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# Biomarker reproducibility in exhaled breath condensate collected with different condensers

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**ABSTRACT: Optimal collection and analysis of exhaled breath condensate (EBC) are prerequisites for standardisation and reproducibility of assessments. The present study aimed to assess reproducibility of EBC volume, hydrogen peroxide ($H_2O_2$), 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 the following four condensers: glass, silicone, EcoScreen® (Erich Jaeger GmbH, Hoechberg, Germany) and an optimised glass condenser. In small EBC samples, $H_2O_2$ was measured by spectrophotometer, 8-isoprostane by enzyme immunoassay, and cytokines by multiplexed xMAP® technology (Luminex Corporation, Austin, TX, USA).**

**The optimised glass condenser yielded significantly more EBC volume (median 2,025 μL, interquartile range 1,600–2,525). The reproducibility of EBC volume, yielded by the new glass condenser, was comparable with EcoScreen® (19–20 coefficients of variation (CV)%), but was significantly better compared with silicone and glass (29–37 CV%). The new condenser was associated with significantly more detections of $H_2O_2$, 8-isoprostane, interleukin-2, -4, -5 and -13, and tumour necrosis factor-α. Isoprostane concentrations were significantly higher using the new condenser, whereas $H_2O_2$ and cytokine concentrations were not. Reproducibility of biomarkers was equally variable for all condenser types.**

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

**KEYWORDS: Condenser, cytokines, exhaled breath condensates, hydrogen peroxide, 8-isoprostane, multiplex array**

The collection of exhaled breath condensate (EBC) is a noninvasive, safe technique with which 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 ($H_2O_2$) 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, both *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

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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 biofluid 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 50-μL condensate samples; however, the detection level did not reach 50% [6]. Liquid bead-based multiplexing xMAP® technology (Luminex Corporation, Austin, TX, 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, it was hypothesised that optimised condensate collection with minimal adhesive properties improves the reproducibility of different measurements in EBC.

The aim of the present study was to assess the effect of four different condensers (glass, silicone, EcoScreen® (Erich Jaeger GmbH, Hoechberg, Germany) and a new, optimised glass condenser) on the reproducibility of breath condensate volume, and the detection, concentration and reproducibility of inflammatory biomarkers, including $H_2O_2$, 8-isoprostane, interleukin (IL)-2, -4, -5, -6, -8, -10 and -13, and tumour necrosis factor (TNF)-α, in EBC in healthy nonasthmatic adults.

## METHODS

### Study subjects

A total of 30 eligible healthy nonasthmatic adult volunteers were recruited (table 1) among medical students and staff at the University Hospital Maastricht (Maastricht, the Netherlands), based on the criterion that each volunteer was able to breath tidally into a mouthpiece for ⩾15 min. Exclusion criteria were as follows: history of asthma, upper or lower airway infection, and use of antibiotics, corticosteroids, cromoglycate, nedocromil, theophylline or leukotriene 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 the following four tests. Test 1: on the first day in the morning; test 2: on the first day in the afternoon; test 3: the second day at the same time as test 1; and test 4: 1 week after test 1 at the same time. In turn, each test consisted of four sequential EBC collections, using random different types of condenser.

**TABLE 1** Subject characteristics of healthy nonasthmatic adult volunteers[#]

| | |
|---|---|
| **Male/female** | 19:11 |
| **Age yrs** | 23 (22–36) |
| **Weight kg** | 75 (62–87) |
| **Height cm** | 180 (171–185) |
| **Nonsmoking** | 28 |
| **History of eczema and/or hay fever** | 6 |

Data are presented as n or median (interquartile range). [#]: n=30.

### EBC collection

EBC was collected using either 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 Dept of Instrument Development Engineering and Evaluation of the Maastricht University (patent number EP 07102586), as described in figure 1. Briefly, the inclined condensation surface is enlarged (using a length of 90 cm), 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 min.

### 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 3 months from sampling time.

$H_2O_2$ was measured in 50-μL EBC, in duplicate by spectrophotometer (UV-VIS Lambda 10 Spectrometer; Perkin Elmer, Shelton/Norwalk, CT, USA) with a lower detection limit of 0.05 μM, as described previously [8].

