

# Inflammatory mediators in exhaled breath, induced sputum and saliva

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## Summary

**Background and Objective** Airway inflammation is assessed to monitor progression, control and treatment of asthma. The collection of exhaled breath condensate (EBC) provides a non-invasive alternative to induced sputum samples for the monitoring of airway inflammation. Both samples can be confounded by salivary contamination. The aim of this study was to compare the levels of inflammatory mediators in samples of EBC, induced sputum and saliva samples from subjects with asthma.

**Method** EBC, saliva and induced sputum samples were collected from subjects with asthma ( $n = 10$ ). Total protein, IL-8, 8-isoprostane and surfactant protein A (SPA) were assessed in each sample.

**Results** Total protein, IL-8, 8-isoprostane and SPA were detected in all sputum samples. Only total protein and SPA were consistently measured in EBC, with levels at least 100-fold lower than those measured in induced sputum. In saliva, total protein, SPA and 8-isoprostane were detected in all samples, with IL-8 detected in 60% of samples.

**Conclusions** Induced sputum is a reliable technique that can be used to assess markers of airway inflammation. While EBC is a simple and inexpensive technique to collect lower airway secretions, the detection of inflammatory mediators is variable, and further work is required to validate this technique to assess inflammatory mediators.

**Keywords** exhaled breath condensate, induced sputum, IL-8, 8-isoprostane, surfactant protein A

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## Introduction

The assessment of airway inflammation is an important tool in the management of airway diseases such as asthma. Strategies adjusting asthma management based on suppressing airway inflammation lead to fewer exacerbations when compared with a guideline-based management strategy using symptoms and lung function [1]. Airway inflammation has been typically assessed using bronchial biopsy, bronchoalveolar lavage (BAL) and/or bronchial washing; however, the invasiveness of these techniques limits their clinical application. In more recent times, the technique of induced sputum has been developed and validated as a reproducible and reliable way to assess airway inflammation that is relatively non-invasive [2].

Induced sputum has been used to investigate airway inflammation in stable asthma and in acute exacerbations of asthma in both adults [3, 4] and children [5, 6], and has been shown to be a safe [7–9], effective and reproducible method [10–13]. It is a simple and useful tool to investigate airway inflammation, providing information on both the

inflammatory cells present in the airways and inflammatory mediators. Studies comparing airway inflammation in BAL with induced sputum have shown them to be similar [14]. However, despite its many advantages, induced sputum is limited by the time required to collect and process the sample prior to measurement, and the expertise required is not readily available in all settings.

The collection of exhaled breath condensate (EBC) has provided a potential new tool for the assessment of airway inflammation. It is a simple technique requiring the patient to breathe tidally via a mouthpiece into a cooled tubing. The fluid that is condensed from exhaled breath can then be collected and used for mediator assessment. Exhaled breath contains many volatile and non-volatile compounds that may provide useful markers of airway inflammation. Several devices have been employed for the collection of EBC, ranging from expensive commercial instruments to more simple inexpensive systems utilizing a mouthpiece, and tubing encapsulated in ice. Most devices allow collection of between 1 and 2 mL of EBC in 10–15 min of tidal breathing, which makes EBC a rapid technique for sampling the airways.

Saliva is a potential contaminant of both sputum and EBC and contains both inflammatory cells and mediators. Contamination of induced sputum samples by saliva may lead to reduced mediator concentrations, but differential cell counts are relatively robust to the effects of saliva contamination

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[15]. The implications for contamination of EBC by saliva are unknown. Selecting lower respiratory portions from the induced sputum samples helps to minimize saliva contamination from induced sputum samples.

Both IL-8 and 8-isoprostane are important markers of airway inflammation and oxidative stress in asthma and other airway diseases. They have been reported to be present in EBC and sputum, thus making a comparison possible. Recently, we have found total protein to be useful in correcting IL-8 levels in sputum for salivary contamination [15]. We assessed the concentration of SPA as a specific protein of lung origin, and therefore it may be expected to be absent from contaminating saliva. Additionally, SPA is an important innate immune receptor, which has been reported to be increased in the BAL of subjects with asthma [16].

The aim of this study was to compare concentrations of inflammatory mediators in samples of EBC and induced sputum from subjects with asthma. We hypothesized that mediators would be detected more readily and in higher concentrations in samples of selected induced sputum. We sought to compare inflammatory mediators in saliva, to assess the potential for confounding of results by salivary contamination.

