

Feasibility of a new method to collect exhaled breath condensate in pre-school children

Rosias PPR, Robroeks CM, van de Kant KD, Rijkers GT, Zimmermann LJ, van Schayck CP, Heynens JW, Jöbsis Q, Dompeling E. Feasibility of a new method to collect exhaled breath condensate in pre-school children.

Pediatr Allergy Immunol 2009.

© 2009 John Wiley & Sons A/S

Exhaled breath condensate (EBC) is a promising non-invasive method to assess respiratory inflammation in adults and children with lung disease. Especially in pre-school children, condensate collection is hampered by long sampling times because of open-ended collection systems. We aimed to assess the feasibility of condensate collection in pre-school children using a closed glass condenser with breath recirculation system, which also collects the residual non-condensed exhaled breath, and subsequently recirculates it back into the condenser. Condensate was collected before and after breath recirculation in 70 non-sedated pre-school children with and without recurrent wheeze. Cytokines (IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF- α) were measured in 50 μ l samples using ultrasensitive multiplexed liquid bead array. The success rate of condensate collection increased from 64% (without recirculation) to 83% (after breath recirculation), and mean condensate volume from 214 to 465 μ l respectively. Detection of cytokines was successful in 95–100% of samples. Cytokine concentrations before and after breath recirculation were not different ($p > 0.232$). In asthmatic children, only TNF- α concentrations were significantly decreased, compared to non-asthmatics. In pre-school children, the collection of EBC is feasible using a new closed glass condenser with breath recirculation system. This new method may help to assess – non-invasively – cytokine profiles in asthmatic and non-asthmatic pre-school children.

Philippe P. R. Rosias^{1,2}, Charlotte M. Robroeks², Kim D. van de Kant², Ger T. Rijkers³, Luc J. Zimmermann⁴, Constant P. van Schayck⁵, Jan W. Heynens¹, Quirijn Jöbsis² and Edward Dompeling²

¹Department of Paediatrics, Orbis Medical Center, Sittard, ²Department of Paediatric Pulmonology, Care and Public Health Research Institute, University Hospital Maastricht, Maastricht, ³Department of Immunology, University Medical Centre, Wilhelmina Children's Hospital, Utrecht, ⁴Department of Paediatrics, University Hospital Maastricht, Maastricht, ⁵Department of General Practice, Care and Public Health Research Institute, Maastricht University, Maastricht, The Netherlands

Key words: asthma; cytokines; exhaled breath condensate; multiplex bead array; pre-school children; wheezing

Philippe P.R. Rosias, Department of Pediatrics, Orbis Medical Center, PO Box 5500, 6130 MB Sittard, The Netherlands

Tel.: +31 88 4597832

Fax: +31 88 4598623

E-mail: p.rosias@orbisconcern.nl

Accepted 17 April 2009

Pre-school wheezing is reported in no less than 30% of children, and may indicate a trivial cold, as well as an ongoing chronic lung inflammation, such as asthma (1–3). Atopic asthma is associated with a cytokine pattern compatible with predominant activation of the T helper (Th)-2 mediated immune response (4). However, in the developing pre-school child, accurate assessment of different wheezing phenotypes is currently difficult (1–3). Moreover, proper diagnosis is hampered by the current lack of a reliable routine technique to assess different types of respiratory inflammation in pre-school children. Ideally, such a technique would be non-invasive, rapid and easy to perform, without any risks, success-

ful at any age, standardized, sensitive, specific and highly reproducible. The collection of exhaled breath condensate (EBC) is a non-invasive technique to obtain direct samples from the lower respiratory tract, without disturbing an ongoing inflammation (5, 6). In children, analysis of EBC reveals the presence of various types of inflammatory biomarkers (7, 8), including cytokines (5, 6, 9–11).

Although general methodological recommendations on EBC collection are published, there are still unresolved questions, resulting in low feasibility of EBC collection in (non-sedated) pre-school children (6, 12–14). Main issues are: (i) the variable efficacy of different condenser

coatings, (ii) the variable efficacy of open-ended collection systems and (iii) the use of suboptimal analytical techniques. At first, different condenser coatings may each have different adhesive interactions with a specific type of biomarker that is to be measured in the collected condensate. Therefore, the efficacy of different breath condenser coatings, such as glass, polypropylene and Teflon™ (DuPont Inc., New Jersey, USA), was studied (15, 16). The use of a newly developed and optimized glass condenser was found to be more efficient, in terms of the volume of EBC, and the detection of cytokines and 8-isoprostane (15, 16). Secondly, all EBC collection systems have an open-ended design. Inevitably, such a design is associated with a variable non-standardized loss of non-condensed exhaled breath. In turn, this loss may contribute to unnecessarily prolonged EBC collection times (from a toddler's point of view), as well as to (intra- and inter-subject) variability of EBC dilution, and thus may influence subsequent analysis (17, 18). Thirdly, feasibility of EBC is also hampered by the use of suboptimal immunoassays with questionable reproducibility, that often require relatively too large sample volumes that often cannot be collected in pre-school children (18, 19).

Taken together, this has led us to make the following methodological adjustments for the purpose of this study in pre-school children: (i) the introduction of a closed optimized glass condenser design with breath recirculation system, which collects all non-condensed residual exhaled breath, and subsequently recirculates it back into the condenser (to optimize condensation and minimize collection time) and (ii) the simultaneous measurement of cytokines in the resulting EBC by means of xMAP® technology (Luminex Corporation, Austin, TX, USA), an ultrasensitive multiplexed liquid bead array that requires only one small (50 µl) sample volume. We hypothesized that the combination of these methodological adjustments enhances the feasibility of EBC collection and analysis in pre-school children. Therefore, the aim of the present study was to assess the feasibility of EBC collection using a modified closed glass condenser in non-sedated pre-school children with and without wheezing.