Isoprostane was measured in 100 μL of EBC, by specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA), which was modified to reach a lower limit of detection of 1.0 pg·mL$^{-1}$. The standard curve of this assay ranged 250–1.95 pg·mL$^{-1}$. It was possible to report 8-isoprostane values as low as 1.0 pg·mL$^{-1}$, 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 3.9 and 7.8 pg·mL$^{-1}$, an isoprostane recovery of 92% (CV of concentration 16%) and 95% (CV of concentration 15%) were found, respectively. A CV (of absorption signals) <15% was considered highly acceptable. Hence, the corresponding CV of concentrations may be higher. Therefore, the CVs found in the 8-isoprostane recovery experiments using these low values were considered good. Standard curves, patient samples and quality control samples of 2.5 and 10 pg·mL$^{-1}$ 8-isoprostane were assayed in triplicate. Intra-assay variation of standard curves, patient samples and quality control samples had to be <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 50-μL EBC, using the liquid bead-based multiplexing xMAP® technology. Multiplex immunoassay was performed as described previously [7, 9, 10]. The corresponding lower limits of cytokine and chemokine detection were as follows (in pg·mL$^{-1}$): IL-2 (1.0), IL-4 (1.2), IL-5 (1.2), IL-6 (0.4), IL-8 (1.1), IL-10 (1.2), IL-13 (1.0) and TNF-α

