthy subjects, smokers, and patients with stable COPD and COPD during exacerbation, respectively. Moreover, ammonium levels in EBC were 7.2 (1.9) μg/ml in 10 smokers and 6.0 (4.0) μg/ml in 10 control subjects ( $p = 0.570$ ). Electrical conductivity of EBC was 58 (63) and 40 (24) μS/cm, respectively ( $p = 0.075$ ). Neither of these two measurements differed significantly between the 2 groups.

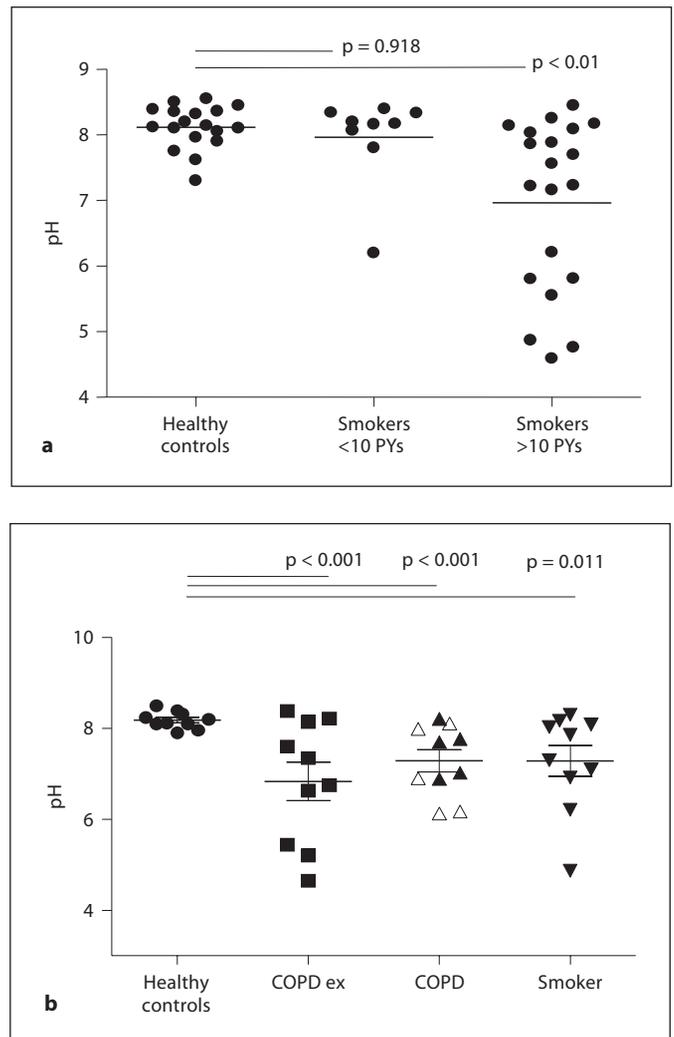
## Discussion

The present study showed that acute smoking causes an increase in conductivity, correlated with higher amounts of ammonium in EBC. Until now, very few data have been collected on the acute effects of smoking on human airways. In vitro models revealed that cigarette smoke caused an inflammatory response in epithelial cells regarding IL-6 and IL-8 [22], while in another model IL-8 production

was inhibited [23]. In the EBC of smokers collected after smoking, we observed no significant increase in pH, although a tendency was noted, but we were able to detect an increase in conductivity. Conversely, there are data on welding fume inhalation, which has been found to increase pH in healthy workers [24]. So far, the mechanisms of pH increase in EBC after inhaling welding fumes have been unclear. However, we consider it likely that nonspecific chemical parameters such as pH and conductivity are closely related in EBC in most circumstances. Noteworthy enough, Do et al. [25] also found a correlation between pH and  $\text{NH}_4^+$ ; however, this group did not use nose clips as recommended [26]. They further used shorter de-aeration periods of only 10 min (we used at least 20 min) and only included 5 current smokers. The most important difference between this and our work is that we measured the influence of acute smoking compared with chronic smokers and not only current smokers.

Cigarette smoke could affect the pH of EBC by changing the buffer capacity of the epithelial lining fluid via physicochemical mechanisms. For example, the hot smoke could raise the local temperature of the lining fluid, thereby increasing the dissociation constant of the  $\text{NH}_4^+$  or bicarbonate buffer, the major buffers in EBC. According to the van't Hoff equation, this would imply a greater buffer capacity. In view of the difference in heat capacities of fluid and gas, such effects are unlikely and should be extremely short-lived. It is also unlikely that smoking-related differences in breathing patterns, with concomitant alterations in  $\text{CO}_2$  levels, had an effect. After de-aerating the samples with argon prior to measurement,  $\text{CO}_2$  was absent as indicated by a blood gas analysis (data not shown). Moreover, smoking did not significantly alter the pH of EBC within half an hour. These observations, as well as the fact that the smokers had refrained from smoking for at least 2 h before the assessments, render it likely that the reduction in pH in asymptomatic smokers truly reflected chronic inflammation or damage of the airways that were present despite the absence of respiratory symptoms and lung function impairment.

