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# Airway antioxidant capacity and pH in chronic obstructive pulmonary disease

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**Key Words**
Bilirubin; Exhaled breath condensate; pH; Trolox

**Abstract**
Oxidant-antioxidant imbalance is implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD). Exhaled breath condensate (EBC) is a novel and non-invasive method of assessing the redox status of the local airway in COPD subjects. It was hypothesized that COPD patients have less antioxidant capacity in their local airway as compared to the control subjects. The method involved collecting EBC from control and COPD patients (stable and during an exacerbation). Concentrations of EBC antioxidants, namely Trolox-equivalent antioxidant capacity (TEAC), bilirubin, urate and ascorbate, were analyzed using ELISA and high performance liquid chromatography. The mean TEAC level in the stable COPD group ($11 \pm 2$ μM) was significantly reduced compared to the ex-smokers & smokers ($45 \pm 11$ μM). The COPD group during an exacerbation had a mean bilirubin level of $0.22 \pm 0.04$ mg/dl, which was significantly lower than the stable COPD group ($0.35 \pm 0.01$ mg/dl), and the non-smoking group ($0.35 \pm 0.02$ mg/dl). Urate and ascorbic acid were undetectable. In conclusion, COPD patients are likely to have increased oxidative stress and reduced antioxidant capacity in their local airways.



## INTRODUCTION

Oxidative stress plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD) via reactive oxygen and nitrogen species, which lead to the activation of nuclear factor kappa-B and activator protein-1. Thus the recruitment and activation of inflammatory cells induces the production of more reactive oxygen and nitrogen species fueling the airway inflammatory processes [1-5]. Many studies have examined a variety of oxidative stress markers and antioxidants in COPD, but much of the work has been done in the systemic circulation instead of in the airway. The level and type of oxidative reactions and the balance of antioxidants present in the extracellular compartments of the airway remain largely unknown. A novel method of assessing the redox status of the airway is exhaled breath condensate (EBC). This technique has not been widely applied to the analysis of the airway redox status in COPD.

Included among the many endogenous antioxidants known to play a pivotal role in the homeostasis are bilirubin, urate, ascorbic acid, as well as many others [6, 7]. While much work has been performed examining the antioxidant capacity of plasma, relatively little data is available relating to these variables in EBC. Bilirubin has been shown to be elevated in the induced sputum of asthmatic subjects, but has not been described in regard to the airways of subjects with COPD [8]. Uric acid or urate has been detected in the bronchoalveolar lavage (BAL) at approximately half the usual serum level of about 400 μM and while plasma urate is reduced in asthma, little is known of the level of urate in the lungs of COPD patients [9, 10]. Furthermore, the severity of COPD has been associated with a decreased serum ascorbate [11, 12]. However, there is relatively little information on the amount of ascorbic acid in the airways of COPD patients, with one study suggesting a reduction of ascorbic acid in the induced sputum of COPD patients [13].

During airway inflammation, hypochlorous, hypobromous, nitrous and peroxynitrous acids are produced by the inflammatory cells and which can contribute to $H^+$ ion burden within the airway [14], thus making pH an indirect index of inflammation, which is associated with the generation of oxidative stress. EBC pH correlates with the extent of oxidative stress (as assessed by $H_2O_2$ and 8-isoprostane) in COPD and asthmatic subjects with the EBC from COPD subjects having the lowest pH compared to the control and asthmatic subjects [15].

This study aimed therefore to determine if the pH and the concentrations of antioxidants above in EBC vary between the COPD and control groups, as well as between those with stable COPD (SCOPD) and an exacerbation of COPD (ECOPD). It was hypothesized that airway acidity (as indicated by EBC hydrogen ion concentration) would be the lowest in controls when compared with patients with SCOPD and during an exacerbation. Antioxidant capacity might be expected to be highest in the EBC of the control group and to be depleted in those with an exacerbation of COPD.

## MATERIALS AND METHODS

### *Study design and subjects*

This was a cross-sectional observational study. Informed consent was obtained from the subjects and ethics approval was obtained from the Prince of Wales' Hospital (SESIAHS) Research Ethics Committee.

