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2015 J. Breath Res. 9 026001

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PAPER

Ultrafine particle content in exhaled breath condensate in airways of asthmatic children

RECEIVED
1 December 2014REVISED
30 January 2015ACCEPTED FOR PUBLICATION
6 February 2015PUBLISHED
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Keywords: ultrafine particles, exhaled breath condensate, asthma

Abstract

Air pollution triggers and exacerbates airway inflammation. Particulate material (PM) in ambient is characterized as being coarse (PM 10, aerodynamic diameter range 2.5–10 μm), fine (PM 2.5, 2.5–0.1 μm) and ultrafine (UFP, nano-sized, <0.1 μm). It is known that smaller inhaled PM produced more inflammation than larger ones. Most data on human exposure to PM are based on environmental monitoring. We evaluated the effect of individual exposure to UFP on functional respiratory parameters and airway inflammation in 52 children aged 6–18 years referred to the Pulmonary and Allergic Diseases Laboratory due to respiratory symptoms. Spirometry, bronchial provocation challenge, induced sputum (IS), exhaled breath condensate (EBC) and fractional exhaled nitric oxide evaluations were performed by conventional methods. UFP content in EBC was analyzed by using a NanoSight Light Microscope LM20. The total EBC UFP content correlated with wheezing ($r = 0.28$, $p = 0.04$), breath symptom score ($r = 0.3$, $p = 0.03$), and sputum eosinophilia ($R = 0.64$, $p = 0.005$). The percent of EBC particles in the nano-sized range also correlated with wheezing ($r = 0.36$, $p = 0.007$), breath symptom score ($r = 0.33$, $p \leq 0.02$), and sputum eosinophilia ($r = 0.72$, $p = 0.001$). Respiratory symptoms and airway inflammation positively correlated to UFP content in EBC of symptomatic children.

1. Introduction

Epidemiologic studies have established an association between exposure to airborne particles in metropolitan areas and population-level increases in morbidity and mortality [1]. Several reports demonstrated that exposure to air pollution triggers and exacerbates airway inflammatory disease in children, causing cough, dyspnea, wheezing and reduced lung function [2–4]. Particulate material (PM) in ambient air pollution is characterized as being coarse (PM 10, aerodynamic diameter range 2.5–10 μm), fine (PM 2.5, 2.5–0.1 μm) and ultrafine (UFP, nano-sized, <0.1 μm). While relatively low levels of exposure to UFP are sufficient to increase the risk of long-term mortality in adults [5], long-term ambient exposure to air pollution

is associated with increased respiratory symptoms in children [3]. UFP exposure in particular was found to be associated with an increased odds ratio of short-term asthma exacerbation among children [2] as well as with increases in airway inflammation in adults, as measured by fractional exhaled nitric oxide (FeNO) and decreased breath pH [6, 7].

Because of their small size, UFP are deposited deeper into the lungs and take longer to be cleared than larger particles [8]. *In vitro* and animal studies have shown that inhaled UFP cause endothelial cell death and produce more inflammation than larger inhaled particles [9, 10], and Warheit *et al* demonstrated *in vitro* that the magnitude of this effect could be underestimated [11]. Most of our knowledge on PM exposure and its effects on humans is based on environmental

monitoring [12]. Environmental monitoring of UFP is particularly challenging due to the short half-life of particles in ambient air [13]. Assessment of individual exposure to PM has been attempted by using personal air samplers as well as by models incorporating environmental pollution sampling and questionnaires [14], but the data from those methodologies are not precise.

