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BIOMARKER REPRODUCIBILITY IN EXHALED BREATH CONDENSATE COLLECTED WITH DIFFERENT CONDENSERS

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ABSTRACT (word count abstract: 200 / 200 words)

Optimal collection and analysis of exhaled breath condensate (EBC) are prerequisites for standardisation and reproducibility of assessments. We aimed to assess reproducibility of EBC volume, hydrogen peroxide, 8-isoprostane, and cytokine measurements using different condensers, including a newly developed glass condenser.

At four points in time, 30 healthy subjects performed sequential EBC collections randomly using four condensers: glass, silicone, EcoScreen®, and an optimised glass condenser. In small EBC samples, hydrogen peroxide was measured by spectrophotometer, 8-isoprostane by enzyme immunoassay, and cytokines by multiplexed xMAP® technology.

The optimised glass condenser yielded significantly more EBC volume (median 2025µL, IQR 1056), reproducibility of EBC volume was comparable with EcoScreen® (19-20 CV%), but was significantly better compared to silicone and glass (29-37 CV%). The new condenser was associated with significantly more detections of hydrogen peroxide, 8-isoprostane, IL-2, IL-4, IL-5, IL-13, and TNF-alpha. Isoprostane concentrations were significantly higher using the new condenser, whereas hydrogen peroxide and cytokine concentrations were not. Reproducibility of biomarkers was equally variable for all condenser types.

In conclusion, significantly more EBC volume and biomarker detections were found using the optimised glass condenser, including higher 8-isoprostane levels. However, biomarker reproducibility in EBC in healthy adults was not influenced by the type of condenser.

Key Words: condenser / cytokines and chemokines / exhaled breath condensates / hydrogen peroxide / 8-isoprostane / multiplex array

INTRODUCTION

The collection of exhaled breath condensate (EBC) is a non-invasive, safe technique to obtain direct samples from the lower respiratory tract, without disturbing an ongoing inflammation (1-3). Analysis of EBC reveals the presence of inflammatory markers such as eicosanoids, hydrogen peroxide, and cytokines (1-3). Although the American Thoracic Society and European Respiratory Society Task Force on EBC published general methodological recommendations on the collection and analysis of EBC, there are still some unresolved methodological pitfalls, as illustrated by the use of various nonstandardised collection systems (1-3).

Optimal condensate collection, and optimal biomarker detection and measurement in EBC are reciprocal prerequisites for any standardisation. However, current condensation systems are suboptimal, with relatively short-measured, open ended designs and loss of noncondensed exhaled breath, as reflected by variable EBC volumes and biomarker reproducibility (1). Logically, modification by using guided breath flows, enlarged condensation surface, and optimised condensate recovery may improve condensation. Moreover, current designs have different inner coatings, featuring different adhesive interactions with exhaled markers (4). Recently, loss of biomarker within the sampling system was demonstrated, *in vitro* and *in vivo*, for 8-isoprostane and albumin, at the expense of nonglass condenser systems, and in favour of glass and silicone condensers (4). To assess clinical relevance, a study on the reproducibility of these biomarker measurements *in vivo,* using different condenser coatings including glass, is needed (4). Furthermore, conventional biomarker assays are not always suitable for use in even large sample volumes of condensate, a bio fluid highly ‘diluted’ by water vapour (5), whereas new analytical techniques are rapidly emerging and may offer new perspectives (6-7). Recently, multiplexed cytometric bead array was used in children to simultaneously measure different cytokines in only 50µL condensate samples, however, the

detection level did not reach 50% (6). Liquid bead-based multiplexing xMAP® technology (Luminex Corporation, Austin, USA), based on flow cytometry and (faster) liquid microspheres reaction kinetics, is less laborious, highly sensitive and specific, allows simultaneous measurements in small sample sizes, and improves interarray reproducibility (7). Therefore, we hypothesised that optimised condensate collection with minimal adhesive properties, improves the reproducibility of different measurements in EBC.

The aim of the present study is to assess the effect of four different condensers (glass, silicone, EcoScreen®, and a new, optimised glass condenser) on the reproducibility of breath condensate volume, and the detection, concentration and reproducibility of inflammatory biomarkers, including hydrogen peroxide, 8-isoprostane, interleukin (IL)-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and tumor necrosis factor (TNF)-alpha, in EBC in healthy nonasthmatic adults.

METHODS

Study subjects

We recruited 30 eligible healthy nonasthmatic adult volunteers (table 1) among medical students and staff at the University Hospital Maastricht, based on the following criteria: each volunteer was able to breath tidally into a mouthpiece for at least 15 minutes; exclusion criteria were: history of asthma, upper or lower airway infection, and use of antibiotics, corticosteroids, cromoglycate, nedocromil, theophylline or leukotrien-antagonist. Nonasthmatic healthy adults were chosen to eliminate possible confounding factors attributable to heterogeneous disease expression and/or variability of disease control.

