ERJ Express. Published on July 26, 2006 as doi: 10.1183/09031936.06.00110305

BREATH CONDENSER COATINGS AFFECT MEASUREMENT OF BIOMARKERS IN EXHALED BREATH CONDENSATE

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Short title: Rosias et al. Breath Condenser Coatings

Word count for body of manuscript: 2896/3000 words

ABSTRACT (word count abstract: 200/200 words)

Exhaled breath condensate collection is not yet standardized. Biomarker measurements are often close to lower detection-limits. We hypothesized that adhesive properties of different condenser coatings interfere with measurements of eicosanoids and proteins in condensate.

In vitro, condensate was derived from a collection-model using two test solutions (8-isoprostane,albumin) and five condenser coatings respectively (silicone,glass,aluminum, polypropylene,teflon). *In vivo*, condensate was collected using these five coatings and the EcoScreen®-condenser to measure 8-isoprostane, and three coatings (silicone, glass, EcoScreen®) to measure albumin.

In vitro, silicone and glass coatings had significantly higher albumin recovery compared with the other coatings (p=0.03). A similar trend was observed for 8-isoprostane recovery (p=0.09). *In vivo,* median (interquartile range) 8-isoprostane concentrations were significantly higher using silicone (9.2 pg/ml(IQR:18.8), p<0.001) or glass (3.0 pg/ml(IQR:4.5), p<0.02) coating compared with aluminum (0.5 pg/ml, IQR:2.4), polypropylene (0.5 pg/ml, IQR:0.5), teflon (0.5 pg/ml, IQR:0.0), and EcoScreen® (0.5 pg/ml, IQR:2.0). Albumin *in vivo* was mainly detectable using glass coating (p<0.008). In conclusion, a condenser with silicone or glass coating is more efficient for measurement of 8-isoprostane or albumin in exhaled breath condensate, than EcoScreen®, aluminum, polypropylene or teflon. Guidelines for exhaled breath condensate standardization should include the most valid condenser coating to measure a specific biomarker.

Key Words: coating, cytokine, exhaled breath condensate, exhaled markers, isoprostanes, methodology

INTRODUCTION

The collection of exhaled breath condensate (EBC) has been rediscovered as a simple and non-invasive technique to measure mediators of airway inflammation. EBC consists not only of water vapor, but also contains aerosolized respiratory fluid droplets released from the respiratory epithelial lining fluid (ELF). These fluid droplets contain traces of non-volatile solutes, which can be recovered in EBC samples (1). EBC is collected by guiding and cooling exhaled air of a tidally breathing subject in a condenser system. EBC does not affect the airways, in contrast to bronchial biopsy, bronchoalveolar lavage and induced sputum. It can be obtained with minimal risk and minimal inconvenience for both adults and children (2). Although the ATS/ERS Task Force on EBC recently published general methodological recommendations on the collection of EBC, there are still some methodological pitfalls and unresolved questions (3-4). Most commonly used condensers are the Ecoscreen®, RTube® and home-made glass or teflon devices.

Two important groups of inflammatory biomarkers in EBC of adults and children are eicosanoids and cytokines (3-10). Eicosanoids, such as 8-isoprostane, are formed by lipid peroxidation of arachidonic acid during oxidative stress (11). 8-Isoprostane (molecular weight 354 Dalton) can be measured in EBC of adults and children, although reproducibility remains controversial (12-13). Cytokines are low molecular weight (<80.000 Dalton) proteins involved in mediating inflammation and tissue repair. Reports on the detection of cytokines in EBC are incidental, with exception of several reports on interleukin (IL)-6 by Carpagnano and co-workers (14). Albumin, a protein with a similar molecular weight (66.000 Dalton), was demonstrated in induced sputum and EBC in asthmatic subjects (15-16).

The reported mean biomarker values in EBC are usually situated in the lower range of detection (3,6). It may be that the biomarker concentration in ELF is intrinsically low, or that only minor amounts are released from ELF into exhaled air. An alternative explanation may

be that adhesive properties of different inner condenser coatings interfere with the detection of various inflammatory markers in EBC (13,17).