In the past, particulate burden in the lung had been assessed by bronchoscopy and bronchoalveolar lavage (BAL) [15, 16]. The invasiveness of the BAL procedure, however, has hampered its application as a tool for screening and for follow-up evaluations, especially in pediatric patients. The search for Noninvasive techniques to monitor the PM burden and inflammation markers has led to the development of a number of approaches, among them the analysis of as induced sputum (IS), FeNO and exhaled breath condensate (EBC) were developed [17]. Extensive work has been invested in comparing IS to BAL findings [18, 19], and there is now solid evidence to show that IS can be used for studying airway inflammatory cell populations [20] and soluble inflammation markers [21, 22] with good reproducibility. We established a correlation between PM exposure and IS eosinophilia in an earlier study in which IS was used to assess individual PM burden [20–23]. We consequently demonstrated that 19 ppb for FENO has good sensitivity and specificity to diagnose asthma in 150 asthmatic children [24]. EBC is a relatively new tool in airway assessment. It requires that the subject breathe tidally via a mouthpiece into cooled tubing and the fluid that is condensed from the exhaled breath is collected and used for mediator assessment. Investigators have detected UFP in the range of 1.0–2.5 μm in the EBC of workers exposed to metal PM [25, 26]. EBC can be used to quantitate exhaled UFP in individuals exposed to cigarette smoke as well as to many compounds that may provide markers of airway inflammation, such as hydrogen peroxide and malondialdehyde [27]. Moreover, levels of H_2O_2 in EBC were shown to be positively correlated to % of neutrophils and eosinophils in the IS of a wide range of pathological conditions the inflammatory markers to be correlated with eosinophil counts in IS [28].

To date, there have been no reports on individual measurements of UFP exposure in the pediatric population. The purpose of the current study was to examine whether exhaled UFP could be assessed by analysis of EBC, and whether exhaled UFP are correlated with symptomatic, functional and/or laboratory measures of airway inflammation in children.

2. Materials and methods

2.1. Study population

We recruited 52 consecutive children between 6–18 years of age who were referred to the Laboratory of Pulmonary and Allergic Diseases in the Tel Aviv Medical Center for diagnostic assessment due to respiratory symptoms. After obtaining approval

from the institutional ethics committee and parental informed consent, the participants' parents filled in a modified International Study of Asthma and Allergies in Childhood questionnaire [29] on their child's symptoms as well as on selected socioeconomic and environmental factors. Severity of the following symptoms was graded on a scale of 0–3: wheeze, rhinorrhea, cough, dyspnea, nocturnal dyspnea and effort-induced dyspnea. Total scores of all respiratory symptoms were calculated for each subject. The children then underwent spirometry, and EBC was collected in the usual manner. Some of the participants were also referred for bronchial provocation challenge, IS and FeNO.

Thirty-seven of the children presented clinical symptoms and with one or more of the following and were therefore considered as being asthmatic: reduced spirometric values (FEV_1 , $FVC \leq 80\%$ $FEV_1/FVC \leq 75$), a positive bronchial provocation study (methacholine or adenosine challenge), a positive FENO result and/or sputum eosinophilia. This group was compared to other 15 children who were free of asthma characteristics.

2.2. Pulmonary function testing (PFT)

PFTs were performed by a Masterlab spirometer (Masterlab E Jaeger, Wurzburg, Germany). Measurements were carried out according to standard protocols of the American Thoracic Society ATS guidelines [30], and the results were expressed as % predictive value. The best of three consecutive measurements was chosen. Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were recorded. The FEV_1 result and the FEV_1/FVC ratio were considered low if they were less than 80% of expected or less than a ratio of 0.75 respectively.

2.3. FeNO

Prior to spirometry, FeNO was measured by a portable chemiluminescence NO analyzer (CLD 88 AM; ECO Medic, Inc., GMBH) according to ATS/ERS recommendations. A FeNO result of more than 19 ppb was considered positive.

2.4. Bronchial provocation challenges

Patients were asked to stop their child's asthma medication before the challenge. Both methacholine and adenosine challenges were performed using a Spira nebulizer and an electronic dosimeter (Pari, Medanz, Starnberg, Germany) with an output of 0.45 μL and a nebulization time of 0.6 s. Methacholine (Provocholine; Methapharm, Geneva, Switzerland) inhalation challenge was performed by the five-breath dosimeter method with a dosing schedule of methacholine concentrations of 0.0625, 0.25, 1, 4, and 16 mg mL^{-1} . Adenosine inhalation challenge was also performed according to the five-breath dosimeter protocol. Sodium salt of adenosine monophosphate (Sigma-Aldrich, St. Louis, MO, USA) was diluted in

saline solution (0.9% sodium chloride) and stored at 4 °C. The dosing schedule was 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 mg mL⁻¹. Positivity of the challenge was established when a decrease in FEV1 of 20% or greater from the lowest post-saline value was achieved. Methacholine challenge was performed according to the ATS guidelines [31].