Study design

To assess within-day, between-day and between-week reproducibility, each volunteer was asked to perform four tests: on the first day in the morning (test 1) and afternoon (test 2), the day after at the same time as test 1 (test 3), and one week later at the same time (test 4). In turn, each test consisted of four sequential EBC collections, using ad random different types of condenser.

Exhaled breath condensate collection

EBC was collected using one of the following condensers: the commercial Teflon-like EcoScreen®, a condenser with exchangeable inner cylinder of silicone or glass, as described previously (4), and a new, optimised glass condenser that was developed in close collaboration with the Department of Instrument Development Engineering & Evaluation (IDEE) of the Maastricht University (patent number EP 07102586), as extensively described in figures 1A-1B-1C. Briefly, the inclined condensation surface is enlarged (using a length of 90cm), condensate recovery is optimised (using a downwards moveable plunger), and breath flows are turbinately directed towards the condenser wall (by the plunger's multiple breath channels).

To perform one EBC collection, each subject was asked to exhale tidally, while using a nose clip, through a mouthpiece and two-way nonrebreathing valve connected with the condenser, during a fixed period of 15 minutes.

Condensate sample processing

Immediately after collection, condensate samples were snap-frozen at -78˚C using dry ice and stored at -80˚C. Analysis was performed within three months from sampling time.

Hydrogen peroxide was measured in 50µL EBC, in duplicate by spectrophotometer (Perkin Elmer® UV-VIS Lambda 10 Spectrometer, Shelton/Norwalk, Connecticut, USA) with a lower detection limit of 0.05µM, as described previously (8).

Isoprostane was measured in 100µL EBC, by specific enzyme immunoassay (Cayman Chemical®, Ann Arbor, Michigan, USA), that was modified to reach a lower limit of detection of 1.0pg/mL. The standard curve of this assay ranged from 250pg/mL to 1.95pg/mL. We were able to report 8-isoprostane values as low as 1.0pg/mL, as a logit/log transformation was used. Isoprostane recovery experiments were performed, and coefficients of variation (CV) of the absorption signals were assessed. When spiking for the lower 8-isoprostane values of 3.9pg/mL and 7.8pg/mL, an isoprostane recovery of 92% (CV of concentration 16%), and 95% (CV of concentration 15%) were found, respectively. A CV (of absorption signals) below 15% was considered highly acceptable. Hence, the corresponding CV of concentrations may be higher. Therefore, the CV's found in the 8-isoprostane recovery experiments using these low values, were considered good. Standard curves, patient samples and quality control samples of 2.5pg/mL and 10pg/mL 8-isoprostane were assayed in triplicate. Intra-assay variation of standard curves, patient samples and quality control samples should be less than 15%, otherwise all samples measured in that assay were excluded and reanalysed. Finally, in all accepted samples, 8-isoprostane concentrations were determined from mean absorption signal intensities.

Cytokines were measured in 50µL EBC, using the liquid bead-based multiplexing xMAP® technology (Luminex Corporation, Austin, USA). Multiplex immunoassay was performed as described previously (7,9-10). The corresponding lower limits of cytokine and chemokine detection were: IL-2 (1.0pg/mL), IL-4 (1.2pg/mL), IL-5 (1.2pg/mL), IL-6 (0.4pg/mL), IL-8 (1.1pg/mL), IL-10 (1.2pg/mL), IL-13 (1.0pg/mL), and TNF-alpha (1.3pg/mL). Measurement and data analysis were performed using the Bioplex 100 system and Bioplex Manager

software version 3.0 (Bio-Rad Laboratories, Hercules, CA). All multiplex immunoassays were performed in 96 well format 1.2μm filter bottom plates (Millipore, Amsterdam, The Netherlands) and a 12 point standard curve in duplicate was included on every plate. In order to minimise interassay variation, positive and negative control samples were included. As far as possible, EBC samples from one donor series of experiments were run on one plate. Three EBC samples were spiked with 100 or 10pg/mL cytokines (IL-2, Il-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF-alpha), and measured them in quadruplicate. The mean CV of these cytokine measurements was 12.2%. The recovery of 100pg/mL spiked cytokine was 103% (range from 71 to 129%). At 10pg/mL, the mean recovery was somewhat lower at 89% (range from 64% in IL-13, to 111% in IL-4). It was concluded that there was a slight matrix effect of EBC, but this did not result in an overestimation of cytokine measurements.