The aim of this study was to investigate the influence of different inner condenser coating surfaces (silicone, borosilicate glass, aluminum, polypropylene, teflon, and EcoScreen® with a teflon-like coating) on the measurement of 8-isoprostane and albumin in EBC using both an *in vitro* and *in vivo* approach.

METHODS

In vitro studies

In close collaboration with the department of Instrument Development Engineering & Evaluation (IDEE) of the University of Maastricht, we developed an *in vitro* EBC collection model consisting of a rechargeable condenser system that was connected in series with an ultrasonic nebulizer (Ultra-Neb™2000, DeVilbiss) and a ventilator (Servo-900C, Siemens) (figure 1A). The rechargeable condenser system consisted of a fixed 30cm long metal outer cylinder (diameter 4cm) (Felix Philips Maatmetaal BV, Maastricht, the Netherlands) connected to a counter current circulating ice-water pump at 0°C, and an exchangeable inner cylinder (diameter 2cm), as shown in figure 1B. The exchangeable inner cylinder was available in five different coatings: silicone, borosilicate glass, aluminum, polypropylene and teflon. The borosilicate glass tubes were manufactured by Louwers Glass and Ceramic Technologies (Hapert, the Netherlands). Identical glass tubes were coated internally with silicone by Imbreglon BV (Beuningen, the Netherlands). The aluminum tubes were manufactured by Felix Philips Maatmetaal BV (Maastricht, the Netherlands). Identical aluminum tubes were coated internally with Tempcoat 1011F (teflon) by Imbreglon BV (Beuningen, the Netherlands). The polypropylene tubes were produced by Applikon BV (Schiedam, the Netherlands). The outer surface of the inner cylinder was in direct contact

with the counter current circulating ice-water, while its inner surface was in direct contact with the exhaled breath or the volatile substances derived from the nebulizer and propulsed by the ventilator. The nebulizer was filled with a biomarker test-solution (42 pg/ml 8-isoprostane or 0.1 mg/ml albumin in a 22 ml saline 0.9% solution), that was nebulized and propulsed by a ventilator through the condenser system, simulating tidal breathing during 15 minutes (ventilator settings: inspiration time 33%, respiratory frequency 20/minute, tidal volume 200 ml). The construction of the collection device only allowed contact between the propulsed air/exhaled breath and one specific type of inner coating that was chosen at that moment (figure 1B). The condensate was collected at the open end of the condenser device directly into vials. For each coating, 15 experiments were performed (see power analysis). After each experiment, the coated tubes were rinsed repeatedly with bi-distilled water and subsequently dried at room air for at least 24 hours. Multiple coated tubes of each type were available to allow a smooth continuation of the experiments, without any delay due to the drying of the coated tube that was used in the preceding test. The biomarker condensate recovery percentage was defined as the ratio of biomarker concentration in condensate, to biomarker concentration in the test-solution.

In vivo 8-isoprostane study

As 8-isoprostane has been detected in EBC of healthy subjects, the influence of condenser coatings on 8-isoprostane measurements *in vivo* was studied in 28 healthy volunteers (see power analysis). Standardized ISAAC questionnaires were completed to exclude the presence of respiratory infections, asthma or allergic disease (18). Each subject was asked to breathe tidally, while wearing a nose-clip, into a mouthpiece connected to a two-way non-rebreathing valve that was in turn connected by tubing to the condenser. The two-way valve and tubing also served as a saliva trap. In this way, each subject exhaled during 15 minutes along each of

the five coatings in random order, as well as along the EcoScreen®, a commercial condenser system with a modified teflon coating.