2.5. IS collection and processing

IS was obtained as previously described in detail elsewhere [19]. After pretreatment with a short-acting β -2 agonist, 3% saline was administered by a nebulizer (U1 Ultrasonic Nebulizer; Omron HealthCare, Henfield, West Sussex, UK) for up to 20 min while the subjects were encouraged to cough and expectorate sputum into a sterile container. Samples were stored at 4 °C and processed within 3 h. All portions with little or no squamous epithelial cells (i.e. a rich non-squamous epithelial cell fraction considered to originate from the lower respiratory tract, hereafter referred to as 'plugs') were collected and treated with dithiothreitol (DTT (Sputalysin); Calbiochem Corp., San Diego, CA, USA). The cell suspension was filtered through a 52 μ m nylon gauze, and the effect of DTT was stopped by diluting the suspension with phosphate-buffered solution to a volume equal to the sputum plus DTT. After centrifugation, the supernatants were resuspended and cytospinned (Shandon Southern Instruments, Sewickley, PA, USA), and the slides were stained with Giemsa.

Sputum was considered eosinophilic if at least 2.7% of the sputum cells were eosinophils, and neutrophilic if at least 65% of cells were neutrophils.

2.6. EBC collection

EBC was collected by a Turbo-Deccs device (Medivac, Parma, Italy) according to the manufacturer's instructions [32]. Briefly, the subjects were asked to breathe into the collecting system for 5 min under conditions of normal tidal volume and without a noseclip. All of the samples were stored at -80 °C until analysis. All EBC collections were performed in an environment with temperature (22–23 °C) and humidity (50%) controlled by a closed air conditioning system.

2.7. Ultrafine particle measurement

The particle size-number distribution of PM_{0.1} was assessed in the EBC samples with the NanoSight LM20 system (Nanosight Ltd., Salisbury) using the Nanoparticle Tracking Analysis (NTA) method of visualizing and analyzing particles in liquids that relates the rate of Brownian motion to particle size. The rate of movement is related only to the viscosity of the liquid, the temperature and the size of the particle, and is not influenced by particle density or refractive index. The particles contained in the sample can be visualized by virtue of the light they scatter when illuminated by a laser light source. The technique retrieves samples

from a volume of 120 × 80 × 20 microns. Sample concentration is adjusted (10⁷–10⁹ particles/ml) such that a significant number of particles is present in the beam path. Approximately 0.3 ml of EBC was introduced into the viewing unit using a disposable syringe. Three videos of 30 s duration were recorded and analyzed (NTA software version 2.0) for each sample. Total particle count, percent of particles that were in the nano-sized range, and total nano-sized particle counts were recorded.

2.8. Statistical analysis

Descriptive statistics are given as means and standard deviations for continuous variables and frequency distribution for categorical variables. The Chi-square or Fisher's exact tests were used in order to examine the difference between groups regarding categorical variables, whereas the two-sample t-test was used to compare groups for continuous variables and the Mann Whitney test for ordinal variables. Spearman and Pearson correlation coefficients were calculated to examine correlations between variables. A multiple regression model was applied in order to assess which parameters were independently associated with nanoparticle levels in EBC. Variables included in the model were: sex, body mass index, exposure to cigarette smoke, length of daily exposure to outside ambient air, incidence of wheezing, a composite score of breath symptoms, and FEV1. The three model building methods were forced entry, forward selection and backward elimination. All statistical analyses were performed using SAS for Windows 9.2. (Cary, NC, USA).

3. Results

Fifty-two children (mean age 12.46 ± 3.7 years, range 8–16 years) were included in the study. Their clinical and demographic characteristics are shown in table 1. Symptoms for which patients were referred were cough ($n = 44$), dyspnea ($n = 37$), wheezing episodes ($n = 36$), exercise-induced dyspnea ($n = 32$) and nocturnal cough ($n = 30$).

All the children underwent FEV1 assessment and the values were low in 12 (23%) of them. Thirty-five children underwent bronchial provocation challenges and the results were positive in 25 (78%) of them. All children underwent EBC UFP assessments and the counts were not significantly different between the participants with positive and negative bronchial challenge results. IS samples were collected from 17 participants. According to the above-mentioned parameters 37/52 (71%) study children were asthmatic. FEV1 and MMF 25–75 were significantly lower in the asthmatic group compared to the non-asthmatic group ($p < 0.03$). The respective eosinophil counts were 10.9% versus 0, the neutrophils were 47.1 versus 16.5 and the FeNO was 64.6 versus 33.5 (table 2(a)). The UFP counts were higher in the EBC samples from the asthmatic participants compared to the non-asthmatic ones but

Table 1. Clinical and demographic parameters of the study population ($n = 52$).