## Methods and materials

### *Subjects and design*

Non-smoking adults with asthma, who had a doctor's diagnosis of asthma plus demonstrated airway hyper-responsiveness to hypertonic saline, were recruited ( $n = 10$ ) from the John Hunter Hospital Asthma Clinic. An analytical cross-sectional study design was used. Subjects gave written informed consent and attended one visit where they underwent clinical assessment, EBC and saliva collection, spirometry, and sputum induction. The Hunter Area Health Service and The University of Newcastle Research Ethics Committees approved this study.

### *Methods*

Samples were collected in the following order:

- Exhaled breath condensate* was collected using the method of Formanek et al. [17]. Briefly, subjects were asked to breathe tidally for 15 min into a 55 cm Teflon tubing (Swagelok, Fluid Systems Technology, Melbourne, Australia) cooled using ice packs.
- Saliva* was collected following the EBC collection by asking participants to chew a cotton roll (Salivette, Becton Dickinson, North Ryde, Australia) for 30 s, after which the saliva was collected by centrifugation. The median (interquartile range (IQR)) yield of saliva was 1.3 (0.6–1.7) mL.
- Sputum induction*: Spirometry (Minato Autospiro AS-600; Minato Medical Science Co Ltd, Osaka, Japan) and combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%) were performed as previously described [18]. The median (IQR) time for sputum induction was 15 (11.3–15) min and a satisfactory sputum sample was obtained from all subjects.

*Sputum analysis*: Sputum was selected from saliva and processed as described [18]. The dispersed suspension was

filtered and a total cell count of leucocytes and viability were performed, and centrifuged, with the supernatant aspirated and stored at  $-80^{\circ}\text{C}$ . Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count obtained from 400 non-squamous cells.

*Fluid-phase mediator assessment* Sputum and saliva total protein content was determined using a commercial kit (Pierce Coomassie Plus Protein Assay Kit, Pierce Rockford, IL, USA). The intra- and interassay coefficient of variation for sputum total protein was 3% and 10%, respectively. The Micro BCA<sup>TM</sup> Protein Assay Kit (Pierce Rockford, IL, USA) was used to determine total protein in EBC samples. The intra- and interassay coefficient of variation for EBC total protein was 7% and 11%, respectively. Both assays employed a standard curve of bovine serum albumin in the range of 100–1500  $\mu\text{g/mL}$  (Coomassie) and 0.5–20  $\mu\text{g/mL}$  (BCA). IL-8 concentration was determined using a commercial ELISA (R&D Systems, Minneapolis, MN, USA). The sensitivity was determined according to the manufacturer's instructions and found to be 5.7 pg/mL.

The concentrations of 8-iso-prostaglandin  $\text{F}_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ) were determined by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA) with standard curves using purified 8-iso-PGF $_{2\alpha}$ . The assay has a 4 ng/L detection limit, uses antiserum that has 100% cross-reactivity with 8-isoprostane and <0.3% with prostaglandin analogues, and has been previously used to measure 8-iso-PGF $_{2\alpha}$  in plasma [19], sputum [20] and expired breath [21].

Surfactant protein A (SPA) was assessed using a previously described method [22]. Briefly, SPA antigen was coated onto plates and incubated overnight. Diluted samples and standards were incubated with primary antibody in a separate plate. A standard curve ranging from 31.2 to 1000 pg/mL was included in quadruplicate on each run. After 90 min, samples/standards were transferred to the coated plate and incubated at room temperature, after which the plate was washed and alkaline phosphatase sheep anti-rabbit IgG added (Sigma, St Louis, MO, USA). The plates were developed using phosphatase substrate (Sigma), and optical density (405 nm) determined. The intra- and interassay coefficient of variation for sputum SPA was 8% and 14%, respectively, and for EBC SPA it was 7% and 32%, respectively.

### *Analysis*

Data were analysed using Stata 7 (Stata Corporation, College Station, TX, USA). Results are reported as median and IQR unless otherwise indicated. Analysis was performed using the two-sample Wilcoxon Rank Sum, Kruskal-Wallis test for more than two groups, and Fischers' exact test for categorical data. Associations between data were determined using the Spearman rank correlation. Results were reported as significant when  $P < 0.05$ .