Statistics

Statistical calculations were performed using SPSS 11.5 (SPSS Inc, Chicago, USA).
Not normally distributed data were expressed as median and interquartile range (IQR). Normally distributed data were expressed as mean and standard error (SE). To estimate variance within a single method of measurement (coating), coefficients of variation (CV = (standard deviation/mean) x100%) were used. The CV was calculated as the mean of individual CV's, calculated over the two or four relevant measurements. Within-subjects, CV's were used as data distribution was normal. When the distribution of individual CV's was not normal, the nonparametric Friedman test was used to see if these CV's were different between coatings. To test for differences among normally distributed repeated measures, analysis of variance (ANOVA) was used. The Chi-square test was used to evaluate statistically the differences between proportions for four groups in a data set. Condensate samples with biomarker concentration below the lower detection limit, *in strictu sensu*

negative detections, were not considered as missing values, because they actually informed us that marker concentrations were below the lower detection limit. Therefore, these negative detections were given an arbitrary value between zero and the lower detection limit: 0.025µM for hydrogen peroxide, 0.1pg/mL for 8-isoprostane, and 0.1pg/mL for cytokines. Samples were defined missing if the EBC volume, yielded after 15 minutes collection time, was zero, or insufficient to analyse.

Power calculation

Ideally, power calculations should be based on the expected changes in biomarker concentrations and on their variability. However, these changes in biomarker concentrations and their variability were unknown. In fact, the objective of this study was to answer this question. Therefore, we took the uncertainty of the estimated variability as a basis for power calculations. For each of the estimated variances (within-day, between-day and between-week) we have data from 30 volunteers. Using standard results for the variance of a chi-square random variable, we infer that, with 30 subjects, the relative confidence limits (relative to the observed value of the variance) will be 0.634 and 1.807. For the standard deviation this implies relative limits of 0.80 and 1.34 (11).

Ethics

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

RESULTS

EBC volume

In 5 out of 480 manoeuvres, we were not able to collect condensate due to erroneously connected tubing. EBC collection by the optimised glass condenser yielded significantly more median condensate volume, compared to the other condensers ($p=0.001$, Friedman) (table 2). Within-day, between-day, between-week and overall reproducibility of EBC volume (expressed as CV%) were comparable in the new condenser and EcoScreen® ($p=0.715$, Chi-square), but was significantly better compared to silicone and glass ($p<0.028$, Chi-square).

Hydrogen peroxide measurements in EBC

Overall, 29% of hydrogen peroxide measurements were missing (table 3). Significantly more positive hydrogen peroxide detections were found using the optimised glass condenser, compared to silicone and glass ($p<0.050$, Chi-square). Median hydrogen peroxide concentrations and reproducibility (expressed as CV%) did not significantly differ between the four condensers ($p=0.286$ and $p>0.080$, respectively, Friedman).

8-Isoprostane measurements in EBC

Overall, 13% of 8-isoprostane measurements were missing (table 4). Significantly more positive 8-isoprostane detections were found using the optimised glass condenser, compared to silicone and glass ($p<0.023$, Chi-square). The median concentration of 8-isoprostane was significantly higher using the new condenser compared with the other three condensers ($p=0.001$, Friedman). Statistically, 8-isoprostane reproducibility (expressed as CV%) did not significantly differ between the four condensers ($p>0.151$, Friedman).

Cytokine measurements in EBC

Overall, 20% of cytokine measurements were missing (table 5.a). Overall, the levels of cytokine detection using xMAP's technology were: IL-2 (68%), IL-4 (97%), IL-5 (73%), IL-6

(46%), IL-8 (46%), IL-10 (61%), IL-13 (70%) and TNF-alpha (64%). The optimised glass condenser had significantly more positive detections of IL-2, IL-4, IL-5, IL-13 and TNF-alpha, compared to silicone and glass ($p<0.050$, Chi-square), and more IL-5 and IL-13 detections, compared to EcoScreen® ($p<0.021$, Chi-square). Cytokine concentrations were not significantly different between the four condenser types ($p>0.113$, Friedman or ANOVA, respectively) (table 5.b). Reproducibility (expressed as CV%) of cytokine measurements did not significantly differ, neither overall (table 5.c), nor within-day, between-day and between-week (data not shown). Best range of overall CV% was found for the measurement of IL-6 (11-14%), IL-8 (2-15%), IL-10 (11-30%) and TNF-alpha (8-22%). When CV's were evaluated using the positive values only (without negative and/or arbitrary values), also no differences between condensers were found (data not shown).

DISCUSSION

We demonstrated that EBC volume, and the detection of biomarkers were significantly influenced by the condenser system, in favour of the new glass condenser, whereas biomarker reproducibility was not influenced by the type of condenser. The proposed optimised glass condenser yielded significantly more condensate volume compared to the silicone, glass and EcoScreen® condensers. Reproducibility of EBC volume was comparable for the new condenser and EcoScreen®, and was significantly better compared to the other two condenser types.