Methods

Study subjects

Eligible pre-school children were recruited at the paediatric outpatient department of the Orbis Medical Center Sittard and the University Hospital of Maastricht. Healthy pre-school chil-

dren were recruited based on a non-respiratory indication for referral to the paediatric outpatient department; the main indications were routine follow-up of growth and development. According to parent-completed questionnaires, medical documents and information of the responsible paediatric pulmonologist, children were allocated to one of the following study groups:

- 1 The *doctor's diagnosed atopic asthma* group characterized by: (i) recurrent wheezing (three or more episodes of wheeze within the preceding 12 months or less), (ii) not 'exclusively' triggered by viral infections, (iii) with a documented history of a favourable corticosteroid effect (clinical improvement, assessed and documented by the child's physician, after at least 3 months of inhaled corticosteroid therapy, or after a short course of oral prednisone therapy during a wheezy episode), (iv) positive family history of atopy in the first degree (parent or sibling with asthma, hay fever, food allergy or eczema) and (v) atopy (positive Phadiatop or radioallergo-sorbent test class ≥ 2 , and/or elevated blood level of total immunoglobulin-E). Current corticosteroid maintenance therapy was not an obligatory criterion.
- 2 The *viral-triggered recurrent wheeze* group characterized by: (i) recurrent wheezing, (ii) 'exclusively' triggered by viral infections, (iii) without a documented history of steroid therapy, or with documented history of no favourable corticosteroid effect and (iv) without corticosteroid maintenance therapy in the preceding 3 months. Lack of atopy was not an obligatory criterion.
- 3 The *healthy control* group characterized by: (i) the absence of recurrent wheezing and other respiratory symptoms (including dyspnoea, recurrent cough and signs of upper airway infection), as excluded by means of standardized ISAAC questionnaires and (ii) without a history of corticosteroid maintenance therapy (20). Lack of atopy was not an obligatory criterion.
- 4 The *unclassifiable recurrent wheeze* group consisted of infants and toddlers with recurrent wheezing, but not eligible for the asthmatic or viral wheeze group. They participated only in the feasibility study.

Overall exclusion criteria were cardiac disease, anatomic or congenital defects of the airways or lungs, syndromes, systemic disease and/or mental retardation. Presumed inability to use a face

Table 1. Characteristics of 70 pre-school children, allocated to one of the four study groups, based on parent-completed questionnaires and available medical documents

Study group	Atopic asthma	Viral wheezing	Healthy control	Unclassifiable wheezing
Number†	23 (33%)	22 (31%)	20 (29%)	5 (7%)
Age (months)‡	31 (21–40)*	28 (21–37)*	13 (8–26)*	25 (22–37)
Male/female	13/10	17/5	12/8	2/3
Weight (kg)‡	14 (12–15)**	13 (12–14)**	11 (8–13)**	12 (11–14)
Z-score weight‡	0 [(-1.0)–0.4]	0 [(-1.5)–1.0]	0 [(-0.6)–0.5]	0 [(-0.5)–0.5]
Height (cm)‡	95 (84–98)	94 (81–98)	85 (70–93)	92 (80–95)
Z-score height‡	0.0 [(-0.5)–1.0]	-0.3 [(-1.0)–1.1]	0.5 [(-1.0)–1.1]	-1.0 [(-2.0)–0.0]
Elevated serum IgE, or positive RAST $\geq$ class 2†	23 (100%)	4 (18%)	0 (0%)	2 (40%)
Current use of steroids†	15 (65%)	–	–	5 (100%)
Inhaled steroid dose‡	400 (300–400)	–	–	400 (200–400)

IgE, immunoglobulin-E; RAST, radioallergo-sorbent test; Steroids, inhaled corticosteroids (and one subject in the atopic asthma group also used a short course of oral prednisone).

* $p = 0.012$ (multiple comparisons, Hochberg correction); ** $p = 0.046$ (multiple comparisons, Hochberg correction).

†Absolute number (%).

‡Median (IQR).

mask did not preclude participation into this study. Subject characteristics are shown in Table 1.

EBC collection

This cross-sectional study comprised one attempt, between wheezing episodes, to collect EBC in a pre-school child. Precedingly, parents received thorough instructions on the collection procedure by one of the coaching childhood asthma nurses and were asked to bring along the child's favourite cartoon, cd-song, booklet and/or puppet.

EBC was collected using a modified closed glass condenser system that was developed in close collaboration with the department of Instrument Development Engineering & Evaluation (IDEE) of the Maastricht University (patent number EP-07102586). The EBC collection system consisted of an inclined and closed glass condenser with a tube length of 90 cm (to enlarge the condensation surface to 1046 cm²), a downwards moveable plunger (to optimize condensate recovery from the inner wall of the condenser), with tangential and axial breath flow channels (to turbulently direct the tidal breath flows towards the cooled inner condenser wall), and an unique breath recirculation system, heated at 37°C (Fig. 1). The breath recirculation system consisted of heated tubing (to guide the non-condensed residual exhaled breath, and to avoid condensation outside the condenser tube), a humidity sensor, a heated inert single use Tedlar™ (SKC Ltd., Dorset, UK) gas sample bag (to collect all non-condensed residual exhaled breath) and a small ventilator system to recircu-