Sex, n (%)	
Male	33 (63.4)
Female	19 (36.6)
Age, years (mean $\pm$ STD)	12.46 $\pm$ 3.7
Height, cm (mean $\pm$ STD)	156.16 $\pm$ 18.7
Weight, kg (mean $\pm$ STD)	50.26 $\pm$ 18.9
Body mass index (mean $\pm$ STD)	19.76 $\pm$ 3.89
Preterm delivery, n (%)	4 (8)
Low birth weight, n (%)	7 (13.8)
Positive history of atopy, n (%)	
Skin	17 (32.6)
Rhinitis	31 (62)
Conjunctivitis	18 (35.3)
Family history	20 (39.2)
Positive parental smoking, n (%)	13 (25.5)
Positive smoking during gestation	7 (13.7)
Hours spent out of the house	8.55 (2.09)
Mean number of children in the family, n (%)	2.33 $\pm$ 0.88
Pets at home, n (%)	20 (39.2)
Clinical parameters, n (%)	
Wheeze ^a	36 (70.6)
Cough	44 (88)
Dyspnea, n (%)	37 (72.5)
Exercise-induced dyspnea ^b	32 (64)
Nocturnal symptoms	30 (58.8)
Controller medication use	34 (65.3)
Inhaled steroid use	23 (46)
Breath severity score ^c (mean $\pm$ STD)	7.23 $\pm$ 3.57
Atopic severity score (mean $\pm$ STD)	4.05 $\pm$ 2.95

^a Wheeze severity on a scale of 0–3.

^b Dyspnea severity on a scale of 0–3.

^c Breath score is the sum of all breath symptom scores on a scale of 0–18.

this did not reach a level of significance (table 2(b)). The percent UFP was significantly higher in the girls than in the boys ($p < 0.001$) (table 2(c)).

The univariate analysis revealed significant correlations between the UFP parameters and the respiratory symptom and inflammation parameters. Specifically, total EBC particle count and percent of particles in the UFP range correlated with the incidence of wheeze, high breath symptom score, and sputum eosinophilia. Shortness of breath was correlated with percent of particles in the UFP range and with the EBC UFP count. FEV1 and FeNO were not correlated to the UFP parameters (table 3). The correlation between sputum eosinophilia and nanoparticle concentrations is depicted in figure 1.

These correlations were further examined in a multivariate regression model. The parameters entered into the model were: sex, body mass index, passive smoking, length of daily exposure to outside ambient air, severity of wheeze, a composite score of breath symptoms and FEV1. After adjustment for these variables, female sex and severity of wheeze were significantly associated with a higher UFP content.

The results of the regression model are presented in table 4.

4. Discussion

The results of the present study demonstrated that the load of exhaled UFP in EBC samples of asthmatic children is correlated to wheezing, dyspnea and breath score severity as well as with IS eosinophilia. To the best of our knowledge, this is the first study to measure UFP in the EBC of asthmatic children, thus providing novel and vitally important information, since UFP are more proinflammatory and thus more harmful in their effect due to their higher oxidative composition compared to larger dust particles [7]. Most of the data on the effect of UFP pollution on the airways had been collected from studies in which PM was measured by environmental monitoring, but none had estimated the internal personal-level burden by means of biological monitoring [2–4]. We measured here the very small fractions of PM matter directly in an EBC sample from each child (i.e. biological monitoring).

We did not find any difference between the content of UFP between the groups. This was in complete correlation with our previous study on asthmatic children in the Tel-Aviv area in which we showed that the content of particles in the micro range size in IS did not differ between groups as well [33]. In this context, given that PFT are not good predictors of disease, there is a need for more sensitive biomarkers. This was clearly apparent in children in the Tel-Aviv area in whom >80% of particles out of the total PM accumulated in the airways had a diameter between 0–2.5 μm , displaying the highest OR 10.7 (CI = 2.052–56.4 $p = 0.005$) for an existing eosinophilic inflammation [33]. That study, however, was performed in IS samples and we had limited the assessment to PM in the micro-sized range. Collecting sputum can be challenging in children [28], while EBC is a simple, inexpensive and noninvasive technique which can be performed on children as young as 6 years of age [34]. Only 17/52 succeeded in producing sputum, but EBC was obtained from them all, the youngest of which was 6 years of age.