Indeed, it seems reasonable to expect acute inflammatory effects immediately after smoking even in chronically exposed subjects who have been smoking >10 cigarettes per day, but the concentration of IL-8 in EBC, a marker related to inflammatory cellular influx and activation, did not change after smoking. However, IL-8 levels were also similar in smokers and healthy nonsmokers, indicating that this marker was less sensitive to alterations occurring within the airways than pH. The negative findings regarding baseline IL-8 are in line with previous



**Fig. 3.** **a** Individual values of EBC pH obtained with RTube™ in healthy control subjects and young asymptomatic smokers with diverse smoking histories in terms of PYs. pH values in smokers with >10 PYs were significantly lower than in control subjects or smokers with <10 PYs ( $p < 0.05$  each). **b** Individual values of EBC pH in healthy control subjects, patients with stable COPD ( $\Delta$  indicating COPD ex-smokers) or exacerbated COPD, and asymptomatic smokers with >10 PYs ( $n = 10$  per group). pH values in control subjects were significantly higher than values of the other three groups, including the smokers ( $p < 0.05$  each). These three groups did not show significant differences in pH values. Horizontal bars indicate median values.

data showing that epithelial cells cultured from bronchial brush biopsies exhibited a similar constitutive and TNF- $\alpha$ -induced IL-8 release in healthy smokers and control subjects [27].

The decreased pH in asymptomatic smokers matches other signs of pulmonary inflammation such as elevated

numbers of neutrophils and increased amounts of inducible nitric oxide synthase, myeloperoxidase, nitrotyrosine and 4-hydroxy-2-nonenal [28]. The known reduction of Fe<sub>NO</sub> in smokers might also indicate inflammation, possibly resulting from the scavenging of NO by reactive oxygen species. Notably, our finding that smokers with a history of >10 PYs showed pH values as low as patients with COPD is in line with data on the increased numbers of inflammatory cells in the small airways of smokers with normal spirometry [29].

The pattern observed for electrical conductivity was different from that of pH. At baseline, conductivity was similar in smokers and nonsmokers, but it showed a marked rise after cigarette smoking, suggestive of direct chemical reactions to cigarette smoke. This was probably not due to increased levels of ammonium ions, which we measured as a major compound of EBC. There was, however, a correlation between these two measurements, at least after smoking. Thus, the increase in conductivity caused by smoking might still be partially attributed to ammonium. In fact, previous observations have suggested that, at baseline, the ammonium levels correlate very well with conductivity in nonlyophilized EBC [30]. It might be speculated that compounds of the inhaled smoke were present in the EBC, or that the production of endogenous aerosols increased due to minor small airway obstruction, thereby increasing EBC conductivity. Although our data are not sufficient to address this question, they suggest that there is a relationship between conductivity and the acute effects of cigarette smoke and pH, which might be relevant for long-term alterations caused by cigarette smoke.

We were able to show that chronic cigarette smoking is associated with a lower EBC pH in smokers without signs of airway disease, compared to nonsmokers. The reduction in pH depends on the duration of smoking, as it occurred only in subjects with a smoking history of greater than 10 PYs, whereas smokers with fewer PYs (on average 3) showed no signs of decreased pH. This finding parallels the previous observation that another marker of inflammation, i.e. the level of IL-6 in EBC, was dependent on daily cigarette consumption [12]. Interestingly, the reduction in pH was similar to that of patients with stable or exacerbated COPD, irrespective of whether they were smokers or ex-smokers. Since the pH of EBC to some extent mirrors the presence of pulmonary inflammation, as described for chronic disorders such as COPD, asthma and bronchiectasis [8, 10, 31], these findings lend support to the assumption of chronic inflammation in asymptomatic smokers.

Based on these considerations, the change in EBC pH in asymptomatic smokers represented an early marker of pulmonary inflammation, which could be determined easily and noninvasively. Future studies might assess whether this measurement has additional uses, e.g. whether it bears the potential for predicting COPD before impairments in lung function might become apparent, or might support the motivation for giving up smoking.

It is apparent that the method of EBC analysis is still fraught with standardization problems. Different devices are used, which may affect sample volume and protein content of EBC [32]. There is no commonly accepted measure for adjusting concentrations in EBC which would help to achieve comparability between studies. It is not known whether the use of the same sampling period or a defined ventilated volume would produce more comparable results in all instances. Moreover, the storage of the samples is a frequently discussed problem. Regarding pH, available data suggest that freezing and thawing does not change the pH of the EBC sample [31]. Whether proteins are lost by freezing and thawing has not been sufficiently clarified. Despite this limitation, the easy collection of samples, the low costs and new, highly sensitive molecular biology tools indicate that EBC analysis is a promising method provided the required standardization is achieved.

In conclusion, the present study demonstrated a reduction in the EBC pH in asymptomatic smokers with a significant smoking history but no lung function impairment. pH values were similar to those of patients with COPD, either with stable disease or exacerbation. It thus appeared that the pH in EBC indicated chronic inflammation in the absence of changes in lung function. In asymptomatic smokers, smoking of a single cigarette induced an increase in electrical conductivity, but not in pH. As an immediate practical consequence, these findings suggest that subjects should refrain from smoking prior to EBC sampling for determinations of conductivity, and possibly also pH.

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