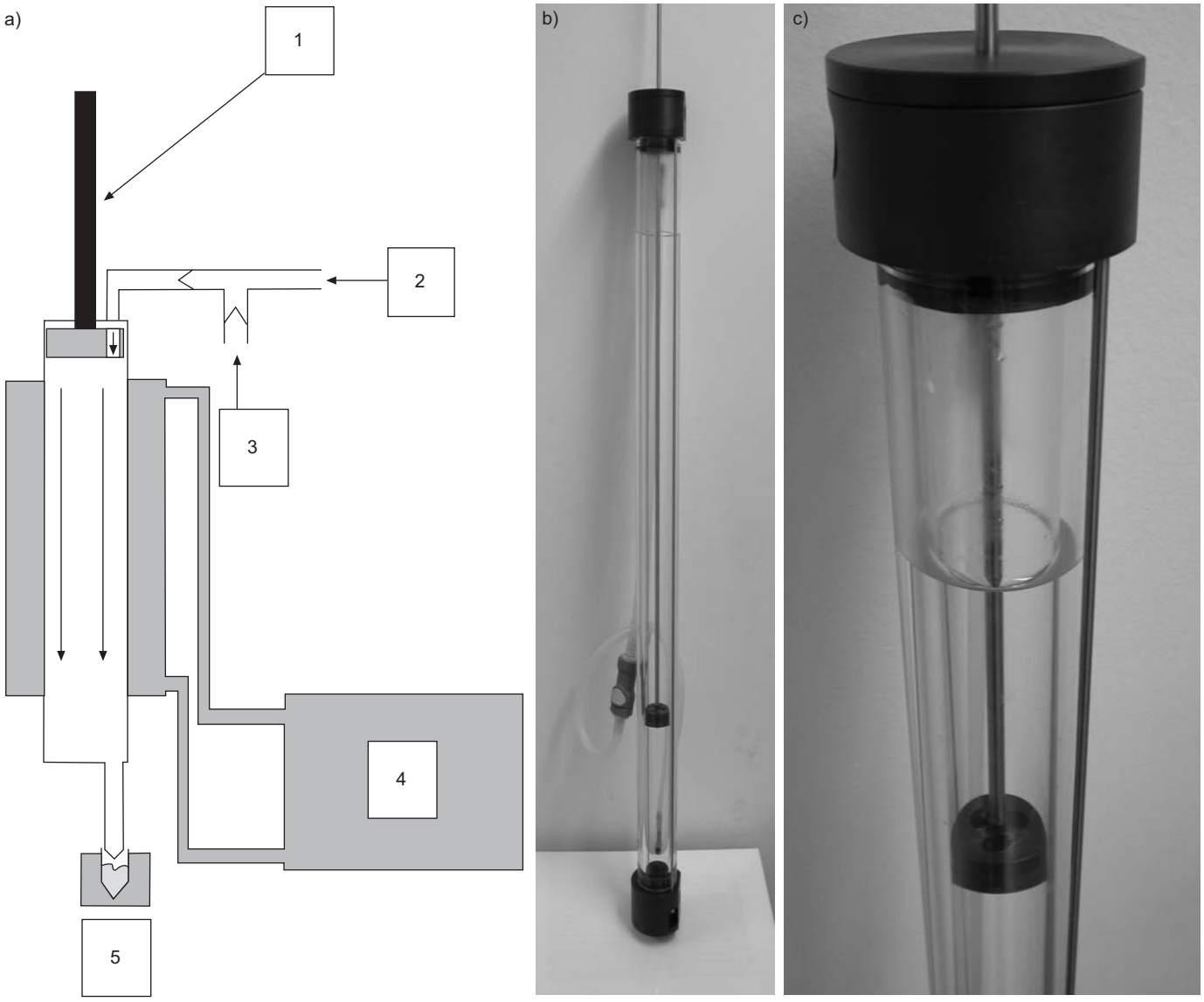


**FIGURE 1.** a) A diagrammatic description of the new, optimised glass condenser system. 1: inclined glass condenser with a tube length of 90 cm (to enlarge the condensation surface) and downwards moveable plunger (to optimise condensate recovery from the inner condenser wall), with tangential and axial breath flow channels (to turbinately direct the tidally exhaled breath flows towards the cooled inner condenser wall). 2: swan-neck tubing (serving as gravitational saliva trap) and two-way nonrebreathing valve, connected to a mouthpiece. 3: entrance of inspired room air. 4: cooling unit consisting of a counter-current circulating ice-water pump (■). 5: removable, cooled glass sample vial (to collect exhaled breath condensate). b) The glass condenser tube with plunger *in situ*, and c) detail of the glass condenser tube with the downwards moveable plunger *in situ*. The plunger has three tangential and axial breath flow channels (to turbinately direct the tidally exhaled breath flows towards the cooled inner condenser wall).

(1.3). Measurement and data analysis were performed using the Bioplex 100 system and Bioplex Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). All multiplex immunoassays were performed in a 96-well format, 1.2-μm 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 either 100 or 10 $pg \cdot mL^{-1}$ cytokines (IL-2, -4, -5, -6, -8, -10 and -13, and TNF-α), and were measured in quadruplicate. The mean CV of these cytokine measurements was 12.2%. The mean recovery of 100 $pg \cdot mL^{-1}$ spiked cytokine was 103% (range 71–129%). At 10 $pg \cdot mL^{-1}$, the mean recovery was somewhat lower at 89% (ranging 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*

Not normally distributed data were expressed as median (interquartile range). Normally distributed data were expressed as mean ± SE. To estimate variance within a single method of measurement (coating), CV were used.

$$CV = (SD/mean) \times 100\% \quad (1)$$

The CV was calculated as the mean of individual CVs, calculated over the two or four relevant measurements. Within-subject CVs were used as data distribution was normal. When the distribution of individual CVs was not normal, the nonparametric Friedman test was used to see if these CVs were different between coatings. ANOVA was used to test for differences among normally distributed repeated measures. The Chi-squared test was used to statistically evaluate the differences between proportions for four groups in a data set. Condensate samples with a biomarker concentration below the lower detection limit, *i.e. in strictu sensu* negative detections, were not considered as missing values because they actually informed 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 as follows: 0.025 μM for $H_2O_2$, 0.1 $pg \cdot mL^{-1}$ for 8-isoprostane and 0.1 $pg \cdot mL^{-1}$ for cytokines. Samples were defined as missing if the EBC volume, yielded after 15 min collection time, was either 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 the present study was to answer this question. Therefore, the uncertainty of the estimated variability was taken as a basis for power calculations. Data from 30 volunteers were thus available for each of the estimated variances (within-day, between-day and between-week). Using standard results for the variance of a Chi-squared random variable, the present authors inferred that, with the 30 subjects, the relative confidence limits (relative to the observed value of the variance) would be 0.634 and 1.807. For the SD, 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 five out of 480 manoeuvres performed, the present authors were unable to collect condensate due to erroneously connected tubing. EBC collection by the optimised glass condenser yielded significantly more median condensate volume compared with the other condensers ($p=0.001$, Friedman test; 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-squared test), but was significantly better compared with silicone and glass ($p<0.028$, Chi-squared test).

### $H_2O_2$ measurements in EBC

Overall, 29% of $H_2O_2$ measurements were missing (table 3). Significantly more positive $H_2O_2$ detections were found using the optimised glass condenser, compared with silicone and glass ($p<0.050$, Chi-squared test). Median $H_2O_2$ concentrations and reproducibility (expressed as CV%) did not significantly differ between the four condensers ($p=0.286$ and $p>0.080$, respectively, Friedman test).