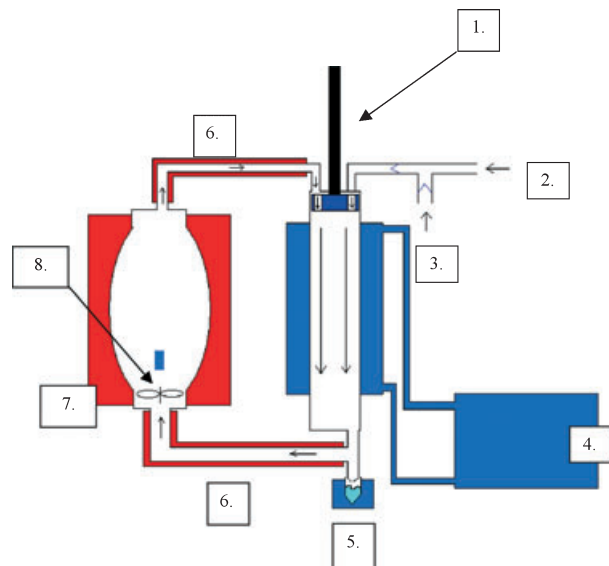


Fig. 1. Schematic representation of the new closed glass condenser system, that enabled condensate collection in pre-school children before and after breath recirculation. 1. Inclined and closed glass condenser, and downwards moveable plunger with tangential and axial breath flow channels. 2. Swan-neck tubing, two-way nonrebreathing valve, and face mask with separated nose and mouth cavity. 3. Entrance of inspired room air. 4. Cooling unit consisting of a counter-current circulating ice-water pump. 5. Removable glass sample vial, to collect exhaled breath condensate. 6. Heated (at 37°C) tubing, to guide the residual noncondensed exhaled breath. 7. Humidity sensor and heated (at 37°C) inert Tedlar™ gas sample bag. 8. Small ventilator system to recirculate the residual noncondensed exhaled breath back into the condenser system.

late the non-condensed residual exhaled breath back into the cooled condenser system (to allow secondary condensation of exhaled breath) (Fig. 1). The connecting tubing was made of

Hytrel® thermoplastic polyester elastomer (Du Pont Plastics, Mechelen, Belgium). The total expiratory resistance of the condenser system with breath recirculation was 2.7 cmH₂O/l/s at a flow of 50 l/min, and 1.9 cmH₂O/l/s at a flow of 30 l/min. The glass condenser was cooled using a counter-current circulating ice-water pump. The cooling temperature was preset at 0°C (Fig. 1).

The child exhaled tidally, as long as possible, through a face mask which separated air from mouth and nasal cavity, and with a two-way non-rebreathing valve (8900 paediatric sizes oro-nasal masks; Hans Rudolph Inc., Kansas City, MO, USA), connected to the glass condenser system by means of a swan-neck tubing (with a length of 38 cm; and serving as gravitational saliva trap). The Hans Rudolph face mask with separated mouth and nasal cavity (which occludes the nasal air flow) was used to sample exhaled breath only from the child's oral cavity (6). It simultaneously served as an alternative noseclip to limit the loss of exhaled air through the nose, and to prevent accidental inhalation–exhalation through the nose during sample collection (6). During the condensation process, the formerly lost and non-condensed exhaled breath was separately being collected in the gradually inflating inert Tedlar™ gas sample bag. When the child refused to proceed and/or started to cry, the procedure was stopped and EBC was collected by pushing the plunger downwards. Subsequently, the plunger was pulled up again, and the separately collected exhaled breath was recirculated from the Tedlar™ gas sample bag back into the condenser system, to perform a secondary breath condensation. The non-condensed exhaled breath was passed again and again through the condenser until the degree of humidity was $\leq 22\%$.

Before and after each EBC collection procedure, the respiratory status was checked by using a clinical asthma score (skin colour changes, breath sounds including coughing and wheeze, breathing rate, retractions and level of alertness). To improve the toddler's coping with distress from the unfamiliar procedure, they were distracted while breathing tidally through a facemask. We primarily combined strict parental cooperation, with an age-appropriate cartoon, that was already started prior to entrance. In case of imminent failure to cope, a more active strategy was used on parent's advice, including children's stories, puppetry and/or children's songs.

EBC collection was defined successful when at least 60 μ l of condensate was produced, comprising the minimal required volume of 50 μ l to perform one multiplexed cytokine analysis, and

an additional 10 μ l to compensate for loss because of sample handling.

After each procedure, the Hans Rudolph face mask and two-way non-rebreathing valve were cleaned with Cidex OPA Solution (Advanced Sterilization Products, Johnson & Johnson Medical BV, Amersfoort, the Netherlands). The non-disposable tubing (Tefa Portanje, Woerden, the Netherlands) and the glass condenser were disconnected, rinsed repeatedly with bi-distilled water and subsequently dried at room air. The non-disposable parts of the heated recirculation system were disconnected, rinsed repeatedly with bi-distilled water and alcohol and subsequently dried at room air.

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. Cytokines in EBC were measured in duplicate using a liquid bead array multiplexing system or xMAP® technology (Luminex Corporation). Multiplex immunoassay was performed as described previously, to measure the following cytokines (with corresponding lower limits of detection): interleukin (IL)-4 (1.2 pg/ml), IL-5 (1.2 pg/ml), IL-6 (2.4 pg/ml), chemokine IL-8 (2.9 pg/ml), IL-10 (2.3 pg/ml), IL-12p70 (1.2 pg/ml), IL-13 (1.0 pg/ml) and tumour necrosis factor-alpha (TNF- α , 1.3 pg/ml) (21–23). 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 96 well format 1.2 μ m filter bottom plates (Millipore, Amsterdam, the Netherlands) and a 12-point standard curve in duplicate was included on every plate. To minimize interassay variation, positive and negative control samples were included. The intrasubject variability of cytokines in EBC was assessed in seven children, aged 2–3 yr, with recurrent wheeze, as follows: at $t = 0$ and 30 min, 100 μ l of condensate was collected using the closed glass condenser system. The short-term intrasubject variability was expressed as the coefficient of variation (s.d./mean $\times 100\%$).