In EBC collected with this new condenser, significantly more positive hydrogen peroxide, 8-isoprostane, IL-2, IL-4, IL-5, IL-13 and TNF-alpha detections were found, supporting improved sampling of EBC. Moreover, 8-isoprostane concentrations were significantly increased in EBC yielded by the new condenser, compared to the other three condensers,

which is in accordance with former findings (4). On the other hand, reproducibility of hydrogen peroxide, 8-isoprostane, and cytokine measurements in EBC did not significantly differ between the four condensers, suggesting no significant influence of the type of condenser coating on reproducibility. In literature and to date, no formal study addressing this issue has been published, although reproducibility using other analytical techniques has been reported (12-14), and/or could not be calculated due to small number of subjects (15-17).

Variations in biomarker measurements in EBC may be attributed to variations in the dilution and/or quality of condensate that is influenced by different collection techniques and procedures, sample processing and storage conditions, and/or sensitivity of the analytical techniques used (17). The levels of the highly volatile hydrogen peroxide in EBC may be susceptible to different cooling temperatures during collection (18), circadian rhythm (19), flow dependency (20), different methods of measurement with widely varying values (even in healthy subjects) close to lower detection limits (3,17,21-22), and high chemical reactivity with salivary and exhaled compounds by which (some) hydrogen peroxide is consumed over time during collection and storage (3,17,22). Isoprostanes are relatively stable end-products of *in vivo* arachidonate peroxidation, and are measured in EBC by immunoassay, that may be influenced by cross reactivity with closely related substances (17). Other confounding factors may be age, diets rich in antioxidants, and smoking habits (17).

The use of CV is not always the ideal way of expressing variability: when mean values are low, CV's can be abnormally high. Therefore, we also expressed our results as standard deviations and intraclass correlation coefficients. When using both of these alternative expression methods, results were comparable: no difference in biomarker variability between condensers was found (data not shown). Furthermore, biomarker levels were assessed in

condensate originating from healthy adults, and thus, may have been more pronounced in a steroid naïve population with documented chronic respiratory inflammation, and comparable levels of disease control. Hence, one may expect increased mean concentrations, and lowered CV's in diseased subjects. On the other hand, with these very low concentrations of cytokines and 8-isoprostane, we cannot rule out an influence of analytical variability.

Cytokines were simultaneously measured by xMAP's technology in small 50µL EBC samples. The overall level of detection was 46-97%, that was much better compared to cytometric bead array in small samples in children (<50%), and in large 1000-2000µL lyophilisated samples in adults (3-100%) (6,23-26). When compared with conventional (solid-phase) immunoassays, multiplexed immunoassays detect bioactive and inactive molecules, have a growing analytical range, are rapid (hours instead of days), have good precision (CV 10-15%), are not interfered by drugs, and have simple protocols (7).

Currently, different nonstandardised techniques to collect EBC are in use (1-3). All systems are based on the cooling of exhaled breath, whereas their design may vary from immersed plastic tubing, over glass distilling columns, to commercial systems, such as the lamellar Teflon-like EcoScreen® condenser (Erich Jaeger GmbH, Hoechberg, Germany), the hand-held disposable polypropylene RTube™ (Respiratory Inc., Charlottesville, Virginia, USA), and the thermostatically-controlled polyethylene Turbo-Deccs (ItalChill, Parma, Italy) (1-3,27). These designs implicate relatively short-measured and open-ended systems that tolerate the needless loss of noncondensed exhaled breath, whether initially, or after a prolonged collection time. Moreover, also biomarkers may be lost within these collection systems, as recently demonstrated for 8-isoprostane and albumin measurements, *in vitro* and *in vivo*, in nonglass condensers (including EcoScreen®) (4). This superiority of glass coatings may be

mainly related to the behaviour of water as bipolar vehicle (4). Three other studies report the influence of sampling systems on biomarkers in EBC (28-30). Tufvesson *et al* proposed to coat EcoScreen® collection surfaces with bovine serum albumin and Tween-20, to measure cytokines and eicosanoids, respectively (28). However, they also reported possible false-positive (eicosanoid) results, and the need of sample concentration, by vacuum-centrifugation, prior to analysis (28). Soyer *et al* found significantly higher cysteinyl leukotrienes and eotaxin using EcoScreen®, compared to RTube™, due to susceptibility of precooled RTube™ sleeves to (increased) ambient temperatures during collection, and due to different materials that could affect sample recovery (29). Prieto *et al* compared RTube™ and EcoScreen®, and reported that EBC pH values are dependent on the collection device used (30). Furthermore, the EcoScreen® has been associated with deposition of frozen condensate on its lamellar walls. For all the above mentioned reasons, we developed an optimised glass condenser system. The new glass condenser had an improved condensation process and condensate recovery, using an inclined and enlarged condensation surface, with a condensate sweeping plunger, having tangentially and axially guiding breath flow channels.