### 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 with silicone and glass ($p<0.023$, Chi-squared test). The median concentration of 8-isoprostane was significantly higher using the new condenser compared with the other three condensers ($p=0.001$, Friedman test). Statistically, 8-isoprostane reproducibility (expressed as CV%) did not significantly differ between the four condensers ($p>0.151$, Friedman test).

### Cytokine measurements in EBC

Overall, 20% of cytokine measurements were missing (table 5). The levels of cytokine detection using xMAP® technology were as follows: IL-2 (68%), IL-4 (97%), IL-5 (73%), IL-6 (46%), IL-8 (46%), IL-10 (61%), IL-13 (70%) and TNF-α (64%). The optimised glass condenser had significantly more positive detections of IL-2, -4, -5, -13 and TNF-α, compared with silicone and glass ($p<0.050$, Chi-squared test), and more IL-5 and -13 detections, compared with EcoScreen® ($p<0.021$, Chi-squared test). Cytokine concentrations were not significantly

**TABLE 2** Volume of exhaled breath condensate (EBC), and reproducibility (expressed as coefficients of variation (CV)) in four different condenser systems

| EBC | Type of condenser | | | | p-value¶ |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| Failed#/total n | 4/120 | 0/120 | 1/120 | 0/120 | |
| Volume of EBC μL | 525 (300–700) | 712 (500–912) | 2025 (1600–2525) | 1237 (950–1800) | 0.001 |
| CV within-day % | 20 (10–37) | 21 (12–31) | 11 (5–20) | 9 (4–25) | 0.002 |
| CV between-day % | 28 (11–47) | 30 (11–37) | 11 (4–30) | 13 (5–28) | 0.027 |
| CV between-week % | 35 (19–55) | 26 (13–41) | 15 (6–28) | 15 (6–27) | 0.003 |
| CV overall % | 37 (27–49) | 29 (22–38) | 20 (10–27) | 19 (11–38) | 0.001 |

Data are presented as median (interquartile range), unless otherwise indicated. EcoScreen® is manufactured by Erich Jaeger GmbH (Hoechberg, Germany). New: optimised glass condenser. #: after 15 min; ¶: Friedman test.

**TABLE 3** Hydrogen peroxide ($H_2O_2$) measurements in exhaled breath condensate (EBC) presented as detection, concentration and reproducibility (expressed as coefficients of variation (CV)) in four different condenser systems

| $H_2O_2$ | Type of condenser | | | | p-value |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| Missing/total n | 59/120 | 49/120 | 10/120 | 23/120 | |
| Ratio of positive to negative detections n/n | 52/9 | 62/9 | 77/33 | 75/22 | 0.003# |
| Concentration of $H_2O_2$ in EBC µM | 2.6 (1.4–4.1) | 2.1 (1.0–3.3) | 1.8 (1.0–2.6) | 2.2 (0.3–2.8) | 0.286¶ |
| CV within-day % | 17 (6–31) | 12 (6–34) | 46 (15–86) | 13 (1–22) | 0.271¶ |
| CV between-day % | 22 (7–40) | 30 (4–46) | 30 (1–119) | 16 (8–60) | 0.821¶ |
| CV between-week % | 18 (3–49) | 26 (5–47) | 32 (13–57) | 12 (5–37) | 0.256¶ |
| CV overall % | 23 (12–37) | 29 (19–40) | 24 (21–73) | 22 (14–27) | 0.080¶ |

Data are presented as median (interquartile range), unless otherwise indicated. EcoScreen® is manufactured by Erich Jaeger GmbH (Hoechberg, Germany). New: optimised glass condenser. #: Chi-squared test; ¶: Friedman test.

different between the four condenser types ($p>0.113$, Friedman test or ANOVA, respectively; table 6). Reproducibility (expressed as CV%) of cytokine measurements did not significantly differ, either overall (table 7) or within-day, between-day and between-week (data not shown). The 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-α (8–22%). When CVs were evaluated using the positive values only (without negative and/or arbitrary values), again no differences between condensers were found (data not shown).

## DISCUSSION

The present authors have 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 with the silicone, glass and EcoScreen® condensers. Reproducibility of EBC volume was comparable for the new condenser and EcoScreen®, and was significantly better compared with the other two condenser types.