Statistics

Statistical calculations were performed using spss 11.5 (SPSS Inc., Chicago, IL, USA). Normally distributed data were expressed as mean and standard deviation. The Student's t -test was used

to test for differences among dependent, paired or repeated samples. Pearson's correlation coefficients were used to assess the degree of association between two normally distributed variables. ANOVA was used to test for differences among normally distributed variables for more than two study groups.

Not normally distributed data were expressed as median with interquartile ranges (IQR). The Wilcoxon-signed rank test was used to test for differences among not normally distributed paired data, whereas the Mann–Whitney *U*-test was used for unpaired data. Spearman's rank correlation coefficients were used to assess the degree of association between two not normally distributed variables. The Kruskal–Wallis test was used to test for differences among non-normal variables for more than two study groups. The Hochberg correction for multiple comparisons was applied. The chi-squared test was used to evaluate the distribution of categorical variables (number of occurrences) in four study groups.

Power calculation

Assuming a success rate of 80%, the standard error of feasibility is 5.1% using $n = 60$ subjects. This was considered sufficiently accurate for the purpose of this study.

Ethics

This study was approved by the Medical Ethics Committee of the University Hospital of Maastricht and the Orbis Medical Center Sittard. All parents gave written informed consent.

Results

Feasibility of EBC collection

A total number of 70 pre-school children participated. Feasibility of the new EBC collection method in pre-school children is shown in Table 2. Before breath recirculation, the mean duration of EBC collection was 11 min (s.d. = 6), the mean EBC volume was 214 μ l (s.d. = 208) and the success rate was 64% in these pre-school children. After breath recirculation, the mean EBC volume was 347 μ l (s.d. = 339). In total, the mean collected EBC volume was 465 μ l (s.d. = 385). The success rate of EBC collection with breath recirculation was 83% in these pre-school children, but differed significantly between the four study groups ($p < 0.001$, χ^2 test).

In the healthy group, the mean duration of EBC collection was 7 min, and mean EBC volume before breath recirculation was 113 μ l, whereas both were significantly decreased compared to the asthmatic and viral wheeze group ($p < 0.031$, ANOVA). A younger age significantly correlated with less duration of EBC collection ($R = 0.481$, $p < 0.001$, Spearman's correlation coefficient). A duration of EBC collection time of 10 min or more, was associated with successful EBC collection before breath recirculation. In due course of the collection procedure, the cooling temperature slightly rose from 0.00°C to a median of 2.15°C (IQR = 0.90–4.85) at the end of EBC collection.

Four children refused to start because of sudden anxiety, associated with insufficient parental stimulation and/or recent ear–nose–throat surgery (adenoidectomy, tonsillectomy).

Table 2. Feasibility of exhaled breath condensate (EBC) collection, before and after breath recirculation in 70 pre-school children

Study group	Atopic asthma	Viral wheezing	Healthy control	Unclass wheezing	p-value
Number*	23 (33%)	22 (31%)	20 (29%)	5 (7%)	–
Age (months)†	31 (21–40)	28 (21–37)	13 (8–26)	25 (22–37)	0.012
Before breath recirculation					
Duration of collection (min)‡	13 (6)	13 (5)	7 (4)	14 (7)	0.004
EBC volume (μ l)‡	199 (169)	291 (223)	113 (168)	320 (295)	0.031
Success rate*	15 (65%)	20 (91%)	6 (30%)	4 (80%)	0.241
After breath recirculation					
EBC volume (μ l)‡	438 (408)	298 (239)	271 (337)	513 (301)	0.385
Total (before and after breath recirculation)					
EBC volume (μ l)‡	491 (444)	447 (354)	383 (372)	730 (223)	0.351
Success rate*	17 (74%)	21 (95%)	15 (75%)	5 (100%)	<0.001

EBC collection was defined successful when at least 60 μ l of condensate was yielded. The unclassifiable (unclass) recurrent wheezing group was excluded for statistical analysis for reasons of disproportionality.

*Absolute number (percentage).

†Median (IQR).

‡Mean (s.d.).

and/or myringotomy with insertion of tympanostomy tube). Distraction during EBC collection was achieved by combined parental cooperation (86%) and watching age-appropriate cartoons (76%), listening to children's stories (36%), using puppetry (15%) and/or listening to children's songs (12%). The Hans Rudolph face mask was applied in 60 children, whereas five preferred the babyhaler® (GlaxoSmithKline BV, Zeist, the Netherlands) and one the nebuchamber® (AstraZeneca BV, Zoetermeer, the Netherlands) face mask. Two collection procedures were not successful because of leakage of exhaled breath from defective Tedlar™ gas sample bags. Neither adverse effects on respiration, nor other side-effects were seen during the collection procedures.

Cytokine measurements before and after breath recirculation

Paired EBC samples were taken from the condensate volume collected before breath recirculation, and from the condensate volume collected after the breath recirculation manoeuvre, separately. Table 3 shows the detection and concentration of IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF- α in EBC collected before, as well as after breath recirculation, in 45 pre-school children. The detection of cytokines in EBC was successful in 95–100% of the collected 50 μ l condensate samples. There was no statistical difference between cytokine concentrations before and after breath recirculation ($p > 0.117$, Wilcoxon-signed rank test).

The coefficients of variation of the short-term intrasubject variability were 13.9% for IL-4, 14.3% for IL-5, 20.0% for IL-6, 33.3% for IL-8, 14.3% for IL-10, 14.3% for IL-12p70, 8.7% for IL-13 and 3.7% for TNF- α .