In vivo albumin study

In contrast to 8-isoprostane, the albumin *in vivo* study was planned in asthmatic children, as the chance on positive albumin detections is larger in a disease population (16). EBC was collected in 40 asthmatic children. Inclusion criteria were doctor-diagnosed mild or moderate persistent asthma requiring for at least 6 months daily inhaled corticosteroids (19). Exclusion criteria were clinical evidence of respiratory infection 4 weeks prior to study, and co-morbidity (mental retardation, respiratory tract anomalies, or cardiac defects). In contrast to the *in vivo* 8-isoprostane study, the asthmatic children in the albumin study were only asked to exhale tidally along only one type of coating (silicone or glass or EcoScreen®), to allow parallel group comparison. Children were randomly allocated to one out of 3 coating groups.

Sample processing

Immediately after collection, the condensate samples were snap-frozen at -78°C using dry ice and subsequently stored at -80ºC until analysis. We did not add a reaction mixture of the immunoassay to the condensate samples before storage. In order to maintain optimal condensate sample preservation, for subsequent 8-isoprostane and albumin measurement, samples were analysed within 6 to 8 weeks of storage at -80ºC. Samples were only defrosted once, at the time of analysis. All isoprostane and albumin concentrations were determined in duplo by specific enzyme-immunoassay (Cayman Chemical®), and sandwich-enzyme-linked-immunosorbent-assay respectively (16). The lower limit of detection was 2.45 and 32 pg/ml respectively.

Statistics

The condensate recovery percentage of a specific coating was the primary outcome measure of the *in vitro* experiments. Mean values and standard deviations of recovery percentages were calculated. A comparison between the five different coatings and between the two biomarkers *in vitro* was made by means of univariate analysis of variance (ANOVA). Power analysis demonstrated a required number of 15 experiments in each group in order to detect a relevant difference between coatings of 25% with a power of 90%, an alpha of 0.05 and a standard deviation of 20% (the standard deviation in the first set of 10 experiments was 20%). In the *in vivo* studies, the number of positive detections, median and mean values, standard deviations (based on the first fifteen subjects) and interquartile ranges were calculated. Power calculation showed that n=23 patients are needed to detect a 10% difference in biomarker concentration with a power of 90%, an alpha of 0.05, and a standard deviation of 14% (paired Student t-test). Because of a number of negative detections, the Wilcoxon paired signed rank test was used to compare data within subjects. A sample with negative detection is not considered as a missing value, because it actually informs us that the measurement of a marker was below the detection limit. Therefore, the negative detection is given an arbitrary value of 0.5.

Ethics

All subjects gave written informed consent. The study was approved by the Medical Ethics Committee of the University Hospital of Maastricht.

RESULTS

In vitro study of albumin and 8-isoprostane

Albumin was detected in 93% of condensate samples (figure 2). Overall, the mean albumin recovery in condensate was only 52% using this experimental model. The albumin recovery percentages varied from 22.9% using a teflon coating, to 69.4% and 94.7% using a glass and silicone coating respectively. The albumin recovery percentage was significantly higher using the silicone and glass coating compared with the other coatings (p=0.03, ANOVA). No other statistically significant differences were present.

Isoprostane was detected in 77/78 condensate samples (figure 3). The isoprostane recovery percentages varied from 93.1% using an aluminum coating to 102.7% and 105.6% using a glass and silicone coating respectively, which did not reach statistical significance (ANOVA, F-test, p=0.09). Overall, the mean 8-isoprostane recovery in condensate was close to 100%, which was significantly higher than the overall albumin recovery percentage of 52% (ANOVA, p=0.0001).

In vivo study of 8-isoprostane

Subject characteristics are shown in table 1. Isoprostane was detected in 41% of condensate samples (figure 4). For each coating, the number of positive / negative isoprostane detections in EBC was 23 / 5 (silicone), 15 / 13 (glass), 11 / 17 (aluminum), 7 / 21 (polypropylene), 5 / 23 (teflon) and 8 / 20 (EcoScreen®). The median (interquartile range) 8-isoprostane concentrations in EBC in 28 healthy subjects ranged from 0.5 pg/ml for aluminum (IQR: 0.5-2.9), polypropylene (IQR: 0.5-1.0), teflon (IQR: 0.5-0.5) and the EcoScreen®(IQR: 0.5-2.5), to 3.0 pg/ml (IQR: 0.5-5.0) and 9.2 pg/ml (IQR: 5.1-23.9) for the glass and silicone coating respectively. EBC 8-isoprostane concentrations were significantly higher using silicone coating compared with each of the other coatings (p<0.001, Wilcoxon signed rank test).