COPD was defined by clinical assessment and lung function tests [16]. COPD subjects were recruited from the Prince of Wales' Hospital with the SCOPD patients recruited from the outpatient respiratory clinic and ECOPD patients as defined according to criteria previously established recruited from the inpatient respiratory wards [17].

Control subjects (healthy non-smokers with less than 1 pack year smoking history, $FEV_1$/FVC ratio of greater than 75%, or ex-smokers & smokers) were recruited from the community with the definition of an ex-smoker being one that had quit active smoking for more than a year. Patients with other respiratory illnesses other than obstructive lung diseases were excluded from the study.

### *Dietary questionnaire*

A validated dietary questionnaire was completed, from which the summed dietary and medication antioxidant intake [18, 19] defined subjects as having either high or low intake [20].

### *Exhaled breath condensate collection, processing and storage*

EBC was collected at 4ºC by 10-20 minutes of tidal breathing in to a condenser with a saliva trap as previously validated [21, 22]. Samples were aliquoted, immediately de-aerated with argon gas, and stored at -80ºC.

### *Measurement of oxidative stress markers and antioxidant capacity*

Not all assays could be performed on all subjects due to a limited EBC sample volume, particularly in those who were severely ill and those with a reduced vital capacity.

*TEAC:* total antioxidant capacity was measured using the Antioxidant Assay Kit (#709001, Cayman Chemical, Ann Arbor, MI, USA) as per protocol. Values approximating zero were replaced by the value 1 μM. The inter-assay and intra-assay coefficient of variation were 3% and 3.4% respectively. The linear dynamic range of the assay was from 0 to 33 μM.

*pH:* EBC pH was measured using a pH meter with silicon chip sensor (Model IQ125, IQ Scientific Instruments, Loveland, CO, USA).

*Total bilirubin:* the modified Jendrassik-Grof method was used in this assay for the standards [23]. Total bilirubin level in EBC was determined using the QuantiChrom Bilirubin Assay Kit (DIBR-180, BioAssay Systems, Hayward, CA, USA). The limit of detection (LOD) and intra-assay coefficient of variation were 0.16 mg/dl (2.74 μM) and 5% respectively.

*Urate and ascorbic acid:* EBC samples were extracted with 10% (w/v) meta-phosphoric acid (MPA). Iso-ascorbic acid (IAA; 10 μM final concentration) was added as an internal standard to confirm efficient recovery of ascorbate from EBC. Samples were de-aerated with argon and stored at -80ºC prior to HPLC analysis. Urate and ascorbic acid were assayed using HPLC with an electrochemical detector (ESA Coulochem III), with a guard (0.45 v) and an analytical cell (0.4 v). The mobile phase comprised 40 mM sodium acetate buffer (pH 4.75), 0.54 mM EDTA, 1.5 mM Q12 ion pair reagent (Regis Technologies, Morton Grove, IL, USA), and 7.5% methanol (v/v). Samples were injected into the LC-18 column (25 cm x 4.6 mm, 5 μ) (Supelco Analytic, Sigma-Aldrich, Castle Hill, NSW, Australia) running at 1 ml/min [24]. The LOD of this HPLC assay was 1 μM for both urate and ascorbic acid.

### *Statistical analysis*

Mean, 95% confidence interval (CI), and standard error (SEM) were calculated for all parametric data, using the Shapiro-Wilk test to determine which data were appropriate for parametric statistical analysis. Unpaired t-tests compared the groups. One-way ANOVA with post-hoc unpaired t-test (Tukey's test) was performed to compare the subgroups. Spearman's rank correlation

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examined the relationship between smoking pack years and pH. Correlation between $FEV_1$ and $FEV_1$/FVC ratio of all subjects excluding non-smokers and each assay were examined via Pearson's coefficient.

## RESULTS

### *Subject characteristics*

There were 39 COPD patients (23 SCOPD; and 16 ECOPD) and 42 controls (31 non- and 11 ex-smokers & smokers) recruited. The subjects' characteristics are illustrated in Table 1.

In terms of gender and the estimated dietary antioxidant intake, the COPD and control subgroups were matched. COPD subgroups were significantly older, had a greater smoking history, and significantly impaired lung function variables. Of those with ECOPD, 68.8% (11/16) had a systemic glucocorticoid administered, but no other subject used systemic glucocorticoids.