In EBC collected with this new condenser, significantly more positive $H_2O_2$, 8-isoprostane, IL-2, -4, -5 and -13, and TNF-α detections were found, supporting improved sampling of EBC. Moreover, 8-isoprostane concentrations were significantly increased in EBC yielded by the new condenser compared with the other three condensers, which is in accordance with former findings [4]. Conversely, reproducibility of $H_2O_2$, 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 the literature to date, no formal study addressing this issue has been published, although reproducibility using other analytical techniques has been reported

**TABLE 4** 8-Isoprostane (IP) measurements in exhaled breath condensate (EBC) presented as detection, concentration and reproducibility (expressed as coefficients of variation (CV)) in four different condenser systems

| 8-IP | Type of condenser | | | | p-value |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| Missing/total n | 27/120 | 16/120 | 9/120 | 10/120 | |
| Positive to negative detections n/n | 89/4 | 98/6 | 110/1 | 109/1 | 0.001# |
| Concentration of 8-IP in EBC $pg \cdot mL^{-1}$ | 2.0 (1.3–3.5) | 2.9 (1.4–3.7) | 3.6 (2.2–4.9) | 2.5 (1.6–3.4) | 0.001¶ |
| CV within-day % | 6 (1–30) | 20 (6–61) | 23 (6–41) | 24 (8–37) | 0.297¶ |
| CV between-day % | 31 (7–72) | 26 (7–64) | 23 (10–42) | 15 (7–39) | 0.559¶ |
| CV between-week % | 22 (9–51) | 22 (2–59) | 15 (8–39) | 25 (8–36) | 0.954¶ |
| CV overall % | 31 (11–68) | 48 (14–69) | 29 (18–40) | 26 (18–44) | 0.151¶ |

Data are presented as median (interquartile range), unless otherwise indicated. EcoScreen® is manufactured by Erich Jaeger Gmbh (Hoechberg, Germany). New: optimised glass condenser. #: Chi-squared test; ¶: Friedman test.

**TABLE 5** Number of positive to negative detections of cytokines in exhaled breath condensate, collected with four different condenser systems

| Cytokine | Type of condenser | | | | p-value# |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| **Missing/total n** | 49/120 | 37/120 | 1/120 | 10/120 | |
| **Positive to negative detections** | | | | | |
| 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-α | 52/19 | 57/26 | 72/47 | 65/45 | 0.050 |

Data are presented as n/n, unless otherwise indicated. EcoScreen® is manufactured by Erich Jaeger Gmbh (Hoechberg, Germany). New: optimised glass condenser; IL: interleukin; TNF: tumour necrosis factor. #: Chi-squared test.

[12–14], and/or could not be calculated due to the 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 $H_2O_2$ 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) $H_2O_2$ 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, which might 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 to express variability: when mean values are low, CV values can be abnormally high. Therefore, results in the present study were also expressed as SD values 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, increased

**TABLE 6** Concentration of cytokine measurements in exhaled breath condensate (EBC), collected with four different condenser systems

| Cytokine | Type of condenser | | | | p-value |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| **Missing/total n** | 49/120 | 37/120 | 1/120 | 10/120 | |
| **Concentration of cytokine in EBC pg·mL$^{-1}$** | | | | | |
| IL-2# | 1.9 (0.5–5.5) | 1.6 (0.8–5.2) | 1.2 (0.9–4.4) | 1.6 (0.5–4.5) | 0.494+ |
| IL-4¶ | 5.8 (5.1–6.6) | 5.1 (4.4–5.8) | 4.7 (4.0–5.4) | 5.4 (4.8–6.0) | 0.129§ |
| IL-5# | 4.4 (2.2–16.0) | 3.0 (1.5–13.6) | 3.2 (2.0–13.4) | 3.1 (1.2–13.3) | 0.544+ |
| IL-6# | 0.9 (0.1–4.4) | 0.9 (0.1–4.6) | 0.7 (0.1–3.9) | 0.6 (0.1–4.5) | 0.113+ |
| IL-8# | 2.0 (0.1–4.7) | 2.1 (0.1–4.1) | 2.7 (0.1–4.9) | 2.1 (0.1–4.0) | 0.341+ |
| IL-10¶ | 1.5 (0.9–2.1) | 1.6 (0.9–2.3) | 1.4 (0.8–1.9) | 1.3 (0.7–1.8) | 0.872§ |
| IL-13¶ | 6.6 (4.2–9.0) | 6.6 (4.4–8.8) | 6.3 (4.8–7.9) | 5.9 (4.3–7.5) | 0.946§ |
| TNF-α¶ | 2.1 (1.2–3.0) | 2.0 (1.1–2.9) | 1.8 (1.2–2.5) | 1.7 (1.0–2.4) | 0.876§ |

Unless otherwise indicated, data are presented as: #: median (interquartile range); or ¶: mean (95% confidence interval). EcoScreen® is manufactured by Erich Jaeger GmbH (Hoechberg, Germany). New: optimised glass condenser; IL: interleukin; TNF: tumour necrosis factor. +: Friedman test; §: ANOVA.

