Association between exhaled breath condensate nitrate + nitrite levels with ambient coarse particle exposure in subjects with airways disease


INTRODUCTION

Exposure to air pollution is associated with deaths from and hospital admissions for chronic respiratory and cardiac disease. The most likely mechanism for these effects is the induction by inhaled particles, particularly in the PM2.5 fraction (particles smaller than 2.5 μm), of lung inflammation involving oxidative stress. However, direct evidence for changes in inflammatory state within the lung in epidemiological studies is scarce as measurement of oxidative stress in the lung in many individuals at multiple locations and times is logistically challenging. Assessment of airway inflammation is now relatively easy using non-invasive means such as induced sputum or exhaled breath condensate (EBC), although neither has been assessed in epidemiological studies. Of the two, EBC collection is theoretically easier to undertake in field studies and allows measurement of pH, ions and small molecules such as glutathione, nitrate and nitrite (markers of oxidative stress) as well as larger molecules such as proteases putatively involved in the inflammatory process (e.g., 8-isoprostane and interleukins). However, the laboratory-based EBC apparatus does not lend itself to epidemiological field studies, while a simpler method, the R-tube, is limited by the need for a freezer to keep the sample cool. EBC NOx (total nitrate + nitrite) is suggested as a reliable marker due to its stability and close correlation with other common markers of oxidative stress.

What this paper adds

- Short-term increases in particulate matter air pollution have been associated with respiratory health.
- Oxidative stress and inflammation may form part of the mechanism of the cardiorespiratory effects of particulate matter air pollution, but the direct evidence in epidemiological studies is scarce.
- We evaluated the association between particle number, fine and coarse particle mass and EBC nitrate and nitrite—a marker of oxidative stress—in adult patients with asthma or chronic obstructive pulmonary disease in four European cities.
- The coarse particle concentration at a central site was significantly associated with increased nitrate and nitrite concentrations in EBC. No associations with other particle metrics were found.
- Our findings add to evidence that coarse particles have health effects that air pollution policies should take into account.
The RUPIOH (Relationship between Ultrafine and fine Particulate matter in Indoor and Outdoor air and respiratory Health) study is an EU-funded study designed to explore the distribution of various particle metrics both indoors and outdoors in conjunction with clinical parameters in patients with asthma or chronic obstructive pulmonary disease (COPD). It also aimed to assess the relationship between exposures for a range of particle metrics and EBC NOx, a marker of oxidative stress. The main findings from the exposure measurements imply that while measurements at a central site reflect temporal changes in particle metrics outside specific homes, they may characterise indoor exposure to ambient particles less well for ultrafine particles than for fine particle mass.11–14 No exposure metric was associated with any index of lung function.13 This does not exclude the possibility of significant inflammatory change in the airways due to pollutant exposure. Which particle metric is best associated with health or physiological end point has been a matter of much discussion, although the fine fraction seems to be where toxicity most resides and there is some evidence that particle numbers (a surrogate of particle surface area) may be a better indicator of health impact than mass.15

In the RUPIOH study, a validated simple method was used for collecting EBC in the field to determine whether glutathione and NOx levels in EBC were associated with exposure to particles both indoors and outdoors.16 A second goal was to evaluate the feasibility of using these markers of exposure to oxidant stress in epidemiological studies.

**MATERIALS AND METHODS**

**Study design**

The study was conducted from October 2002 to March 2004 in four European cities: Helsinki (Finland), Athens (Greece), Amsterdam (the Netherlands) and Birmingham (UK). The full methodology has been described elsewhere.11–14 During the whole study period, a reference site at an urban background location in each city was used to monitor particle mass concentration and particle number concentration (PNC). At various locations (urban and suburban) covering the participating cities, homes of subjects on streets of both low and high traffic density with either asthma or COPD were selected for the study. Air pollution monitoring was done for 1 week in these homes both indoors (living room) and outdoors (garden or balcony). During this week, respiratory health was characterised by spirometry, a symptom diary and EBC collection. EBC was collected three times during that week in each subject. This paper focuses on collection of EBC and levels in EBC of NOx and glutathione in relation to pollutant exposure.