The dietary intake of antioxidants of all the subjects was lower than the recommended daily intake as stated in the Australian Guide to Healthy Eating [20].

### *pH*

pH was measured in 33 COPD subjects (14 ECOPD, 19 SCOPD), and 35 healthy controls (24 non-smokers, 11 ex-smokers & smokers).

The COPD group had a mean EBC pH of 7.1 ± 0.2, which was significantly lower by -0.5 (95% CI -0.89 to 0.14, p = 0.0074) when compared to the mean pH of the control group (7.6 ± 0.1, p = 0.0074; Fig.1).

One-way ANOVA also revealed a significant difference between the mean pH of the subgroups (F value = 3.8, p = 0.0044). The SCOPD group had the lowest mean pH among all subgroups (6.8 ± 0.2), and was significantly reduced when compared to non-smokers (7.6 ± 0.1, $p < 0.05$) by 0.8 (95% CI 1.4 to 0.12; $p < 0.05$), and smokers & ex-smokers (7.7 ± 0.1, $p < 0.05$, Fig.2). There was no significant difference between the mean pH within the COPD or within the control subgroups.

When pooling all subjects together, there was a significant negative correlation between EBC pH and the total amount of cigarette smoking (Spearman r = -0.48; 95% CI -0.66 to -0.25; p = 0.0001). pH demonstrated a statistically significant positive correlation with $FEV_1$/FVC ratio of all subjects excluding non-smokers (Pearson r = 0.41; 95% CI 0.067 to 0.67; $p < 0.05$) but not with $FEV_1$.

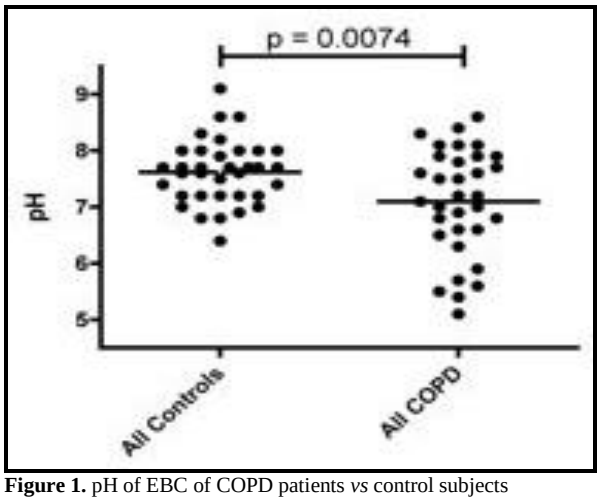


**Figure 1.** pH of EBC of COPD patients *vs* control subjects

**Table 1.** Characteristics of subjects with COPD versus healthy controls

| | Control subjects (n = 42) | | COPD (n = 39) | |
|---|---|---|---|---|
| | **Non-smokers** | **Ex-smokers & Smokers** | **Stable** | **Exacerbation** |
| **Number** | 31 | 11 | 23 | 16 |
| **Age (years)*** | 39.3 ± 3.5 | 37.7 ± 3.8 | 68.7 ± 2.4 | 75.3 ± 1.4 |
| **Gender (male/female)** | 15/16 | 9/2 | 12/11 | 11/5 |
| **$FEV_1$*** | 3 ± 0.2 | 3.04 ± 0.43 | 1.26 ± 0.17 | 0.81 ± 0.09 |
| **% pred $FEV_1$*** | 92.8 ± 3.9 | 86.8 ± 8.6 | 50.89 ± 4.73 | 34.12 ± 3.52 |
| **FVC*** | 3.48 ± 0.21 | 3.56 ± 0.54 | 2.49 ± 0.22 | 1.62 ± 0.15 |
| **% pred FVC*** | 89.98 ± 3.64 | 83.7 ± 6.05 | 76.0 ± 5.38 | 51.25 ± 3.98 |
| **% FEV1/FVC*** | 85.62 ± 1.15 | 79.9 + 2.27 | 51.88 ± 4.27 | 51.11 ± 3.69 |
| **Systemic corticosteroid use** | 0/31 | 0/11 | 0/23 | 11/16 |
| **Dietary antioxidants (high/low)**** | 0/31 | 0/11 | 0/23 | 0/16 |
| **Smoking (pack years)*** | 0 ± 0 | 10.9 ± 4.3 | 51.4 ± 6.7 | 53.4 ± 8.1 |

Data are normally distributed and presented as mean ± SEM. $FEV_1$, forced expiratory volume in 1 second; FVC, forced vital capacity.
*****There is statistically significant difference between the subgroups.
******The threshold between "high" and "low" is defined by the recommended dietary intake as set by the Australian Guide to Healthy Eating.