Cytokines in subgroups with and without wheeze

For further cytokine analysis between the different study groups, the separately collected condensate samples before as well as after breath recirculation in each subject, were considered as one donor series. For reasons of disproportion, data of the children that did not use the Hans Rudolph face mask (with separated mouth and nasal cavity) were excluded. Between the asthmatic ($n = 17$), viral wheeze ($n = 21$) and healthy ($n = 10$) group, the median cytokine concentrations of IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70 and IL-13 did not differ (data not shown), whereas the median concentration of TNF- α was significantly decreased in the asthmatic group [median = 38.8 pg/ml (IQR = 37.1–41.3) in asthma, 43.1 pg/ml (IQR = 40.2–46.1) in viral wheeze and 40.9 pg/ml (IQR = 39.7–41.9) in healthy controls, respectively] ($p = 0.018$, Kruskal–Wallis). Only a weak correlation existed between age and cytokine concentrations ($-0.120 < R < 0.392$, Spearman's correlation coefficient).

In atopic asthmatic children, inhaled corticosteroids did in general not influence cytokine levels in EBC, with one exception: TNF- α concentrations were significantly lowered in children with inhaled corticosteroid therapy, compared with steroid naïve children (median = 37.1 pg/ml vs. 41.7 pg/ml, respectively) ($p = 0.013$, Mann–Whitney U -test).

When asthmatic and non-asthmatic (viral wheeze and healthy) children were compared, TNF- α concentrations in the asthmatic group (median = 38.8 pg/ml, IQR = 37.1–41.3) were significantly decreased vs. non-asthmatic children (median = 41.7 pg/ml, IQR = 40.2–45.4) ($p = 0.013$, Mann–Whitney U -test) (Table 4, Fig. 2a). The ratios of IL-5/TNF- α and IL-13/

Table 3. Detection (%) and concentration (pg/ml) of cytokines in exhaled breath condensate (EBC) collected before and after breath recirculation in 45 pre-school children

EBC	Before breath recirculation		After breath recirculation		p-value Wilcoxon-signed rank	Lower limit of detection (pg/ml)
	Detection %	pg/ml Median (IQR)	Detection %	pg/ml Median (IQR)		
IL-4	100	2.1 (1.7–2.4)	100	2.2 (1.7–2.6)	0.346	1.2
IL-5	98	16.7 (16.7–18.3)	97	16.7 (16.7–18.3)	0.278	1.2
IL-6	96	3.3 (2.5–3.4)	95	2.9 (2.9–3.3)	0.611	2.4
IL-8	100	3.9 (3.4–4.4)	100	4.4 (3.9–5.0)	0.117	2.9
IL-10	98	4.7 (4.5–5.2)	100	4.7 (4.7–5.2)	0.455	2.3
IL-12p70	100	3.4 (3.4–3.8)	100	3.4 (3.4–3.7)	0.301	1.2
IL-13	100	8.5 (7.6–9.4)	100	8.5 (8.0–9.4)	0.603	1.0
TNF- α	96	40.9 (39.0–44.8)	95	40.9 (37.1–43.2)	0.746	1.3

IL, interleukin; IQR, interquartile range; TNF, tumour necrosis factor.

Table 4. Cytokine profile in exhaled breath condensate (EBC) in 48 doctor's diagnosed atopic asthmatic and non-asthmatic pre-school children (consisting of the viral-triggered recurrent wheezing group and healthy control group)

EBC	Doctor's diagnosed atopic asthma	Non-asthmatic pre-school children	p-value (MWU)	Lower limit of detection (pg/ml)
Number	17	31	—	—
TNF- α *	38.8 (37.1–41.3)	41.7 (40.2–45.4)	0.013	1.3
IL-5/TNF*	0.46 (0.42–0.52)	0.40 (0.38–0.47)	0.018	—
IL-13/TNF*	0.24 (0.20–0.26)	0.20 (0.18–0.23)	0.034	—
IL-4*	2.1 (1.9–2.5)	2.0 (1.7–2.5)	NS	1.2
IL-5*	17.5 (16.1–19.1)	16.7 (15.9–18.3)	NS	1.2
IL-6*	3.3 (2.8–3.6)	3.1 (2.7–3.5)	NS	2.4
IL-8*	4.2 (3.9–5.0)	4.2 (3.9–4.5)	NS	2.9
IL-10*	4.7 (4.5–5.9)	5.0 (4.7–5.2)	NS	2.3
IL-12p70*	3.6 (3.4–4.0)	3.4 (3.4–3.7)	NS	1.2
IL-13*	9.0 (8.0–9.9)	8.5 (7.6–9.4)	NS	1.0

IL, interleukin; MWU, Mann–Whitney *U*-test; NS, not significant; TNF, tumour necrosis factor.

*Median (IQR).

TNF- α in the asthmatic group (median = 0.46, IQR = 0.42–0.52; median = 0.24, IQR = 0.20–0.26, respectively) were significantly higher compared with non-asthmatic children (median = 0.40, IQR = 0.38–0.47; median = 0.20, IQR = 0.18–0.23, respectively) ($p = 0.018$ and 0.034 , respectively, Mann–Whitney *U*-test) (Table 4, Fig. 2b,c).

Discussion

We report an enhanced feasibility of EBC collection in pre-school children with and without wheezing, using a novel closed glass condenser with breath recirculation system, and using multiplexed liquid bead array: (i) the overall success rate increased from 64% to 83%, and the mean EBC volume increased from 214 to 465 μ l, indicating that breath recirculation indeed improved the feasibility of EBC collection, (ii) the detection of cytokine profiles in 50 μ l EBC was successful in 95–100% of the collected samples, (iii) similar cytokine concentrations were found in EBC collected before as well as after breath recirculation and (iv) in the atopic asthmatic group, only TNF- α concentrations in EBC were significantly decreased, compared with pre-school children with viral wheeze and healthy controls.