**Study population**

Subjects were aged 35 years or older, had a doctor diagnosis of chronic respiratory disease (asthma, COPD, as defined by Global Initiative for Asthma17 or Global Initiative for Chronic Obstructive Lung Disease18 criteria) and had experienced respiratory symptoms in the past 12 months. Those patients who had not received a definite diagnosis of asthma or COPD (especially in the Netherlands) were classified as non-specific chronic lung disease.19 Because of difficulties to recruit a sufficient number of subjects (especially in the Netherlands, some subjects without lung disease were included. Patients with severe disease, defined as use of bronchodilating reliever medications more than three times a day, nebulised bronchodilators or long-term oxygen treatment, were excluded. Preference was given to non-working subjects to eliminate potential confounding by occupational exposures and to obtain a closer approximation of personal exposures in the indoor home environment. Subjects were non-current smokers living in a household without a smoker. There was no criterion for years since quitting smoking. Past smokers quittd smoking on average 19.5 years before recruitment. Two subjects quittd in the year before recruitment. Details of treatment were obtained for each subject, in particular dose of inhaled corticosteroid (ICS).

**Ethical review**

Written informed consent was obtained from each subject. The study was approved by local medical ethics committees in all centres before the start of the fieldwork.

**Exposure assessment**

Airborne concentrations of particles smaller than 10 µm (PM10), smaller than 2.5 µm (PM2.5), coarse particles (PMcoarse) and total PNCs were measured at a central monitoring site for each centre during the entire approximately 18 months study period and both outside and inside each subject’s home. The central urban background site measurements were continuous for the duration of the project and were selected to avoid local sources of air pollution. The measurements in and near the subjects’ homes were performed for the week of observation for each individual. Each individual was visited three times in that week. Harvard impactors were used for sampling of PM10 and PM2.5 (set at a flow rate of 10 litres/min) as integrated 24-h averages from noon to noon using timers. Noon to noon samples were taken to increase efficiency of obtaining seven daily samples given limited equipment. The coarse fraction was taken as the difference between PM10 and PM2.5. For indoor sampling, silent pumps specifically designed for home measurements were used. TSI model 5022A Condensation Particle Counters (TSI Incorporated, Shoreview, Minnesota, USA) were used to obtain total PNCs for all particles >7 nm in diameter. Continuous measurements (PNC) were averaged to the same noon to noon time intervals.11 12 14 There were 11%, 14% and 10% observations with missing exposure measurements for the indoor, outdoor and central site, respectively. Traffic count data obtained from the municipality were used to classify a home as a major road (>10,000 vehicles per day).11

**EBC collection**

EBC samples were collected using a validated system developed in Birmingham.16 This comprised two Teflon® tubes of 7.9 mm diameter (Du Pont, Wilmington, Delaware, USA) submerged in a container of ice/ice packs of 8 cubic litre capacity. Subjects were asked to breathe continuously through a mouthpiece via a two-way non-rebreathing valve (Intersurgical Ltd, Berkshire, England) attached to the tubes while wearing a nose clip for 15 min. At the end of the sampling period, samples were tipped from the end of the tubes into sample tubes, which were kept on dry ice until being returned to the local laboratory where they were kept at −70°C for later central testing in Birmingham as a single batch. All collections were made under direct supervision. This method produces an average volume of 1.5 ml of condensate in laboratory studies.16 Saliva contamination is potentially a problem. We addressed this by discarding those that were clearly contaminated on visual inspection (frothy, opaque appearance with more viscous properties).

**Glutathione and NOx measurement**

**Total glutathione**

Total glutathione was determined by use of a validated assay kit (Trevigen Inc®, Gaithersburg, Maryland, USA).16 20 This used an enzymatic recycling method, employing glutathione reductase,
for the quantification of total glutathione. Measurement of absorbance at 405 nm was completed using a spectrophotometric plate reader (Labsystems Multiskan MS, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). The standard limit of detection of the glutathione assay was >3.12 picomoles/50 µL. Following repeatability experiments and further assay development in our own laboratory, a limit of detection of >1.56 picomoles/50 µL was established. Samples were thoroughly defrosted and mixed using a vortex mixer prior to analysis.