Similarly, glass coating was better than polypropylene, teflon and the EcoScreen® ($p<0.02$, Wilcoxon test). The 8-isoprostane concentrations obtained with aluminum, polypropylene, teflon and the EcoScreen® did not differ from each other ($p=0.763$, Friedman test). As median values and interquartile ranges in smokers and non-smokers were comparable, smoking was not a confounder in this study.

In vivo study of albumin

Subject characteristics are shown in table 1. Albumin was detected in 8/40 condensate samples (0.04-0.24 microgram/ml). Positive detections were almost exclusively found with a glass coating: 7/13 samples using a glass coating, 1/14 samples using the EcoScreen® and 0/13 samples using a silicone coating ($p<0.008$, Chi-square test). Disease stability, lung function and atopic status were not a confounding factor, as these characteristics were equally distributed across the 3 coating groups (data not shown).

DISCUSSION

Both *in vitro* and *in vivo*, this study demonstrates a significant influence of the different inner condenser coatings on the measurement of eicosanoid and protein biomarkers in EBC. In particular, silicone and glass coatings were superior to aluminum, polypropylene and teflon with respect to the recovery of both 8-isoprostane and albumin in EBC *in vitro* and *in vivo.*

A careful MEDLINE on-line database search for all available studies on 8-isoprostane in EBC demonstrates data in line with the results of our study. When considering an identical assay (EIA or RIA), higher 8-isoprostane concentrations were reported using a glass coating compared with the EcoScreen®. *In vivo* using the EcoScreen®, our 8-isoprostane

concentrations (median 3.5 pg/ml, range 2.5-4.8 pg/ml) were comparable to the values reported by Van Hoydonck (mean 4.6 pg/ml, range 3.9-7.7 pg/ml). Specific data on detectability of isoprostane in EBC are scarce. We report 41% of detectability in volunteers, which is in agreement with the findings (36%) of Van Hoydonck et al in smokers (20).

In vitro, the overall mean albumin condensate recovery percentage was 52%, compared to 100% isoprostane recovery. This suggests a more difficult condensate recovery of protein biomarkers, which is supported by our limited number of positive albumin detections *in vivo*. Also, the relatively few positive studies on cytokines or proteins in EBC versus several positive reports on 8-isoprostane and leukotrienes in EBC may suggest that the recovery of proteins in EBC is less easily than of eicosanoids. Recently, Tufvesson also showed that the detection of eicosanoids and cytokines in EBC in asthmatic subjects may be facilitated by coating of all collection surfaces with Tween 20 and bovine serum albumin respectively, combined with vacuum-centrifugation of condensate samples (21). Moreover, 30-fold differences in condensate levels of IL-4 in asthmatic children were found using different condenser systems and enzyme immunoassays (22-24). The latter finding indicates that variability of biomarker levels in condensate may also be attributed to intrinsic analytical problems of currently used immunoassays, as illustrated recently for thromboxane A2 metabolite measurements in EBC (24).

In vitro, the mean 8-isoprostane condensate recovery percentages did not differ significantly between the condenser coatings ($p=0.09$). An explanation may be the higher overall mean condensate recovery of isoprostane, in contrast to albumin, which may illustrate a different behavior of these biomarkers during the expiratory phase of the EBC collection. In turn, this may be a reflection of difficulties experienced by molecules with a higher molecular weight in their transition from respiratory fluid droplets and exhaled air to condensate. On the other

hand, overload in the 8-isoprostane *in vitro* tests seems unlikely when considering that the absolute amount in the test tubes must have been very close to the concentrations found *in vivo.* Hence, it may be that the nebulizer set-up is producing 8-isoprostane aerosols that are more effectively condensed as compared to the real EBC *in vivo*. Therefore, further studies on the optimal condenser coating should always include *in vivo* experiments.