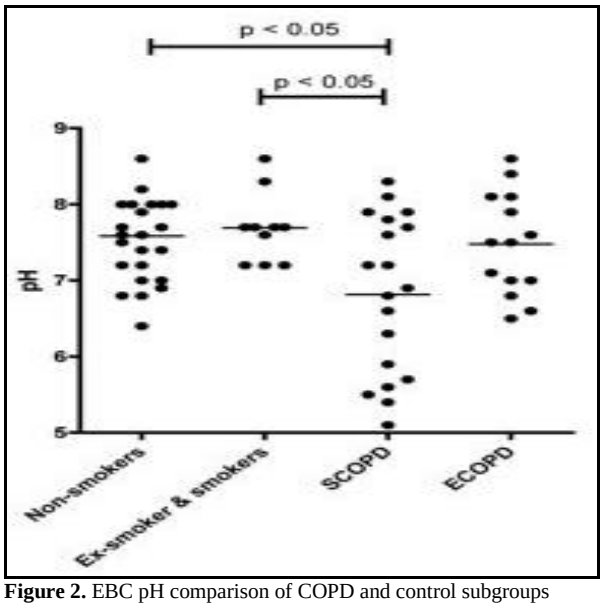


**Figure 2.** EBC pH comparison of COPD and control subgroups

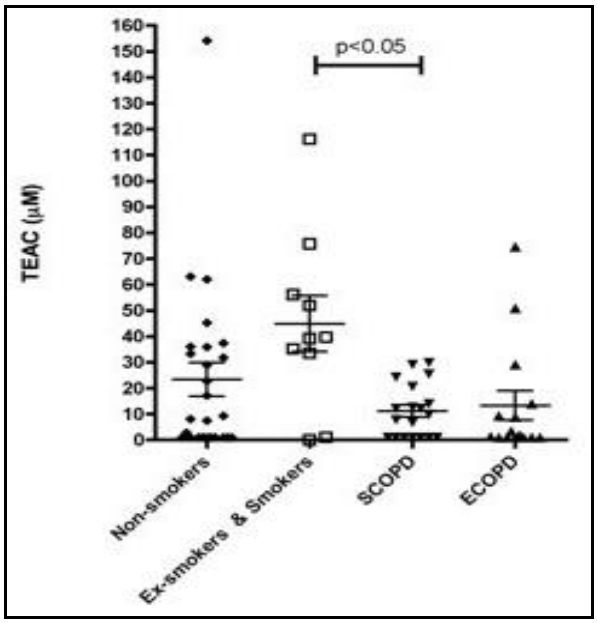


**Figure 3.** EBC Trolox equivalent antioxidant capacity (TEAC) of COPD *vs* control subjects

### *TEAC*

Overall, 34 COPD (19 SCOPD and 15 ECOPD patients) and 36 control samples (26 non-smokers; 10 ex-smokers & smokers) were analyzed.

The COPD group had a mean TEAC of 12 ± 2.8 µM, which was significantly lower than that of the control group (29 ± 5.7 µM, $p < 0.01$) by 17 ± 6.5 µM ($p < 0.01$).

ANOVA and subgroup analysis indicated significant differences (One-way ANOVA; F value = 3.7, $p = 0.005$) with a significantly reduced mean TEAC level in the SCOPD group (11 ± 2.4 µM) compared to the ex-smokers & smokers (post-hoc Tukey's comparison test; 45 ± 1.1 µM, $p < 0.05$) (Fig.3) by 34 mM (95% CI 65 to 2.0, $p < 0.05$). There was no statistically significant difference between the mean TEAC of ECOPD and that of SCOPD groups or within the control subgroups. There was also no statistically significant correlation between TEAC and $FEV_1$ or $FEV_1$/FVC ratio.