In the present study, these improvements resulted in significantly increased EBC volumes, and increased biomarker detections with the new glass condenser, compared to silicone, glass and EcoScreen®. This suggests both an improved condensation process, and an increased opportunity to perform a broad spectrum of analyses. Moreover, the optimised glass and EcoScreen® condenser were both significantly associated with less variation in the generated EBC volume, compared to the other condensers. Hereby, reducing a possible confounding influence of the variable quantity of EBC collected over a given time, and even within individuals.

Optimisation of EBC collection, using the modified new glass condenser with statistically equivalent CV's compared to the commercial EcoScreen®, and optimisation of EBC analysis, using rapid, multiplexed measurement of cytokines in small EBC sample sizes, may all together open a window of opportunities, even in strained collection procedures, such as in young or dyspnoeic subjects (with less sustained efforts to cope with sampling procedures), by allowing the search for, and identification of, particular profiles of different exhaled markers, involved in the regulation of chronic respiratory inflammation, for diagnostic and monitoring purposes.

In conclusion, the optimised glass condenser yielded significantly more EBC volume, with good reproducibility. Furthermore, significantly more positive detections of hydrogen peroxide, 8-isoprostane, IL-2, IL-4, IL-5, IL-13 and TNF-alpha were found in EBC collected with the new condenser, hereby offering an increased capacity to analyse for complex biomarker profiles. Moreover, concentrations of 8-isoprostane were significantly increased using the optimised glass condenser, compared to the other three condensers. However, reproducibility of biomarker measurements in EBC was not influenced by the type of condenser.

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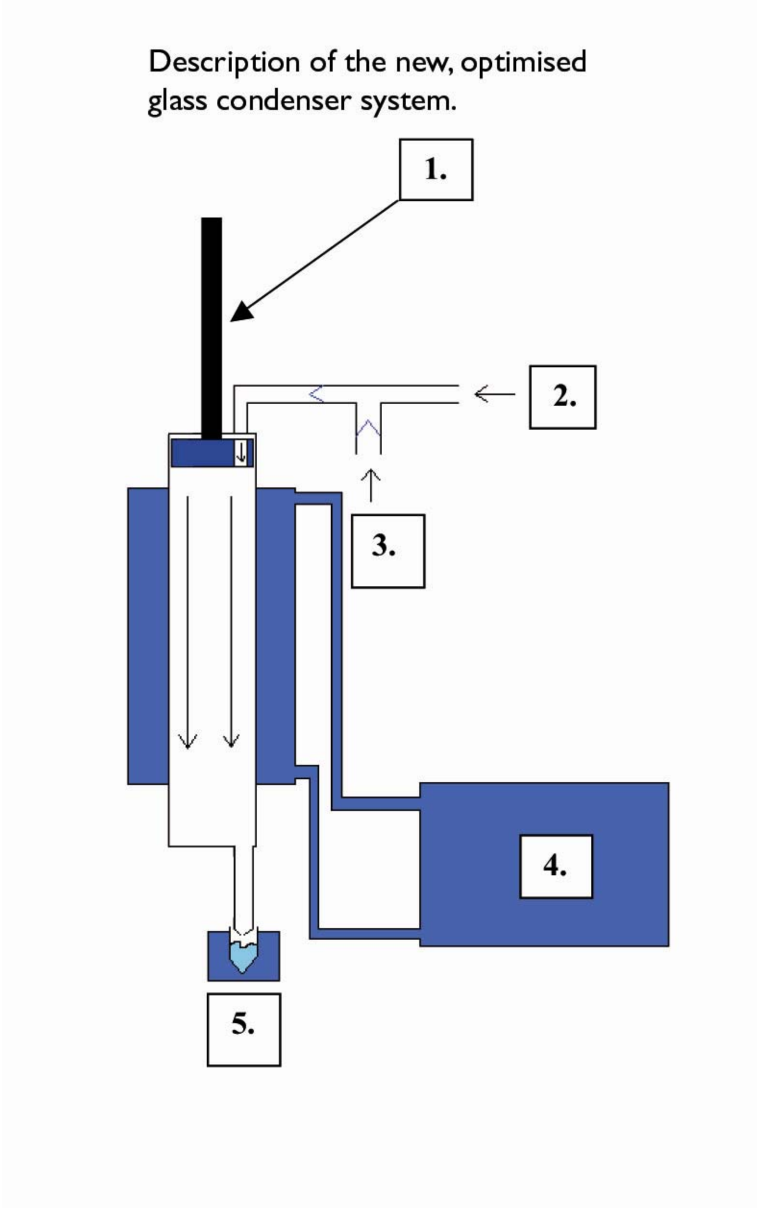