The EcoScreen® is a commonly used and commercially available condenser system with distinctive physical characteristics. In contrast to our collection system and the RTube that collect EBC in the liquid phase, the EcoScreen® collects EBC mainly as ice, which may be associated with more drastic freeze-thaw cycles that can affect the molecules thus collected (23,25). The EcoScreen® is a condenser system based on a modified inner teflon coating. The 8-isoprostane EBC concentrations were similar for the EcoScreen® and the teflon coating, suggesting no significant influence from the specific physical characteristics of the EcoScreen® condenser device *in vivo*.

A different design of the *in vivo* 8-isoprostane and albumin study was chosen, although ideally both study parts should have had a similar design. However, several considerations not to do so were decisive: (1) subjects with asthma are more likely to have positive albumin detection in EBC than healthy controls (16); (2) a 90 minutes EBC collection period, like in the *in vivo* 8-isoprostane study, was not realistic nor feasible in the asthmatic children of the *in vivo* albumin study. Therefore, not an intra-subject comparison but a between-subject comparison (parallel groups) of condenser coatings was chosen for the *in vivo* albumin study.

Standardization of the EBC collection method implicates more than applying one identical collection technique. It implicates the development of a condenser system with the highest and least varying recovery percentages. Optimalization of condensate recovery percentages may lead to an improvement of biomarker reproducibility. Therefore, we recommend the use of a condenser system with an inner glass or silicone coating for the measurement of 8-

isoprostane and albumin in EBC. The reproducibility of measurements by using different condenser coatings should be evaluated *in vivo*.

An explanation for this superiority of glass and silicone coatings may be that water acts as a bipolar vehicle. The pH of exhaled breath ranges from 6.50 to 7.80, whereas the isoelectrical point (pI) of human albumin is situated at pH 4.80 (3,26). In other words, at higher pH values (such as reported in EBC) albumin acts as a negatively charged molecule. Therefore, the negatively charged albumin in exhaled breath may be repulsed by glass that is negatively charged as well. This may promote the recovery of albumin in EBC. In contrast, the same albumin molecule may be attracted by aluminum metal ions that are positively charged. This may hamper the recovery of albumin in EBC in this case. Furthermore, 8-isoprostane acts extremely hydrophobic. It will stick to neutral surfaces (such as teflon) in the presence of aqueous solutions, to lipids and to detergents.

Not only adhesive and electrical properties of the condensation equipment may affect whether a compound is collected in EBC, but also issues such as the length of the condensation tube, thermal conduction of the coating, and solute solubility, volatility, and its dissociation constant ($pK_a = -\log K_a$).

Finally, these findings in 8-isoprostane and albumin do not necessarily hold for all other biomarkers. Every biomarker has its own chemical and physical characteristics. Currently, one may speculate that most eicosanoids act in a similar way. Likewise, small proteins such as cytokines may act differently from eicosanoids, but similarly when compared one cytokine to another.

We conclude that a condenser system with a silicone or glass coating is more efficient for the measurement of 8-isoprostane or albumin in EBC, compared with the EcoScreen®, or condensers with aluminum, polypropylene or teflon coating. The increased efficiency of the borosilicate glass and silicone coatings may be due to less adhesive properties resulting in

more biomarker recovery in the condensate. To establish methodological standardization of the collection of EBC, the most valid and inert condenser coating has to be applied for the measurement of a specific inflammatory biomarker.

ACKNOWLEDGEMENTS

The authors wish to thank Mieke Dentener and Roy Cloots of the department of Respiratory Medicine, University Hospital of Maastricht, for their technical support in the analysis of the albumin condensate samples; M'hamed Hadfoune of the General Surgery, Nutrition and Toxicology Research Institute Maastricht, Maastricht University, for his technical support in the analysis of the 8-isoprostane condensate samples; Paul Laeven of the department of Instrument Development Engineering & Evaluation, University of Maastricht, **paul.laeven@id.unimaas.nl**, for his technical support in the development of the rechargeable condenser system; Jildou Sijbrandij of the department of Epidemiology, University of Maastricht, for her statistical support; and last but not least the volunteers for their participation.