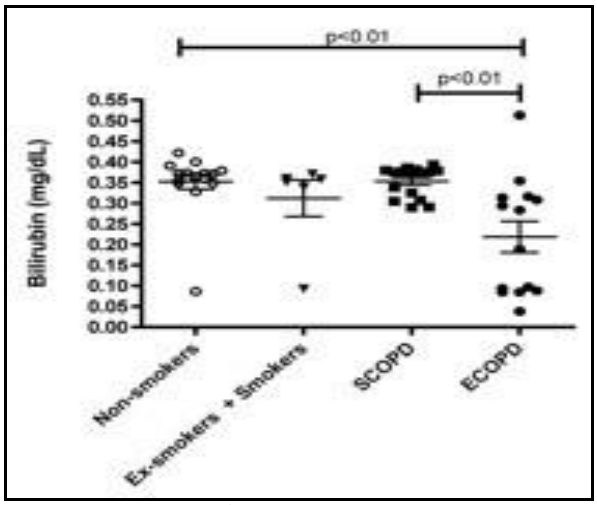


**Figure 4.** EBC bilirubin of COPD and control subgroups

### *Bilirubin*

A total of 30 COPD (16 with SCOPD and 14 ECOPD) and 22 control samples were analyzed.

Bilirubin was detectable in EBC. One-way ANOVA of the COPD and control subgroups demonstrated significant differences in mean bilirubin level (F value = 4.5, $p = 0.0017$; Fig.4). The ECOPD group had a mean bilirubin level of 0.21 ± 0.04 mg/dl (3.6 ± 0.6 µM), which was significantly lower than the SCOPD group (0.35 ± 0.01 mg/dl (6 ± 0.2 µM, $p < 0.01$) by 0.14 mg/dl (95% CI 0.24 to 0.03, $p < 0.01$), and the non-smoking control group (0.35 ± 0.02 mg/dl (6 ± 0.3 µM), $p < 0.01$) by 0.13 mg/dl (95% CI 0.24 to 0.03, $p < 0.01$). There was no statistically significant difference among the control subgroups nor between the mean bilirubin levels of the COPD group (0.29 ± 0.02 mg/dl; 5 ± 0.3 µM) and the control group (0.34 ± 0.02 mg/dl; 5.8 ± 0.3 µM) ($p = 0.093$). There was also no statistically significant correlation between bilirubin and $FEV_1$ or $FEV_1$/FVC ratio.

### *Urate and ascorbate*

Samples from 3 ECOPD patients and 6 control subjects were analyzed for urate and ascorbate. No urate or ascorbate could be detected in any EBC samples (LOD 1 µM).

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**Table 2**. Mean TEAC, bilirubin and pH levels in COPD and control subgroups

| | Mean TEAC (μM) | Mean bilirubin (mg/dL) | Mean pH |
|---|---|---|---|
| **All COPD** | 12.1 ± 2.8 | 0.29 ± 0.02 | 7.1 ± 0.2 |
| **All controls** | 29.3 ± 5.7 | 0.34 ± 0.02 | 7.6 ± 0.1 |
| **SCOPD** | 11.2 ± 2.4 | 0.22 ± 0.04 | 7.5 ± 0.2 |
| **ECOPD** | 13.3 ± 5.7 | 0.35 ± 0.01 | 6.8 ± 0.2 |
| **Non-smokers** | 23.3 ± 6.5 | 0.35 ± 0.02 | 7.6 ± 0.1 |
| **Ex-smokers & smokers** | 44.9 ± 11 | 0.31 ± 0.04 | 7.7 ± 0.1 |

Data are normally distributed and presented as mean ± SEM

**Table 3.** Correlations between $FEV_1$, $FEV_1$/FVC and TEAC/bilirubin/pH in all subjects excluding non-smokers

| | Pearson R value | |
|---|---|---|
| | **$FEV_1$** | **$FEV_1$/FVC** |
| **TEAC** | -0.18 (95% CI -0.49 to 0.17) / p = 0.309 | -0.18 (95% CI -0.49 to 0.17) / p = 0.309 |
| **Bilirubin** | -0.066 (95% CI -0.39 to 0.27) / p = 0.701 | -0.041 (95% CI -0.36 to 0.29) / p = 0.813 |
| **pH** | 0.34 (95% CI -0.016 to 0.62) / p = 0.061 | 0.41* (95% CI 0.067 to 0.67) |