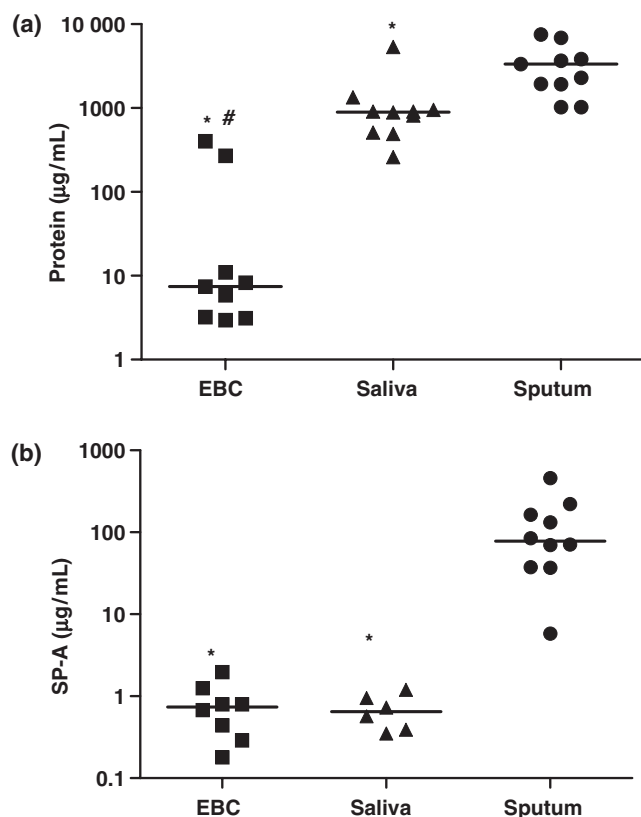
## Results

The analysis consisted of 10 subjects with asthma with a mean (range) age of 49 (22–65) years. Seven (70%) subjects were female and six (60%) were atopic. Eight (80%) participants

**Table 1.** Inflammatory mediators detected in EBC, saliva and sputum samples

| Mediator      | EBC    | Saliva   | Sputum   | P-value |
|---------------|--------|----------|----------|---------|
| Total protein | 9 (90) | 10 (100) | 10 (100) | >0.05   |
| IL-8          | 2 (25) | 6 (60)   | 10 (100) | 0.003   |
| 8-isoprostane | 1 (11) | 8 (100)  | 10 (100) | <0.001  |
| SPA           | 8 (89) | 6 (100)  | 10 (100) | >0.05   |

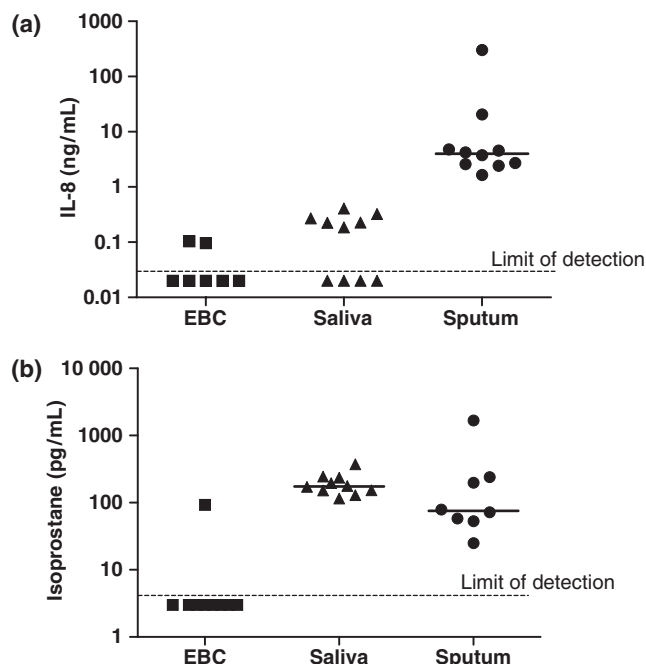
Data are number (percent) of samples with a detectable result. EBC, exhaled breath condensate; SPA, surfactant protein A.

**Fig. 1.** (a) Total protein levels in exhaled breath condensate (EBC), saliva and sputum samples. (b) Surface protein A (SPA) levels in EBC, saliva and sputum samples. #  $P < 0.01$  vs. saliva; \*  $P < 0.01$  vs. sputum.

were taking inhaled corticosteroid treatment with a median (IQR) daily dose of 1000 (1000–1250) µg fluticasone. Three (30%) subjects were ex-smokers with an average 31-pack year history. Participants had a mean (SD) forced expiratory volume in 1 s percentage predicted of 87% (23%). Half ( $n = 5$ ) of the participants had moderate persistent asthma, two had severe persistent asthma, one mild persistent asthma and the remaining two had intermittent asthma according to global initiative for asthma guidelines.