We simultaneously introduced novel techniques to optimize the condensation process (see Fig. 1), and to improve small condensate sample analysis, in non-sedated pre-school children with and without recurrent wheezing. Cumulatively, this new closed glass collection

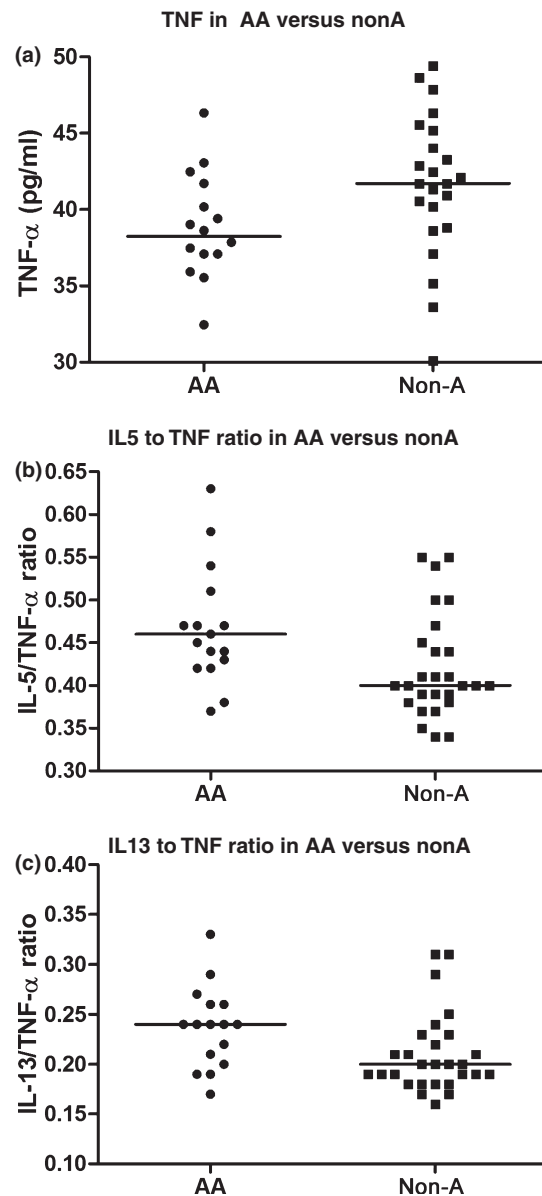


Fig. 2. (a,b,c) T helper 2/T helper 1 cytokine profiles in exhaled breath condensate in doctor's diagnosed atopic asthmatic (AA) and non-asthmatic (non-A) pre-school children. (a) Condensate TNF-alpha concentration was significantly decreased in the doctor's diagnosed atopic asthmatic group (median 38.8 pg/mL, IQR 37.1–41.3) compared to nonasthmatic (median 41.7 pg/mL, IQR 40.2–43.4) preschool children ($p = 0.013$, MWU). (b) Condensate IL-5/TNF-alpha ratio was significantly increased in the doctor's diagnosed atopic asthmatic group (median 0.46, IQR 0.42–0.52) compared to nonasthmatic (median 0.40, IQR 0.38–0.47) preschool children ($p = 0.018$, MWU). (c) Condensate IL-13/TNF-alpha ratio was significantly increased in the doctor's diagnosed atopic asthmatic group (median 0.24, IQR 0.20–0.26) compared to nonasthmatic (median 0.20, IQR 0.18–0.23) preschool children ($p = 0.034$, MWU). IL, interleukin; IQR, interquartile range; MWU, Mann Whitney U test; TNF, tumor necrosis factor.

method with breath recirculation resulted in an overall success rate of 83%, compared with 64% when using the 'usual' (without or before breath recirculation) procedure and in a mean total EBC volume of 465 μl . In the literature on EBC in pre-school children, Moeller et al. (14) proposed a method to collect, both orally and nasally, a median EBC volume of 282 μl , using two disposable Plastipak syringes (BD Plastipak, Franklin Lakes, NJ, USA), in tidal breathing infants, however, during sedated sleep and without assessment of markers in the collected condensate. Griesse et al. presented a nasal polypropylene collection system with air suction pump, to measure hydrogen peroxide and 8-isoprostane, and reported 85 $\mu\text{l}/\text{min}$ of nasally collected EBC in healthy children aged 4 wk to 18 yr, however, with contamination by nasal secretions (24, 25). The present study reports a correlation between age and duration of EBC collection, which is in accordance with the significantly increased volume of collected EBC with age, reported by both Moeller et al. (14) and Griesse et al. (24). Irrespective of the duration of EBC collection, breath recirculation allowed condensation of the totally exhaled breath volume within the closed glass system, in contrast to maximally 30% condensation using the open-ended nasal suction system of Griesse et al. (24, 25), and in contrast to the open-ended non-standardized EBC collection techniques that are available for use in older children and adults, and, that all have a variable loss of non-condensed exhaled breath, as condensation does not immediately occur, and as condensation does not continue once the surface is saturated (5, 6, 26–29). Hence, our concept of breath recirculation enabled not only the collection of an increased volume of EBC, but also, in the younger toddlers, with limited cooperation in time (often less than 10 min), and often not familiar with the (repeated) use of a face mask, breath recirculation was often crucial to yield a minimal but sufficient volume of condensate. Furthermore, cytokine concentrations in EBC collected before breath recirculation, and after breath recirculation did not differ significantly, demonstrating that condensate cytokine concentrations were not affected by the breath recirculation process and illustrating an efficiency to generate additional and equivalent condensate.