*marks a value of statistical significance

## DISCUSSION

This study found that the mean TEAC level in the stable COPD group was significantly reduced compared to the ex-smokers & smokers, and that the COPD exacerbation group had a significantly lower mean bilirubin level than the stable COPD group and the non-smoking group. This is one of the first reports of the TEAC being measured in EBC of COPD and control subjects, confirming the presence of radical scavengers in EBC as previously hypothesized [21, 25]. It establishes that while EBC TEAC does not measure antioxidant efficiency, it can provide a useful method to measure the total free radical scavenging capacity in the airways of COPD patients, overcoming some of the difficulties in sampling sputum and BAL. In the past, it has been difficult to measure the antioxidant status in the lung compartments. While plasma TEAC levels of ECOPD patients have been shown to be lower than that of the SCOPD or normal control subjects , it does not necessarily reflect local airway antioxidant status, and EBC could therefore provide a more direct and relevant method using a minimally invasive procedure [26, 27]. This study has detected TEAC in EBC at the level of 10~60 μmol/l, which is significantly lower than the plasma TEAC measured in other studies (in the range of 300 to 1300 μmol/l) [26, 27].

A previous study by Liu *et al* showed decreased TEAC levels in EBC of asthmatic subjects but failed to show a significant difference between COPD and healthy controls, possibly due the small sample size [21]. This paper has extended those observations by demonstrating that COPD patients indeed have a lower level of TEAC in their EBC. Thus, COPD patients are likely to lack antioxidants not only in their diet and systemic circulation but also in the airways [1, 11, 12, 26, 28].

An unexpected finding was that ECOPD group did not have a significantly reduced EBC TEAC level compared to SCOPD group and control subgroups as hypothesized. This might possibly be attributed to the sample size as well as those with an exacerbation of their COPD already having received, prior to sampling, high dose systemic glucocorticoids, which could induce a decrease in airway oxidative stress [14, 15]. Prospective studies could investigate whether systemic glucocorticoid use would cause an increase in the EBC TEAC level of patients admitted for exacerbations of COPD, but obviously this is difficult in subjects who are very breathless. EBC as a technique can be useful for assessing the drug effects in patients with stable COPD [29].

TEAC represents an overall measure of antioxidant capacity and a minor contributor to this activity may be bilirubin. This is one of the first time that bilirubin has been reported in EBC, although bilirubin has been detected in the induced sputum of other patient groups [8]. The levels of EBC bilirubin (0.246 mg/dl - 0.379 mg/dl, corresponding to 4.2 μM - 6.47 μM) were similar to those in induced sputum (2.82 μM to 6.89 μM) measured by timed-endpoint diazo method [8] while few studies have determined bilirubin in BAL. Nonetheless, the use of these ELISAs in measuring bilirubin has been validated using HPLC [30, 31].

It has been postulated that the pathogenesis of COPD increases the burden of oxidative stress in the lung via smoking or infection, which leads to an up-regulation

of heme oxygenase-1 (HO-1) [32]. This causes an increased production of exhaled carbon monoxide and biliverdin, which subsequently is converted to bilirubin, an antioxidant which counters the elevated oxidative stress [32]. In an exacerbation of COPD, the excessive oxidative stress may deplete the bilirubin, or there may be a reduction of synthesis as suggested by decreased HO-1 activity in the alveolar macrophages of severe COPD patients [33]. This is the first time that a statistically significant reduction in bilirubin derived from the airways has been found in association with an exacerbation. The low level in EBC may suggest that it is not the main contributor to the total radical scavenging ability in EBC. Nonetheless, these findings suggest a role for bilirubin in the pathogenesis of COPD and warrant more study in this field. Our study was limited by the difficulty in recruiting older control subjects who had no comorbidities, smoking history or past lung disease. In addition, it was very difficult to find subjects who had a comparable smoking history to those with COPD, yet who were healthy, leading to a poor match for smoking history in the control group.