EBC has been widely proposed to assess and to bio-monitor airways inflammation in children [35, 36]. However, there are several drawbacks to its use, among them the lack of validated normal reference values and some methodological problems like lack of full standardization of EBC markers. In contrast to those studies that mainly measured volatile and non-volatile components in EBC fluid, we now investigated the PM content in the EBC sample. Other studies had also looked at the effect of PM on airways inflammation. One of them dealt with domestic airborne black carbon levels and 8-isoprostane in EBC among children in New York City and showed that those particle fractions activate oxidative stress and that their measurement can be used as a biomarker for airway inflammation

Table 2. (a) Functional and inflammatory parameters. (b) Mean ultrafine particle load in children with asthma. (c) Mean ultrafine particle load in boys and girls.

(a)			
Functional parameters (mean $\pm$ SD)	Asthmatic $n = 37$	Non-Asthmatic $n = 15$	p value
FVC (%)	84.9 $\pm$ 21.2	95.4 $\pm$ 15.9	0.06
FEV1 (%)	85.3 $\pm$ 15.5	95.6 $\pm$ 15	0.03
FEV1/FVC (%)	98.2 $\pm$ 13.5	99.3 $\pm$ 11.1	0.77
Small Airways (MMF25-75)	68.8 $\pm$ 27.2	86.9 $\pm$ 23.5	0.03
Inflammatory Parameters (mean $\pm$ SD)	$n = 16$	$n = 1$	
Eosinophils (%)	10.9 $\pm$ 73	0	—
Neutrophils (%)	47.1 $\pm$ 25.5	16.5	—
FENO (ppb)	$n = 36$	$n = 6$	0.13
	64.6 $\pm$ 73	33.5 $\pm$ 27.2	
(b)			
Parameters measured by nanosight	Asthmatic $n = 37$	Non-asthmatic $n = 15$	p value
Total EBC UFP particle content, mean $\pm$ SD ($10^8/\text{ml}$) ^a	2.92 $\pm$ 4.3	2.7 $\pm$ 2.95	0.83
Percent of EBC particles in UFP range, mean % $\pm$ SD	15.32 $\pm$ 2.8	16.23 $\pm$ 4.3	0.86
(c)			
Parameters measured by nanosight	Boys $n = 33$	Girls $n = 19$	p value
Total EBC particle content, mean $\pm$ SD (10^8 ml^{-1}) ^b	3.2 $\pm$ 3.1	2.3 $\pm$ 5.2	0.51
Percent of EBC particles in UFP range size, mean % $\pm$ SD	7 $\pm$ 9.5	20 $\pm$ 18.4	0.001

^a In 0.3 ml of EBC as described in section 2.

^b In 0.3 ml of EBC as described in section 2.

FVC—forced vital capacity, FEV1—flow expiratory volume in one second, MMF 25–75—maximum mid expiratory flow between 25% and 75% of the FVC, FENO—fractional exhaled nitric oxide, ppb—parts per billion.

The T -test was used to compare groups: $p \leq 0.05$ was considered significant.

EBC, exhaled breath condensate; UFP, ultrafine particles.

Note: the T -test was used to compare groups: $p \leq 0.05$ was considered significant.

EBC, exhaled breath condensate; UFP, ultrafine particles.

The T -test was used to compare groups p value ≤ 0.05 was considered significant.

Table 3. Relationships between ultrafine particle load and clinical parameters.

Parameters measured by NANOSIGHT	Wheezing ^a	Dyspnea ^b	Breath score ^c	FEV1	FeNO	Percent of sputum eosinophils ^d
Number	51	51	52	52	26	17
Total EBC UFP particle content (10^8 ml^{-1})						
R	0.28	0.24	0.30	−0.18	−0.25	0.64
p value	0.04	0.09	0.03	0.19	0.21	0.005
Percent of EBC particles in UFP range (%)						
R	0.36	0.34	0.33 0.02	−0.21 0.13	−0.01 0.97	0.72
p value	0.007	0.01	0.33 0.02	−0.21 0.13	−0.01 0.97	0.001

^a Wheezing severity on a scale of 0–3.