We acknowledge that we cannot exclude a possible influence of the observed small rise in cooling temperature, during the collection procedure. Furthermore, we acknowledge that the use of a face mask without partitioning (mouth–nose)

in six out of 66 children, is less ideal, although we did not find a statistical influence on the volume of EBC, or the condensate cytokine concentrations ($p \geq 0.296$ and 0.188 , respectively, unpaired t -test). In the present study, we did not compare several other collecting devices. The following reasons have led us to decide *not* to include other devices. At first, pre-school children might have refused to proceed with again another test, which would have biased feasibility. Secondly, we recently demonstrated, in adults, that an open-ended prototype of the glass condenser without breath recirculation yielded significantly more EBC volume (median = 2025 μl), compared with three other condensers (e.g. the EcoScreen® (Erich Jaeger GmbH, Hochberg, Germany) yielded 1237 μl), and the glass coating was associated with significantly more detections of IL-2, IL-4, IL-5, IL-13 and TNF- α in EBC (15, 16). In pre-school children, the feasibility of EBC collection using the most efficient condenser, i.e. the open-ended glass prototype, or, in other words, the condenser without breath recirculation was 64%. Hence, we did not find it appropriate to include other, less optimal condensers in these children as this would have resulted in an efficiency even lower than 64%, based on the comparative study in adults (16). However, to further increase the feasibility, the condenser was equipped with the breath recirculation system, which further increased success rate up to 83%, whereas, cytokine concentrations before and after breath recirculation did not differ significantly, indicating no additional loss of cytokines in the recirculation system.

Multiplexed liquid bead array was used to measure cytokines more accurately in only 50 μl sample volumes of EBC. The detection level of cytokines was high, ranging from 95% to 100%. Multiplexed liquid bead array is based on flow cytometry and microspheres (with faster and more reproducible liquid reaction kinetics, than solid planar assays), and allows multiplexing of up to 100 assays within one sample (21). Reasons to introduce this technique were: (i) lack of sensitivity of conventional enzyme-linked immunosorbent assays to detect cytokines in 200–500 μl EBC and (ii) variable level of simultaneous cytokine detection by cytometric bead array ranging from 0% to 25% in 50 μl EBC in children and 3–100% in 1000–2000 μl lyophilized EBC in adults (10, 30, 31). When compared with conventional assays, multiplexed immunoassays are highly sensitive and specific, detect bioactive and inactive molecules, are rapid [hours instead of day(s)], have good (but not yet excellent) precision with coefficients of variation ranging

from 10% to 15%, are not interfered by drugs, have a simple protocol, require small sample volumes and allow simultaneous detection (21).

In pre-school children with different wheezing phenotypes, the central issue consists of finding a non-invasive measure to identify underlying (persisting) airway disease, such as atopic asthma. In contrast to pre-school children with viral wheeze and healthy children, atopic asthma is characterized by a predominantly Th2-mediated chronic eosinophilic airway inflammation (1, 4). Statistical analysis only revealed a significantly decreased TNF- α in EBC in the atopic asthmatic group, compared with viral wheeze and healthy controls. Steroid-treated atopic asthmatic children had lowered levels of TNF- α in EBC. This is in accordance with studies on bronchial biopsies in atopic asthmatic inflammation showing significantly decreased levels of TNF- α after 8 wk of budesonide treatment (32). The other EBC cytokine levels measured in the present study were similar in all subject groups, and were close to, but not below the lower limits of detection, in contrast to the few published data in older children and adults (11, 31, 33). As supported by the findings of Shahid et al. (11), who reported increased levels of IL-4 and decreased IFN- γ in EBC in asthmatic children, the ratio's of IL-5/TNF- α and IL-13/TNF- α in EBC in our study were significantly increased in the asthmatic group, compared with non-asthmatics. However, the difference between these fractions of IL-5 and IL-13 to TNF- α was mainly caused by the lowered levels of TNF- α in EBC in asthmatic pre-school children. Obviously, further evaluation of repeated cytokine profile measurements in distinct wheezing phenotypes is warranted. Finally, we speculate that the proposed method of EBC collection may open a new window of opportunities in paediatric research, as it may allow non-invasive inflammatory profiling in non-sedated pre-school children.

In conclusion, we demonstrated the feasibility of EBC collection in non-sedated pre-school children with and without wheezing illness using a novel closed glass condenser with breath recirculation system. Multiplexed liquid bead array enabled more accurate cytokine measurement in only small EBC sample sizes. The presented method may help to assess – non-invasively – cytokine profiles in asthmatic and non-asthmatic pre-school children.

Acknowledgments

The authors wish to thank the childhood asthma nurses, Irene Breugelmans, Jacqueline Frenken and Paula Heggen, for their technical support in the collection of EBC

(Department of Paediatrics, Orbis Medical Center, Sittard, the Netherlands); and last but not least the parents and children for their participation.