### Total nitrate + nitrite (NOx)

Nitrate and nitrite combined is referred to here as NOx as in previous studies,21 but this is not to be confused with NOx in ambient air (NO2 plus NO). EBC NOx was determined as a coloured azo-dye product of the Griess reaction, using a spectrophotometric plate reader (Labsystems Multiskan MS) at 540 nm. The concentration of total NOx is indirectly measured by determination of nitrate (NO3−) and nitrite (NO2−) in EBC. The concentration of total NOx is indirectly measured by determination of nitrate and nitrite in EBC. The concentration of total NOx is indirectly measured by determination of nitrate and nitrite in EBC. The concentration of total NOx is indirectly measured by determination of nitrate and nitrite in EBC. The concentration of total NOx is indirectly measured by determination of nitrate and nitrite in EBC.

#### Questionnaire and lung function data

Respiratory symptoms, medication use and time spent outdoors were recorded three times daily in a diary for the study week. ICS dose was expressed as the daily dose in microgram equivalents of beclometasone, taking fluticasone as twice as potent as beclometasone and budesonide as equipotent. ICS dose data were split into steroid naive (0 g), low-dose (1–999 µg) and high-dose (>999 µg) groups, using the median as the cut-off point to define high and low dose. Exposure to indoor sources of pollution (eg, cooking, smoking) during sampling was obtained from a time—activity diary kept by participants during the 1-week measurement period with a 30 min resolution. Lung function was measured three times daily using a home spirometer.13

#### Statistical analysis

Associations between air pollution exposure and EBC NOx were assessed by linear regression. All analyses were performed with data for all cities combined, as the number of observations per city was limited. Our main model adjusted for city, outdoor temperature and season. An indicator variable for city was included in the regression model to adjust for potential systematic differences in EBC NOx between cities. Outdoor temperature on the day of EBC collection was included as a potential confounder. Outdoor temperature data were collected from the nearest station of the respective meteorological offices. We additionally adjusted for season, defined as the four seasons corresponding to the meteorological seasons, for example, December, January and February were defined as winter. To account for repeated measurements within subjects, a random intercept approach was used.

We additionally investigated a model having city-specific terms for season and temperature, by including interaction terms of city and these variables. Instead of indicator variables for season, we also included trend terms (linear, quadratic, cubic). Finally, we performed an analysis adding temperature squared to the model to accommodate potential non-linear temperature effects.

EBC NOx data were transformed using a log10 function due to deviations from the normality assumption. We assessed the effects of same day (lag 0, from yesterday noon to today noon) and previous days’ (lag 1–2 days) air pollution. Effect estimates were calculated for an increase of 10 µg/m3 PM10, 10 µg/m3 PM2.5, 10,000 particles per cm3 PNC and 10 µg/m3 coarse particles. We assessed effect modification by disease classification, inhaled steroid usage, sex, age, season, city, time spent outside the home (continuous) and traffic near the home (major road yes/no) using an interaction term of the exposure variable and the effect modifier. Analysis of the air pollution effects was done using mixed-effects models (PROC MIXED) in SAS (V9.2, SAS Institute Inc.). Analysis of the association between subject average EBC NOx and subject characteristics (eg, disease status) was performed with linear regression.

### RESULTS

#### Subject characteristics

EBC was collected in 147 individuals. Data from 14 subjects were excluded from analysis as samples were contaminated with saliva. One hundred and thirty-three individuals remained for EBC analysis, of which 111 subjects with more than one valid