Hydrogen ion content or pH has been reported to be correlated with and may be an indirect indicator of oxidative stress [15] as it has been suggested to be indicative of neutrophilic inflammation [15, 34]. It is a relatively stable marker, resistant to degradation and dilution but pH may decrease with age. Breath $CO_2$ lowers the pH of EBC, therefore de-aeration of EBC samples with argon gas immediately after collection, freezing and minimizing the length of time the samples remained at room temperature was undertaken before analysis in order to standardize EBC pH conditions [14, 35]. This study showed a significantly negative correlation between the EBC pH and the amount of smoking, emphasizing the oxidative damage by cigarette smoking on the airway. Furthermore, this study showed a significantly positive correlation between the degree of airway obstruction ($FEV_1$/FVC ratio) and pH in all subjects excluding non-smokers without COPD. This study confirmed a significantly lower EBC pH in severe and stable COPD subjects [36, 37], and it also extends the finding by showing a significant difference between the stable COPD group and the ex-smokers & smokers subgroups. Moreover, this study suggested that severity of airway obstruction in subjects with COPD and cigarette exposure is related to a higher degree of oxidative stress. The mean pH of the ECOPD group was higher than that of the SCOPD group. This was unexpected as it would be anticipated that during an exacerbation of obstructive lung diseases, there would be a greater infiltration of inflammatory cells and therefore more acid production (*e.g.* HOCl, $HNO_2$, and HOBr), thus lowering the pH [15]. Again, these results could be due to those with exacerbations being treated prior to sampling by the administration of high dose systemic glucocorticoids [14]. In the future, incorporation of additional oxidative stress such as that of HOCl- and HOBr-mediated oxidative reactions (*i.e.*, 3-chlorotyrosine, 3-bromotyrosine) would assist in clarifying which acid moieties were contributing to the acidosis.

A limitation of this study is that due to the sample size the stable COPD group could not be further divided into the different disease severity groups according to the GOLD classification. In stable COPD patients, there is a difference between smoking status and EBC pH measured, as well as a trend of lower pH with disease severity in the stable COPD group [36]. It would be interesting in the future to explore EBC pH in different degrees of disease severity in both stable COPD and exacerbations with smoking as an independent factor. This may further elucidate the role of oxidative stress and smoking in the pathogenesis of COPD. Furthermore, it would also be worth studying the relationships between other antioxidants such as secretory leucocyte protease inhibitor [38], 8-isoprostane, a stable marker of oxidative stress [39], and leucotriene B4, a potent pro-inflammatory mediator [40, 41], in the EBC.

Uric and ascorbic acids are antioxidants which have been detected in the BAL [9, 10], which might be expected to comprise the same components as EBC. Neither urate nor ascorbate could be detected using HPLC with electrochemical detection. Lack of detection of these antioxidants is supported by the finding that these antioxidants exist in low level in the other body compartments. Urate levels in the BAL and bronchial wash are in the range of 0.4 to 0.97 μM [10, 42] although higher values have been reported [9]. Ascorbic acid has been detected in BAL in concentrations ranging from 0.01 to 0.21 μM [10]. A low level of urate and ascorbate in EBC might indicate that they are not the major antioxidants in the local airway, unlike their significant role in the systemic circulation [9, 43, 44]. It is possible, however, that ascorbate degradation could occur during the collection of the condensate, as it is readily converted by many redox-active agents, such as the transition metals.

Overall, there is clear evidence of significant oxidative stress in the local airway of COPD patients. COPD patients have a decreased level of TEAC in the exhaled breath condensate, and a more acidic environment in the local airways. In particular, those with COPD exacerbations have a reduced level of bilirubin. Not only does oxidative stress occur in the airways of patients with COPD exacerbations, oxidative stress also occurs in patient with stable COPD. The underlying issue of oxidative imbalances therefore may be an important area that needs to be addressed in patients with stable COPD, with the aim to reduce disease

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progression or further exacerbations. The detection of bilirubin in the EBC suggests that bilirubin potentially plays a role in modulating the pathogenesis of COPD and warrants further investigation. However, the low level, along with the undetectable levels of urate and ascorbate may suggest that they are not the main contributors of TEAC in EBC. Thiols and other unknown agents may therefore be responsible for the TEAC in EBC. Nonetheless, EBC antioxidants (bilirubin and TEAC) and oxidative stress markers may be useful in monitoring the effects of COPD interventions in the future, particularly if the use of antioxidant strategies is shown to be useful.

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