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List of figures

Figure 1A.

In vitro EBC collection model consisting of a rechargeable condenser system connected in series with an ultrasonic nebulizer and a ventilator. The nebulizer could be filled with a test solution of a known concentration of 8-isoprostane or human albumin. The nebulized test solution was propulsed by the ventilator through the condenser system.

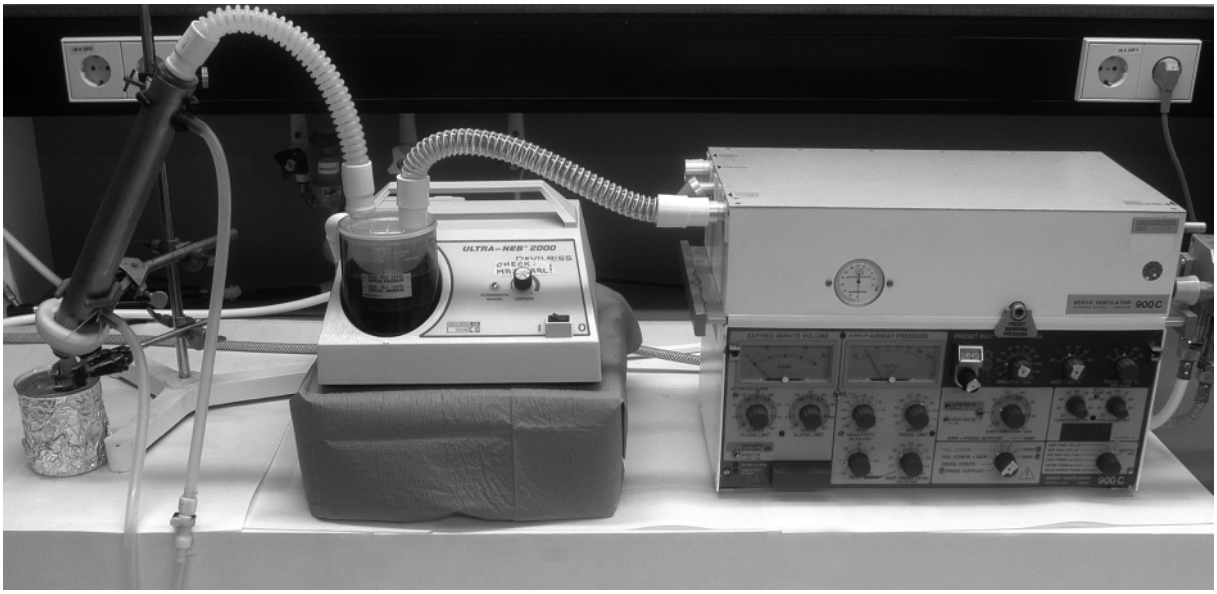

Figure 1B.

Schematic representation of the rechargeable condenser system, that consisted of a fixed 30cm long metal outer cylinder (diameter 4cm) connected to a counter current circulating ice-water pump at 0°C, and an exchangeable inner cylinder (diameter 2cm). The exchangeable inner cylinder was available in five different coatings: silicone, borosilicate glass, aluminum, polypropylene and teflon. The outer surface of this inner cylinder was in direct contact with

the counter current circulating ice-water, while its inner surface was in direct contact with the exhaled breath or the volatile substances derived from the nebulizer and propulsed by the ventilator.