^b Dyspnea severity on a scale of 0–3.

^c Breath score: the sum of all breath symptom scores on a scale of 0–18.

^d Percent of total sputum cell count.

R = Spearman correlation coefficients: $p \leq 0.05$ was considered significant.

FEV1, flow expiratory volume in 1 s, FeNO, fractional exhaled nitric oxide (parts per billion), EBC, exhaled breath condensate.

[37]. Similar data emerged from a study on primary school children in an urban area in Antwerp [38]. Both of those studies, however, involved exogenous black carbon combustion-derived particles mainly around $2.5 \mu\text{m}$ in size and taken from sampling air either in the

child's home (the NY study) or by monitoring stations located ~2–3 km away from the two schools which the children attended (the Belgian study). Notably, measurements derived by environmental monitoring neither consider nor are correlated to individual exposure

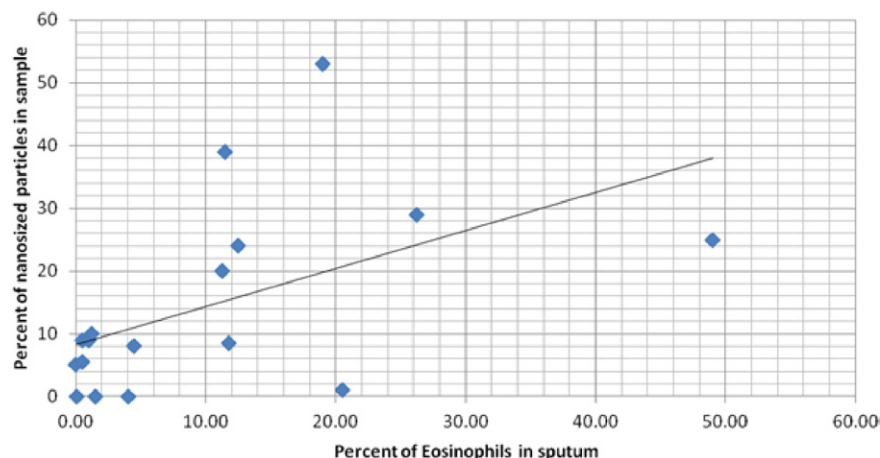


Figure 1. Percent of EBC particles in the UFP range (%) sputum eosinophils (%). There is a positive correlation between the percent of eosinophils in sputum cells and the percent of EBC particles in the UFP range. By Spearman Correlation $r = 0.56$, $p = 0.02$. There is a similar correlation for the percent of eosinophils and total UFP count in EBC samples.

levels that take in account many factors, such as age, respiratory rate, outdoor activity, and housing effect exposure levels [33]. Moreover, those studies evaluated PM that are not ultrafine in size (i.e. >100 nm). Indeed, the ultrafine fraction was shown to escape clearance by alveolar macrophages resulting at long interaction with epithelium and therefore induction of epithelium injury and influx of inflammatory cells (i.e. neutrophils and eosinophils) [39].

In this context, we found a positive correlation between percent of EBC particles in UFP range size (%) and IS eosinophils (%). These results are in agreement with our previous studies that showed high and significant odds ratios for an association between the accumulation of particles and the severity of eosinophilic inflammation [33]. Suppression of eosinophil infiltration in clinical disease by glucocorticoids is usually associated with the amelioration of symptoms and disordered airway function [40], and implementing a treatment strategy directed towards normalizing the eosinophil level in IS reportedly reduced exacerbations of asthma [41].

We did not find any correlation between functional parameters and the UFP content in the total EBC, but we did find a significant correlation between UFP content and clinical symptoms, wheezing, dyspnea and breath score. Moreover, the UFP content in EBC levels significantly predicted severity of wheeze in a multivariate analysis model. Although the severity of wheezing was only borderline in our model, wheezing had been found to be an outstanding symptom as a final predictor in a very recent study that showed that VOC biomarkers in EBC together with gene expression and early PFT can predict and perhaps follow-up wheezing in preschool asthmatic children [42].