EBC collection resulted in a median (IQR) of 1.5 (1–2.45) mL of condensate. The pH was determined within 10 min of collection and was median (IQR) 7.3 (5.4–8.3).

Total protein was detectable using the Coomassie method for all saliva and induced sputum samples. No samples of EBC had detectable protein using the Coomassie method even with the micro-adaptation, which allows measurement of protein concentrations as low as 1 µg/mL. Nine (90%)

**Fig. 2.** IL-8 levels in exhaled breath condensate (EBC), saliva and induced sputum samples. 8-isoprostane in EBC, saliva and induced sputum samples. #  $P < 0.01$  vs. saliva, \*  $P < 0.01$  vs. sputum. The dashed line denotes the limit of detection for each assay.

EBC samples had detectable levels of protein using the micro-BCA protein assay (Table 1). EBC contained significantly less protein compared with saliva and induced sputum, while saliva contained significantly less protein than sputum (Fig. 1a).

SPA was detected in all sputum, saliva and in 8/9 (89%) of EBC samples tested ( $P > 0.05$ , Table 1). One sample of EBC and four saliva samples were of insufficient volume to have the SPA concentration assessed. Levels of SPA when detected were significantly higher in induced sputum samples compared with both EBC and saliva ( $P = 0.0002$ , Fig. 1b). Levels in EBC were not different to those in saliva.

IL-8 was detectable in all sputum, saliva and in 8/9 (89%) of EBC samples tested ( $P = 0.003$ , Table 1). Two EBC samples were of insufficient volume to have the IL-8 concentration assessed. The median (IQR) concentration of IL-8 in samples when detected was increased in sputum 4 (2.6–4.8) compared with saliva 0.25 (0.22–0.33) and EBC 0.1 (0.1–0.1) pg/mL, (Fig. 2a, *kwallis*,  $P = 0.001$ ).

All sputum and saliva samples assessed had detectable concentrations of 8-isoprostanes, but only 1/9 (11%) EBC sample had a detectable quantity of 8-isoprostane (Table 1). One EBC sample and two saliva samples had insufficient volume to have the 8-isoprostane concentration assessed. The median (IQR) concentration of 8-isoprostane in induced sputum and saliva was 75.5 (55.5–219.5) and 174.5 (152–235) pg/mL, respectively. There was no difference in 8-isoprostane levels measured in sputum and saliva samples (Fig. 2b, *kwallis*,  $P > 0.05$ ).

There were no correlations between condensate and sputum mediators except for SPA, where a significant negative correlation was observed ( $r = -0.78$ ,  $P = 0.02$ ,  $n = 8$ ).

## Discussion

This study assessed the concentrations of inflammatory mediators in samples of EBC, saliva and saline-induced selected sputum. We found that the levels of total protein and SPA were significantly lower in samples of exhaled breath compared with both sputum and saliva samples. Mediator levels of IL-8 and 8-isoprostane were detected in small numbers of EBC samples, but were detected in all sputum samples and most saliva samples.

Levels of protein in EBC samples have not been reported for subjects with asthma; however in healthy subjects levels of protein in EBC are similar around 5.7 µg/mL [23]. It is not clear why protein was undetected using the Coomassie assay. This may have been because of interfering substances in the exhaled breath samples, which did not affect the BCA assay. One of the common substances known to cause interference in the Coomassie assay is detergent, and the kit protocol recommends using the BCA assay if the samples show lower values than expected (Pierce Coomassie product insert). The other key difference between the two protein assays is the molecular weight cut-off. The Coomassie protein assay can only detect proteins of molecular weight > 3000 Da, while the BCA assay can measure peptides as small as 2000 Da. This indicates that the proteins collected by the two techniques are not similar.

This is the first report of SPA assessment in EBC. SPA is a lung-specific collectin that is produced and stored by alveolar type II cells and non-ciliated epithelial cells and secreted into the airways [24]. SPA levels were higher in samples of induced sputum than saliva and EBC samples, which contained similar concentrations (Fig. 2). The difference in concentration observed between sputum and EBC may represent the dilution of the epithelial lining fluid (ELF) in EBC and would indicate a dilution factor of approximately 100 times.