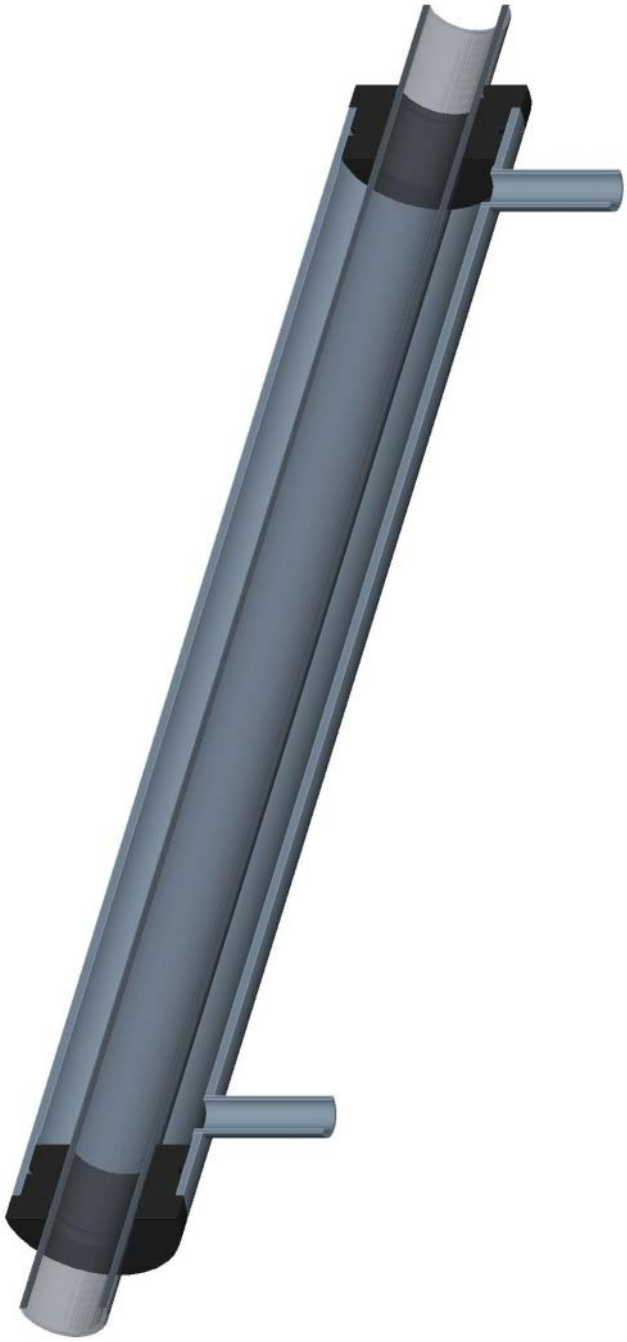

Figure 2.

In vitro albumin condensate recovery percentages (%) (mean ± SEM) using five different condenser coatings. Albumin was detected in 65 out of 70 condensate samples (one negative sample using glass, four negative samples using teflon). Silicone and glass coatings had significantly higher albumin condensate recovery percentages compared with the other coatings (p=0.03).

Legend to figure 2:

coatings: silicone (S), borosilicate glass (G), aluminum (A), polypropylene (P) and teflon (T)

SEM: standard error of the mean

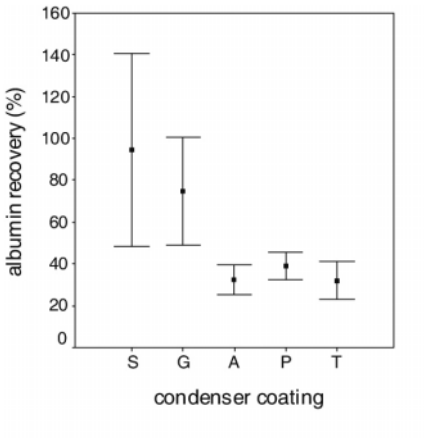

Figure 3.

In vitro 8-isoprostane condensate recovery percentages (%) (mean ± SEM) using five different condenser coatings. Isoprostane was detected in all (n=78) but one condensate sample (one negative sample using polypropylene). Silicone and glass coatings had higher 8-isoprostane condensate recovery percentages, although statistically not different compared with the other coatings (p=0.09).