References

1. GRIGG J, SILVERMAN M. Wheezing disorders in young children: one disease or several phenotypes? In: FREY U, GERRITSEN J, eds. *Respiratory Diseases in Infants and Children*. Eur Respir Mon 2006; 37: 153–69.
2. DE JONGSTE J. To wheeze or not to wheeze. Am J Respir Crit Care Med 2006; 174: 1281–2.
3. MARTINEZ F, WRIGHT A, TAUSSIG L, HOLBERG C, HALONEN M, MORGAN W. Asthma and wheezing in the first six years of life. N Eng J Med 1995; 332: 133–8.
4. ROBINSON D, HAMID Q, YING S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Eng J Med 1992; 326: 298–304.
5. ROSIAS P, DOMPELING E, HENDRIKS J, HEIJNENS J, DONCKERWOLCKE R, JÖBSIS Q. Exhaled breath condensate in children: pearls and pitfalls. Pediatr Allergy Immunol 2004; 15: 4–19.
6. HORVÁTH I, HUNT J, BARNES P, ON BEHALF OF THE ATS/ERS TASK FORCE. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 2005; 26: 523–48.
7. LINKOSALO L, LEHTIMÄKI L, LAITINEN J, KAILA M, HOLM K, MOILANEN E. Increased bronchial NO output in severe atopic eczema in children and adolescents. Pediatr Allergy Immunol 2008; 19: 426–32.
8. KIELBASA B, MOELLER A, SANAK M, et al. Eicosanoids in exhaled breath condensates in the assessment of childhood asthma. Pediatr Allergy Immunol 2008; 19: 660–9.
9. ROBROEKS C, ROSIAS P, VAN VLIET D, et al. Biomarkers in exhaled breath condensate indicate presence and severity of cystic fibrosis in children. Pediatr Allergy Immunol 2008; 19: 652–9.
10. ROBROEKS C, JÖBSIS Q, DAMOISEAUX J, et al. Cytokines in exhaled breath condensate of children with asthma and cystic fibrosis. Ann Allergy Asthma Immunol 2006; 96: 349–55.
11. SHAHID S, KHARITONOV S, WILSON N, BUSH A, BARNES P. Increased interleukin-4 and decreased interferon-gamma in EBC of children with asthma. Am J Respir Crit Care Med 2002; 165: 1290–3.
12. BARALDI E, GHIRO L, PIOVAN V, CARRARO S, ZACCHELLO F, ZANCONATO S. Safety and success of exhaled breath condensate collection in asthma. Arch Dis Child 2003; 88: 358–60.
13. CHEAH F, DARLOW B, WINTERBOURN C. Problems associated with collecting breath condensate for the measurement of exhaled hydrogen peroxide from neonates on respiratory support. Biol Neonate 2003; 84: 338–41.
14. MOELLER A, FRANKLIN P, HALL G, HORAK F, WILDHABER J, STICK S. Measuring exhaled breath condensate in infants. Pediatr Pulmonol 2006; 41: 184–7.
15. ROSIAS P, ROBROEKS C, NIEMARKT H, et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. Eur Respir J 2006; 28: 1036–41.
16. ROSIAS P, ROBROEKS C, KESTER A, et al. Biomarker reproducibility in exhaled breath condensate collected with different condensers. Eur Respir J 2008; 31: 934–42.

17. ROSIAS P, ROBROEKS C, HENDRIKS J, DOMPELING E, JÖBSIS Q. Exhaled breath condensate: a space odyssey, where no one has gone before... *Eur Respir J* 2004; 24: 189–90.
18. ROSIAS P, JÖBSIS Q, VAN DE KANT K, et al. Global condensation: a 'climate change' towards better standardisation of exhaled breath condensate measurements. *Eur Respir J* 2008; 31: 684–5.
19. HUSZÁR É, SZABÓ Z, JAKAB A, BARTA I, HERJAVECZ I, HORVÁTH I. Comparative measurement of thromboxane A2 metabolites in exhaled breath condensate by different immunoassays. *Inflamm Res* 2005; 54: 350–5.
20. ASHER M, KEIL U, ANDERSON H, et al. International study of asthma and allergies in childhood (ISAAC): rationale and methods. *Eur Respir J* 1995; 8: 483–91.
21. DE JAGER W, RIJKERS G. Solid-phase and bead-based cytokine immunoassay: a comparison. *Methods* 2006; 38: 294–303.
22. DE JAGER W, PRAKKEN B, BIJLSMA J, KUIS W, RIJKERS G. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J Immunol Methods* 2005; 300: 124–35.
23. DE JAGER W, TE VELTHUIS H, PRAKKEN B, KUIS W, RIJKERS G. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 2003; 10: 133–9.
24. GRIESE M, LATZIN P, BECK J. A noninvasive method to collect nasally exhaled air condensate in humans of all ages. *Eur J Clin Invest* 2001; 31: 915–20.
25. LATZIN P, BECK-RIPP J, HARTL D, BARTENSTEIN A, NOSS J, GRIESE M. 8-Isoprostane in nasally exhaled breath condensate in different pediatric lung diseases. *Eur J Med Res* 2007; 12: 21–5.
26. CAGLIERI A, GOLDONI M, ACAMPA O, et al. The effect of inhaled chromium on different exhaled breath condensate biomarkers among chrome-plating workers. *Environ Health Perspect* 2006; 114: 542–6.
27. HORVÁTH I, DONNELLY L, KISS A, et al. Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. *Am J Respir Crit Care Med* 1998; 158: 1042–6.
28. SOYER O, DIZDAR E, KESKIN O, LILLY C, KALAYCI O. Comparison of two methods for exhaled breath condensate collection. *Allergy* 2006; 61: 1016–8.
29. TUFVESSON E, BJERMER L. Methodological improvements for measuring eicosanoids and cytokines in exhaled breath condensate. *Respir Med* 2006; 100: 34–8.
30. SACK U, SCHEIBE R, WÖTZEL M, et al. Multiplex analysis of cytokines in exhaled breath condensate. *Cytometry A* 2006; 69: 169–72.
31. MATSUNAGA K, YANAGISAWA S, ICHIKAWA T, et al. 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.
32. WILSON S, WALLIN A, DELLA-CIOPPA G, SANDSTRÖM T, HOLGATE S. Effects of budesonide and formoterol on NF- κ B, adhesion molecules, and cytokines in asthma. *Am J Respir Crit Care Med* 2001; 164: 1047–52.
33. ROBROEKS C, DOMPELING E, RIJKERS G, DAMOISEAUX J, JÖBSIS Q. Inflammatory markers in exhaled breath condensate, assessed by means of Luminex® technology, can differentiate between children with asthma, cystic fibrosis, and healthy controls. *Eur Respir J* 2007; 30 (Suppl. 51): 498s–9s.