#### Table 1 Patient characteristics: baseline data for all subjects and for individual cities

<table>
<thead>
<tr>
<th>City</th>
<th>No. of subjects</th>
<th>Disease status, N (%)</th>
<th>Sex (F:M)</th>
<th>Age (years)</th>
<th>Pack-years smoked</th>
<th>Inhaled steroids daily dose, N (%)</th>
<th>ICS dose (µg)</th>
<th>FEV1 (% predicted)</th>
<th>FEV1/FVC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>All centres</td>
<td>111</td>
<td>A=63 (57)</td>
<td>77.24</td>
<td>62.3±10.6</td>
<td>13.3±29.5</td>
<td>SN=25 (23)</td>
<td>795±743</td>
<td>80.6±27.7</td>
<td>76.6±11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=24 (22)</td>
<td></td>
<td>(35.7–84.7)</td>
<td>(0–192)</td>
<td>&lt;999=38 (35)</td>
<td>(0–4000)</td>
<td>(27.7–137.2)</td>
<td>(44–100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS=24 (22)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;999=45 (42)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helsinki</td>
<td>32</td>
<td>A=29 (91)</td>
<td>26.6</td>
<td>63.4±9.4</td>
<td>3.6±10.3</td>
<td>SN=2 (6)</td>
<td>850±514</td>
<td>81.8±21.5</td>
<td>77.3±12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=2 (6)</td>
<td></td>
<td>(36.2–84.7)</td>
<td>(0–41)</td>
<td>&lt;999=14 (44)</td>
<td>(0–2000)</td>
<td>(35.1–121.4)</td>
<td>(46–98)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS=1 (3)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;999=16 (50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Athens</td>
<td>24</td>
<td>A=10 (42)</td>
<td>10.14</td>
<td>64.2±12.5</td>
<td>27.9±40.8</td>
<td>SN=6 (28)</td>
<td>797.5±685</td>
<td>71.0±22.1</td>
<td>76.6±9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=12 (54)</td>
<td></td>
<td>(35.7–79.7)</td>
<td>(0–148)</td>
<td>&lt;999=9 (26)</td>
<td>(0–2000)</td>
<td>(27.7–104.3)</td>
<td>(50–90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS=1 (4)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;999=11 (48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amsterdam</td>
<td>37</td>
<td>A=8 (22)</td>
<td>25.12</td>
<td>62.4±9.6</td>
<td>16.3±35.0</td>
<td>SN=16 (44)</td>
<td>546.5±846.9</td>
<td>85.3±21.7</td>
<td>77.8±10.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=8 (22)</td>
<td></td>
<td>(44.5–77.3)</td>
<td>(0–192)</td>
<td>&lt;999=13 (36)</td>
<td>(0–4000)</td>
<td>(33.5–131.5)</td>
<td>(49–100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS=21 (57)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;999=7 (19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birmingham</td>
<td>18</td>
<td>A=16 (89)</td>
<td>16.2</td>
<td>57.8±11.2</td>
<td>5.3±10.6</td>
<td>SN=1 (6)</td>
<td>1205.5±785.5</td>
<td>82.0±30.8</td>
<td>77.4±10.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=1 (6)</td>
<td></td>
<td>(37.6–75.8)</td>
<td>(0–34)</td>
<td>&lt;999=5 (29)</td>
<td>(0–2000)</td>
<td>(28.9–137.2)</td>
<td>(49–97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS=1 (6)</td>
<td></td>
<td></td>
<td></td>
<td>&gt;999=11 (65)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented are mean ± SD, with minimum and maximum in parentheses unless otherwise indicated. FEV1 = forced expiratory volume in one second, comparing measured versus predicted, see reference.13

A, asthma; C, COPD; FVC, forced vital capacity; ICS, inhaled corticosteroid; NS, non-specific lung disease; SN, inhaled steroid naive.
EBC measurement (below) were included in the final data analysis (table 1). Sixty-three subjects were labelled as asthma and 24 as COPD. Twenty-four subjects were labelled as nonspecific chronic lung disease or were without chronic respiratory disease (25 from the Dutch centre). The 22 excluded subjects did not differ from the 111 subjects in distribution over the cities and disease status. For the main analysis, the three groups were analysed together.

Pollution data
Data for each subject’s home monitoring results and the central site information pertain to their individual study week. Full information on the particle measures and their spatial distribution have been published elsewhere, but the mean results for each metric are summarised in table 2. PM concentrations differed significantly between the cities, with the highest concentrations observed in Athens and the lowest in Helsinki. Concentrations also differed between central site, residential outdoor and indoor locations. Within each location, significant temporal variability of air pollution was present. There were low correlations between indoor and outdoor/central site measurements for coarse particles and PNC especially.