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Table 1. Subject characteristics of healthy nonasthmatic adult volunteers (n=30).

male : female ratio	19 : 11
age (in years)*	23 (14)
weight (in kg)*	75 (25)
height (in cm)*	180 (14)
non smoking	28
history of eczema and/or hay-fever	6

legend to table 1:

* median and (interquartile range)

Table 2. Volume of exhaled breath condensate, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

EBC	Silicone	Glass	New	EcoScreen	p-value
missings	4/120	0/120	1/120	0/120	-
volume *	525 (319)	712 (297)	2025 (1056)	1237 (759)	**0.001** †
CVin-day *	20 (27)	21 (19)	11 (15)	9 (21)	**0.002** †
CVbe-day *	28 (36)	30 (26)	11 (26)	13 (23)	**0.027** †
CVbe-week *	35 (36)	26 (28)	15 (22)	15 (21)	**0.003** †
CVoverall *	37 (22)	29 (16)	20 (17)	19 (27)	**0.001** †

legend to table 2:

EBC	exhaled breath condensate
New	optimised glass condenser
missings	number of failed EBC collections, after 15 minutes, to total number of tests
volume	volume of EBC, in μL
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Friedman test

Table 3. Hydrogen peroxide measurements in exhaled breath condensate: detection, concentration, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

H_2O_2	Silicone	Glass	New	EcoScreen	p-value
missings	59/120	49/120	10/120	23/120	-
detections	52/9	62/9	**77/33**	75/22	**0.003** †
conc *	2.6 (2.7)	2.1 (2.3)	1.8 (1.6)	2.2 (2.5)	0.286 ‡
CVin-day *	17 (25)	12 (28)	46 (71)	13 (21)	0.271 ‡
CVbe-day *	22 (33)	30 (42)	30 (118)	16 (52)	0.821 ‡
CVbe-week *	18 (46)	26 (42)	32 (44)	12 (32)	0.256 ‡
CVoverall *	23 (25)	29 (21)	24 (52)	22 (13)	0.080 ‡

legend to table 3:

H_2O_2	hydrogen peroxide
New	optimised glass condenser
missings	number of missing measurements to total number of tests
detections	number of positive to negative detections
conc	concentration of H_2O_2 in EBC, in µM
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Chi-square test
‡	Friedman test

Table 4. Isoprostane measurements in exhaled breath condensate: detection, concentration, and reproducibility (expressed as coefficients of variation) in four different condenser systems.

8-IP	Silicone	Glass	New	EcoScreen	p-value
missings	27/120	16/120	9/120	10/120	-
detections	89/4	98/6	**110/1**	109/1	**0.001** †
conc *	2.0 (2.2)	2.9 (2.3)	**3.6 (2.7)**	2.5 (1.8)	**0.001** ‡
CVin-day *	6 (29)	20 (55)	23 (35)	24 (29)	0.297 ‡
CVbe-day *	31 (65)	26 (57)	23 (32)	15 (32)	0.559 ‡
CVbe-week *	22 (42)	22 (57)	15 (31)	25 (28)	0.954 ‡
CVoverall *	31 (57)	48 (55)	29 (22)	26 (26)	0.151 ‡

legend to table 4:

8-IP	8-isoprostane
New	optimised glass condenser
missings	number of missing measurements to total number of tests
detections	number of positive to negative detections
conc	concentration of 8-IP in EBC, in pg/ml
CV	coefficient of variation, in percentage
in-day	within day
be-day	between day
be-week	between week
*	expressed as median (interquartile range)
†	Chi-square test
‡	Friedman test

Table 5.a. Number of positive to negative detections of cytokines in exhaled breath condensate, collected with four different condenser systems.

detections	Silicone	Glass	New	EcoScreen	p-value *
missings	49/120	37/120	1/120	10/120	-
IL-2	50/21	60/23	**82/37**	68/42	**0.001**
IL-4	69/2	81/2	**113/6**	108/2	**0.001**
IL-5	58/13	65/18	**87/32**	70/40	**0.001**
IL-6	41/30	45/38	48/71	43/67	0.811
IL-8	44/27	45/38	44/75	44/66	0.999
IL-10	54/17	57/26	63/56	61/49	0.654
IL-13	53/18	58/25	**88/31**	71/39	**0.001**
TNF-alpha	52/19	57/26	**72/47**	65/45	**0.050**

legend to table 5.a:

detections	number of positive to negative detections
New	optimised glass condenser
missings	number of missing measurements to total number of tests
IL	interleukin
TNF	tumor necrosis factor
*	Chi-square test