Mediator detection may be limited in EBC samples by the dilution of the sample and the detection limit of conventional assays. The non-volatile components of EBC are thought to be transported as respiratory droplets of ELF which are diluted by water vapour [25]. The degree of dilution may be variable making it difficult to determine whether increases in mediator concentrations are entirely because of the disease process or are simply increased because of increased droplet formation. Attempts to calculate the dilution of the ELF by water vapour in EBC suggest that there is <0.1 µL of ELF in every millilitre of EBC or a dilution factor of up to 20 000 times [26].

IL-8 was detected in 25% of EBC samples from subjects with asthma. When IL-8 levels are expected to be high, such as in acute lung injury, higher proportions of EBC samples have been shown to have detectable IL-8. However, in subjects without lung injury, most samples had no IL-8 detected [27, 28]. IL-8 levels are correlated with pH in EBC samples from patients with lung injury [27], which supports the idea that in diseases where EBC pH has shown to be reduced such as acute asthma and cystic fibrosis (CF), higher EBC IL-8 levels might be expected. Increased IL-8 in acute asthma [3, 6] and CF [29] are features well established in induced sputum. If it were assumed the EBC samples are 100-fold more dilute than induced sputum, then concentrations of 3 pg/mL would need to be readily detectable. This unfortu-

nately is below the limit of detection of many commercial assays. For the IL-8 assay used in this study the minimum detectable concentration was 5.3 pg/mL.

Several studies have examined airway oxidative stress using EBC and reported increased 8-iso-PGF2α concentrations in chronic obstructive pulmonary disease [30], CF [31], acute lung injury/acute respiratory distress syndrome [32], obstructive sleep apnoea [33] and asthma [21, 34]. The concentrations of 8-iso-PGF2α in EBC have also been shown to increase with asthma severity [21] and to fall following resolution of acute asthma exacerbations [35]. Other groups, however, have been unable to reliably measure 8-iso-PGF2α concentrations in EBC samples and some concerns have been raised over reproducibility [36]. Our data show that 8-iso-PGF2α was detected in less than 10% of EBC samples, with the remaining samples having concentrations below the limit of detection of the EIA. Other researchers have also shown that 8-iso-PGF2α concentrations in EBC were below the limit of detection in the majority of samples [37, 38]. The study by Rosias et al. indicated that the coating on the condenser system was critical. Silicon coating led to the detection of 8-iso-PGF2α in over 80% of samples. Teflon coating, as used in our study, resulted in detectable 8-iso-PGF2α concentrations in only 17% of samples [37]. This highlights that there are several technical issues regarding EBC collection that need to be resolved before this method can be reliably used to monitor airway oxidative stress. The recently developed radioimmunoassay for 8-iso-PGF2α measurement has the potential to expand research in this area, because of its improved sensitivity and specificity [39]. Our data also show that 8-iso-PGF2α concentrations in induced sputum are significantly higher than EBC levels, and 8-iso-PGF2α is detected in 100% of samples. This concurs with another recent report showing that other markers of oxidative stress, including malondialdehyde, are found in higher concentrations in induced sputum than EBC [40]. We have carefully evaluated the measurement issues [20] associated with induced sputum 8-iso-PGF2α and found the measurement to be valid and reproducible providing a useful tool to monitor airway oxidative stress.

Saliva contamination does not appear to be an issue in EBC samples in this study. If the EBC samples were contaminated with even a small amount of saliva (10% for example), then the Coomassie Protein Assay would have been able to detect these low levels using the microadaptation of the method, which can determine protein concentrations as low as 1 µg/mL. No protein was detectable in EBC samples using the micro-Coomassie assay. Similarly, this level of contamination would have resulted in higher SPA levels than those reported here. The low levels of SPA reported are likely to be the result of the dilution of the ELF by water rather than its contamination with saliva. A number of studies have measured salivary amylase in EBC samples in order to evaluate salivary contamination and have found no evidence of salivary amylase [31, 39, 41–43]. It is interesting to note that in their study of nasal EBC, oral EBC and saliva, Griese et al. [43] found no evidence of salivary amylase or phosphorus in the EBC samples, but on two-dimensional electrophoresis found evidence of similar proteins in both EBC and saliva; but compared with saliva the range and amount of protein was small.

In summary, our investigation shows that when detected, mediator levels in EBC are lower than in induced sputum samples. Saliva contains similar levels of total protein and 8-isoprostane but lower levels of IL-8 and SPA compared with induced sputum. Thus, we conclude that while induced sputum can be used to detect inflammatory mediators, the measurement mediators in EBC are variable and require further investigation.

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