Legend to figure 3:

coatings: silicone (S), borosilicate glass (G), aluminum (A), polypropylene (P) and teflon (T)

SEM: standard error of the mean

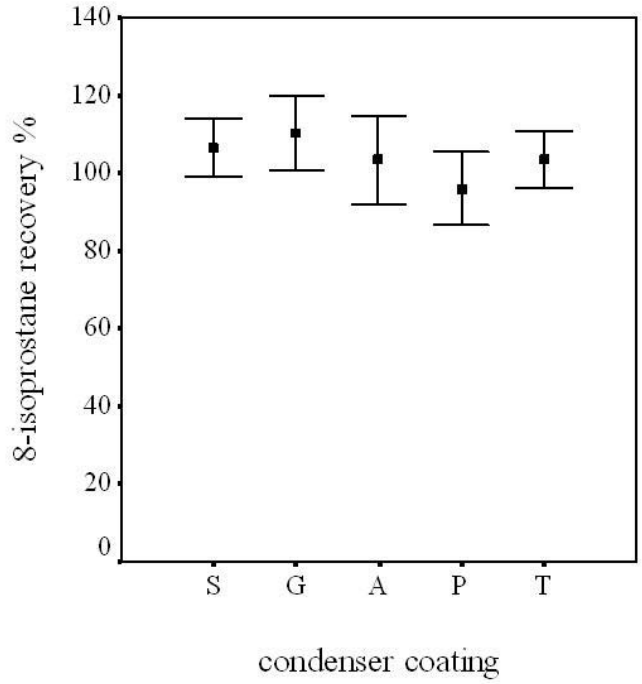

Figure 4.

In vivo 8-isoprostane condensate concentrations (pg/ml) (median and interquartile range) in 28 healthy volunteers using six different condenser coatings. Isoprostane was detected in 69 out of 168 condensate samples. Isoprostane condensate concentrations were significantly

higher using a silicone coating ($p<0.001$) or a glass coating ($p<0.02$) compared with the other coatings.

Legend to figure 4:

coatings: silicone (S), borosilicate glass (G), aluminum (A), polypropylene (P), teflon (T), and the EcoScreen® condenser (E)

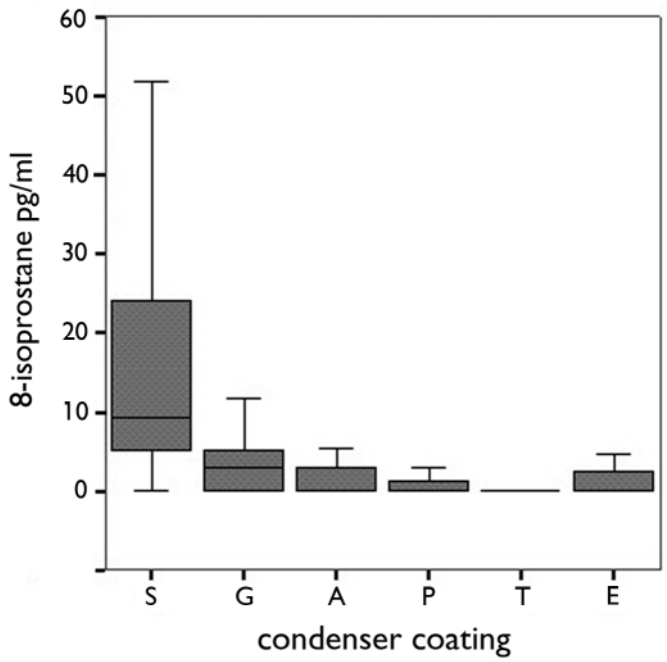

Table 1. Subject characteristics.

	Healthy subjects 8-isoprostane study	Asthmatic subjects albumin study
number	28	40
male (n)	10	27
smoking (n)	5	0
atopic (n)	0	36
median age (yrs)	26	11
range of age (yrs)	20-57	5-17

Legend to table 1:

n number

yrs years