Table 5.b. Concentration of cytokine measurements in exhaled breath condensate, collected with four different condenser systems.

conc	Silicone	Glass	New	EcoScreen	p-value
missings	49/120	37/120	1/120	10/120	-
IL-2 *	1.9 (5.0)	1.6 (4.4)	1.2 (3.5)	1.6 (4.0)	0.494
IL-4 †	5.8 (0.3)	5.1 (0.3)	4.7 (0.3)	5.4 (0.3)	0.129
IL-5 *	4.4 (13.8)	3.0 (12.1)	3.2 (11.4)	3.1 (12.1)	0.544
IL-6 *	0.9 (4.3)	0.9 (4.5)	0.7 (3.8)	0.6 (4.4)	0.113
IL-8 *	2.0 (4.6)	2.1 (4.0)	2.7 (4.8)	2.1 (3.9)	0.341
IL-10 †	1.5 (0.3)	1.6 (0.9)	1.4 (0.8)	1.3 (0.7)	0.872
IL-13 †	6.6 (1.1)	6.6 (1.1)	6.3 (0.7)	5.9 (0.8)	0.946
TNF-alpha †	2.1 (0.4)	2.0 (0.4)	1.8 (0.3)	1.7 (0.3)	0.876

legend to table 5.b:

conc	concentration in EBC, in pg/ml
New	optimised glass condenser
missings	number of missing measurements to total number of tests
IL	interleukin
TNF	tumor necrosis factor
*	expressed as median (interquartile range), Friedman test
†	expressed as mean (standard error), analysis of variance

Table 5.c. Overall reproducibility (expressed as coefficients of variation) of cytokine measurements in exhaled breath condensate, collected with four different condenser systems.

CV overall	Silicone	Glass	New	EcoScreen	p-value
missings	49/120	37/120	1/120	10/120	-
IL-2 †	80 (12)	75 (11)	84 (10)	70 (11)	0.802
IL-4 †	33 (7)	32 (6)	36 (5)	26 (4)	0.494
IL-5 †	74 (13)	71 (12)	73 (11)	81 (13)	0.941
IL-6 *	12 (77)	12 (68)	14 (78)	11 (69)	0.690
IL-8 *	15 (28)	12 (21)	2 (18)	9 (25)	0.766
IL-10 *	30 (48)	17 (48)	16 (39)	11 (53)	0.719
IL-13 †	51 (11)	68 (12)	73 (10)	63 (10)	0.574
TNF-alpha *	17 (45)	22 (64)	19 (62)	8 (68)	0.878

legend to table 5.c:

CV	coefficient of variation, in percentage
New	optimised glass condenser
missings	number of missing measurements to total number of tests
IL	interleukin
TNF	tumor necrosis factor
*	expressed as median (interquartile range), Friedman test
†	expressed as mean (standard error), analysis of variance

REFERENCES

1. Rosias PPR, Dompeling E, Hendriks JJE, Heijnens JWCM, Donckerwolcke RAMG, Jöbsis Q. Exhaled breath condensate in children: pearls and pitfalls. *Pediatr Allergy Immunol* 2004; 15: 4-19.

2. Jöbsis Q, Rosias PPR. Analysis of exhaled breath condensate in children. In: New perspectives in monitoring lung inflammation. Montuschi P, editor. Taylor & Francis Publishers, London, 2004. (ISBN 0415324653)

3. Horváth I, Hunt J, Barnes PJ, on behalf of the ATS / ERS Task Force, Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005; 26: 523-548.

4. Rosias PP, Robroeks CM, Niemarkt HJ, Kester AD, Vernooy JH, Suykerbuyk J, Teunissen J, Heynens J, Hendriks HJ, Jöbsis Q, Dompeling E. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. *Eur Respir J* 2006; 28: 1036-1041.

5. Huszar E, Szabo Z, Jakab A, Barta I, Herjavecz I, Horvath I. Comparative measurement of thromboxane A2 metabolites in exhaled breath condensate by different immunoassays. *Inflamm Res* 2005; 54: 350-355.

6. Robroeks CMHHT, Jöbsis Q, Damoiseaux JGMC, Heijmans PHM, Rosias PPR, Hendriks HJE, Dompeling E. Cytokines in exhaled breath condensate of children with asthma and cystic fibrosis. *Ann Allergy Asthma Immunol* 2006; 96: 349-355.

7. de Jager W, Rijkers GT. Solid-phase and bead-based cytokine immunoassay: a comparison. *Methods* 2006; 38: 294-303.

8. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PN, van Herwaarden CL, Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Resp Crit Care Med* 1996; 154: 813-816.

9. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J Immunol Methods* 2005; 300: 124-135.

10. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 2003; 10: 133-139.

11. Rosner B. Fundamentals of Biostatistics. 6th Edn. Dixbury Press, Belmont, CA, 2006; p.200.

12. Brooks WM, Lash H, Kettle AJ, Epton MJ. Optimising hydrogen peroxide measurement in exhaled breath condensate. *Redox Rep* 2006; 11: 78-84.

13. Gerritsen WB, Zanen P, Bauwens AA, van den Bosch JM, Haas FJ. Validation of a new method to measure hydrogen peroxide in exhaled breath condensate. *Respir Med* 2005; 99: 1132-1137.

14. Gonzalez-Reche LM, Musiol AK, Muller-Lux A, Kraus T, Goen T. Method optimization and validation for the simultaneous determination of arachidonic acid metabolites in exhaled breath condensate by liquid chromatography-electrospray ionization tandem mass spectrometry. J Occup Med Toxicol 2006, doi:10.1186/1745-6673-1-5.

15. Van Hoydonck PG, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EH. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. *Eur Respir J* 2004; 23: 189-192.

16. Kharitonov SA. Exhaled markers of inflammatory lung diseases: ready for routine monitoring? *Swiss Med Wkly* 2004; 134: 175-192.

17. Rahman I, Biswas SK. Non-invasive biomarkers of oxidative stress: reproducibility and methodological issues. *Redox Rep* 2004; 9: 125-143.

18. Goldoni M, Caglieri A, Andreoli R, Poli D, Manini P, Vettori MV, Corradi M, Mutti A. Influence of condensation temperature on selected exhaled breath parameters. *BMC Pulmonary Medicin*e 2005, doi: 10.1186/1471-2466-5-10.

19. Nowak D, Kalucka S, Bialasiewicz P, Krol M. Exhalation of H_2O_2 and thiobarbituric acid reactive substances (TBARs) by healthy subjects. *Free Radic Biol Med* 2001; 30: 178-186.

20. Schleiss MB, Holz O, Behnke M, Richter K, Magnussen H, Jorres RA. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate. Eur Respir J 2000; 16: 1115-1118.

21. Rahman I. Reproducibility of oxidative stress biomarkers in breath condensate: are they reliable? *Eur Respir J* 2004; 23: 183-184.

22. American Thoracic Society. Workshop Proceedings: Exhaled nitric oxide and nitric oxide oxidative metabolism in exhaled breath condensate. *Proc Am Thorac Soc* 2006; 3: 131-145.

23. Sack U, Scheibe R, Wötzel M, Hammerschmidt S, Kuhn H, Emmrich F, Hoheisel G, Wirtz H, Gessner C. Multiplex analysis of cytokines in exhaled breath condensate. *Cytometry A* 2006; 69: 169-172.

24. Gessner C, Scheibe R, Wötzel M, Hammerschmidt S, Kuhn H, Engelmann L, Hoheisel G, Gillissen A, Sack U, Wirtz H. Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. *Respir Med* 2005; 99: 1229-1240.

25. Matsunaga K, Yanagisawa S, Ichikawa T, Ueshima K, Akamatsu K, Hirano T, Nakanishi M, Yamagata T, Minakata Y, Ichinose M. Airway cytokine expression measured by means of protein array in exhaled breath condensate: correlation with physiologic properties in asthmatic patients. *J Allergy Clin Immunol* 2006; 118: 84-90.

26. Schumann C, Triantafilou K, Krueger S, Hombach V, Triantafilou M, Becher G, Lepper PM. Detection of erythropoietin in exhaled breath condensate of nonhypoxic subjects using a multiplex bead array. *Mediators Inflamm* 2006, doi:10.1155/MI/2006/18061.

27. Caglieri A, Goldoni M, Acampa O, Andreoli R, Vettori MV, Corradi M, Apostoli P, Mutti A. The effect of inhaled chromium on different exhaled breath condensate biomarkers among chrome-plating workers. *Environ Health Perspect* 2006; 114: 542-546.

28. Tufvesson E, Bjermer L. Methodological improvements for measuring eicosanoids and cytokines in exhaled breath condensate. *Respir Med* 2006; 100: 34-38.

29. Soyer OU, Dizdar EA, Keskin O, Lilly C, Kalayci O. Comparison of two methods for exhaled breath condensate collection. *Allergy* 2006; 61: 1016-1018.

30. Prieto L, Ferrer A, Palop J, Domenech J, Llusar R, Rojas R. Differences in exhaled breath condensate pH measurements between samples obtained with two commercial devices. *Respir Med* 2007, doi: 10.1016/j.rmed.2007.02.023.