These results are in correlation with other studies that had shown that while exhaled H_2O_2 concentrations in the EBC of children with acute asthma exacerbation are significantly elevated, the H_2O_2 levels in the short-term follow-up remain at high levels

Table 4. Summary of multivariate analysis model to predict levels of EBC UFP.

Parameter estimates for total EBC UFP particle content			
Variable	Parameter estimate	<i>t</i> Value	<i>p</i>
FEV1 (%)	0.00555	0.67	0.5044
Age (years)	0.03331	0.68	0.5040
BMI	-0.00899	-0.23	0.8206
Sex (F/M)	0.56002	2.17	0.0368
Exposure to cigarette smoke (yes/no)	0.28981	0.99	0.3265
Outside time ^a (h)	0.04835	0.86	0.3962
Severity of wheeze ^b	0.32054	1.98	0.0555
Breath symptom score ^c	-0.08605	-1.55	0.1300
Atopic score ^d	0.42892	1.48	0.1476

^a Outside time: length of time spent out of doors.

^b Severity of wheeze on a scale of 0–3.

^c Breath score: the sum of all breath symptom scores on a scale of 0–18.

^d Positive history of atopy: skin, rhinitis, conjunctivitis, family history.

UFP, ultrafine particles; EBC, exhaled breath condensate; FEV1, low expiratory volume in 1200 s; BMI, body mass index.

Note: data are presented as regression parameter estimates, *t* and *p* values.

with no correlation to lung function [43]. Finally, De Prins *et al* [44] reported that exhaled NO and nasal tryptase are associated only with clinical symptoms, such as wheeze, rhinitis and nasal allergy in primary school children. Taken together, these data support the hypothesis that inflammation precedes functional changes.

Lung function measurements are used to monitor disease activity but test results are not closely related to inflammatory processes which may well precede changes in lung function. IS analyses in patients with symptoms suggestive of asthma and signs of eosinophilic airway inflammation had normal lung func-

tion test results and responded favorably to inhaled beclomethasone dipropionate treatment [45]. Moreover, eosinophils, T cells, mast cells, and interleukin-5 levels were significantly elevated in the airway mucosa of asthmatic subjects in remission [46].

The percent of UFP particles in EBC samples of the girls in our study was significantly higher compared to the boys (20% versus 7%, $p = 0.001$). We assume that several factors can predict levels of UFP in the EBC, and this was demonstrated in the multivariate analysis model. Gender and wheezing significantly predicted levels of UFP in the EBC samples. These results are in agreement with those of previous studies that showed a differential deposition of particles in airways according to gender due to differences in the anatomy of airways [47]. Jaques *et al* [48] measured a total lung deposition of inhaled UFP in healthy men and woman and demonstrated a clear gender difference. Moreover, they found that the total number of inhaled (Ni) and exhaled (Ne) UFP varied according to six different breathing patterns [49]. We had also observed that 'parent-reported wheeze' is an abnormal type of breath sound [50]. In our current study, the extent of wheezing reach a borderline significant value that should be confirmed in a larger population.

The first limitation of the present study is the lack of information about the nature of particles measured (i.e. nanomaterials, diesel engine emissions, natural component). The second limitation is the lack of knowledge regarding the origin of the particles. There are several studies that looked at 'endogenous' particles in the EBC of smokers [51] and occupational expose workers [52] as a biomarker of inflammatory changes in airway disease that should reflect alterations in the respiratory tract lining fluid, whose main constituents are lipids and proteins. Those studies measured the particles exhaled on an impactor filter for coarse (PM10) particles. For this range of particles we have clearly demonstrate in previous occupational set-ups using induced sputum samples that the origin of the particles are 'exogenous' [19, 23]. Now, using EBC samples, we believe that we are also measuring exogenous UFP that first deposit on the fluid of the respiratory tract lining and then migrate to inner compartments that are sampled by IS samples. This migration had been demonstrated in animal models [53].

5. Conclusions

To the best of our knowledge, this is the first report that high EBC UFP content is associated with severity of wheezing, sputum eosinophilia and female sex among children. We provide evidence to substantiate the feasibility of measuring UFP in the EBC of asthmatic children as a potential method for screening and biological monitoring in a pediatric population. This and the reproducibility of the detecting markers should be confirmed in future studies with a larger cohort of children.

Acknowledgment

This work was supported by the Environment and Health Fund. Esther Eshkol is thanked for editorial assistance.

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