**TABLE 7** Overall reproducibility (expressed as coefficients of variation) of cytokine measurements in exhaled breath condensate, collected with four different condenser systems

| Cytokine | Type of condenser | | | | p-value |
|---|---|---|---|---|---|
| | Silicone | Glass | New | EcoScreen® | |
| **Missing/total n** | 49/120 | 37/120 | 1/120 | 10/120 | |
| **Overall reproducibility %** | | | | | |
| IL-2# | 80 (54–106) | 75 (53–97) | 84 (64–104) | 70 (47–94) | 0.802+ |
| IL-4# | 33 (19–47) | 32 (20–44) | 36 (25–48) | 26 (17–34) | 0.494+ |
| IL-5# | 74 (47–102) | 71 (47–96) | 73 (51–94) | 81 (54–108) | 0.941+ |
| IL-6¶ | 12 (0–77) | 12 (0–68) | 14 (3–81) | 11 (0–69) | 0.690f |
| IL-8¶ | 15 (0–28) | 12 (0–21) | 2 (0–18) | 9 (0–25) | 0.766f |
| IL-10¶ | 30 (6–54) | 17 (4–52) | 16 (5–44) | 11 (10–63) | 0.719f |
| IL-13# | 51 (27–74) | 68 (42–93) | 73 (53–93) | 63 (42–84) | 0.574+ |
| TNF-α¶ | 17 (5–50) | 22 (10–74) | 19 (7–69) | 8 (0–68) | 0.878f |

Unless otherwise stated, data are presented as #: mean (95% confidence interval) or ¶: median (interquartile range). New: optimised glass condenser; IL: interleukin; TNF: tumour necrosis factor. +: ANOVA; f: Friedman test.

mean concentrations and lowered CVs in diseased subjects could be expected. Conversely, with these very low concentrations of cytokines and 8-isoprostane, an influence of analytical variability cannot be ruled out.

Cytokines were simultaneously measured by xMAP® technology in small, 50-μL EBC samples. The overall level of detection was 46–97%, which was much better compared with cytometric bead array in small samples in children (<50%), and in large 1,000–2,000-μL 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 (take hours instead of days to perform), have good precision (CV 10–15%), are not interfered with 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, the hand-held disposable polypropylene RTube™ (Respiratory Inc., Charlottesville, VA, 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, biomarkers may also be lost within these collection systems, as recently demonstrated for 8-isoprostane and albumin measurements, both *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 and BJERMER [28] proposed to coat EcoScreen® collection surfaces with bovine serum albumin and Tween-20, to measure cytokines and eicosanoids, respectively. However, they also reported possible false-positive (eicosanoid) results, and the need of sample concentration, by vacuum centrifugation, prior to analysis. SOYER *et al.* [29] found significantly higher cysteinyl leukotrienes and eotaxin using EcoScreen®, compared with RTube™, due to susceptibility of pre-cooled RTube™ sleeves to (increased) ambient temperatures during collection, and due to different materials that could affect sample recovery. PRIETO *et al.* [30] compared RTube™ and EcoScreen®, and reported that EBC pH values are dependent on the collection device used. Furthermore, the EcoScreen® has been associated with deposition of frozen condensate on its lamellar walls. For these reasons, an optimised glass condenser system was developed by the present authors. 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 with 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® condensers were both significantly associated with less variation in the generated EBC volume compared with the other condensers, thereby 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 CVs compared with the commercial EcoScreen®, and optimisation of EBC analysis using rapid, multiplexed measurement of cytokines in small EBC sample sizes may, in combination, 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 exhaled breath condensate volume, and with good reproducibility. Furthermore, significantly more positive detections of hydrogen peroxide, 8-isoprostane, interleukin-2, -4, -5 and -13, and tumour necrosis factor-$\alpha$ were found in exhaled breath condensate collected with the new condenser, thereby offering an increased capacity to analyse for complex biomarker profiles. Moreover, concentrations of 8-isoprostane were significantly increased using the optimised glass condenser compared with the other three condensers. However, reproducibility of biomarker measurements in exhaled breath condensate was not influenced by the type of condenser.

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