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**Environmental**

### Table 2 Mean particle levels indoors, outside the homes and at the central sites for the four centres separately and combined

<table>
<thead>
<tr>
<th>Indoor levels</th>
<th>Outdoor levels</th>
<th>Central site levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PM2.5</strong></td>
<td><strong>PM10</strong></td>
<td><strong>PMcoarse</strong></td>
</tr>
<tr>
<td>Helsinki</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6 ± 4.9</td>
<td>12.8 ± 6.4</td>
<td>5.3 ± 3.9</td>
</tr>
<tr>
<td>Athens</td>
<td>23.1 ± 9.7</td>
<td>36.3 ± 13.9</td>
</tr>
<tr>
<td>Amsterdam</td>
<td>17.2 ± 11.8</td>
<td>25.6 ± 14.5</td>
</tr>
<tr>
<td>Birmingham</td>
<td>14.0 ± 23.8</td>
<td>22.8 ± 25.3</td>
</tr>
<tr>
<td>All centres</td>
<td>15.3 ± 13.8</td>
<td>23.8 ± 17.0</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD. Mass metrics are in microgram per cubic metre. PNC = particle number count given as number of particles/cubic centimetre air. PM<sub>2.5</sub> and PM<sub>10</sub> are particles smaller than 10 μm and 2.5 μm, respectively. PM<sub>coarse</sub> is particles between 2.5 and 10 μm.

**EBC Nox**
From the 133 subjects, we had 310 valid NOx analyses available. EBC NOx was above the DL in 88.4% of samples tested (273/310). Values below the DL were set to 0.78 μmol/l (0.5 × DL). We had three valid observations per subject for 68 subjects, two valid observations per subject for 45 subjects and one valid observation per person for 20 subjects and two subjects with no valid observations. These 22 observations were excluded as we cannot analyse within subject temporal variability in relation to temporal variability of air pollution. The between-days coefficient of variation of the assay was 9% for concentrations near the population mean.

The mean value for the whole group (all cities) was 9.5 μmol/l (table 5). EBC NOx levels were statistically significantly different between the four cities, with the highest levels found in Athens and the lowest level in Amsterdam and Helsinki. For asthma and COPD (all cities), the mean levels were 9.6 and 11.2 μmol/l, respectively. Differences in EBC NOx between disease status were, however, statistically non-significant. In a regression model with both city and disease status, only city was a significant predictor of EBC NOx. We could not identify any significant predictor of baseline EBC, except city. Age, sex, disease, medication and smoking status (former−never), pack-years smoked, traffic near the home, educational level, lung function were not associated with EBC NOx and did not explain the higher values in Athens.

**EBC Nox and pollutant associations**
Associations between air pollutant levels and EBC NOx are shown in table 4. PNC and PM<sub>2.5</sub> concentrations were not related to EBC NOx for any of the evaluated locations and lags (0, 1, 2 days). The coarse particle concentrations measured at the central site and to some extent the home outdoor location was related to EBC NOx. The most significant association was found with a 1-day lag for central sites and 2-day lag for the home outdoor measurements.

**Effect modification**
An analysis limited to subjects with asthma and COPD showed very similar associations as in the main analysis, for example, for previous day (lag 1) coarse particles at the central site, the effect estimate was 13.8% (−0.3 to 29.9%). Interaction analyses showed no differences in the effect estimates for coarse particles between the three disease categories (table 5). There was a tendency towards more significant associations between coarse particle levels and EBC NOx in subjects that did not take inhaled steroid medication in particular in those with asthma (table 5). However, the differences between subgroups were not statistically significant. There were also no significant differences in effect estimates between city, season and above and below the median (3 b) values for time spent out of the home, traffic near the home (major road yes/no) and temperature on the day before the test.

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**Table 3** Exhaled breath condensate NOx levels (in micromoles/litre) for all cities and stratified by city and disease status

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean ± SD</th>
<th>Minimum - Maximum</th>
<th>N subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>9.5 ± 10.5</td>
<td>0.8 - 65.4</td>
<td>111</td>
</tr>
<tr>
<td><strong>By city</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helsinki</td>
<td>6.2 ± 7.6</td>
<td>0.8 - 44.4</td>
<td>32</td>
</tr>
<tr>
<td>Athens</td>
<td>17.7 ± 13.0</td>
<td>1.8 - 55.6</td>
<td>24</td>
</tr>
<tr>
<td>Amsterdam</td>
<td>6.7 ± 8.1</td>
<td>0.8 - 65.4</td>
<td>37</td>
</tr>
<tr>
<td>Birmingham</td>
<td>9.1 ± 9.3</td>
<td>0.8 - 43.7</td>
<td>18</td>
</tr>
<tr>
<td><strong>By disease status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>9.6 ± 10.7</td>
<td>0.8 - 55.6</td>
<td>63</td>
</tr>
<tr>
<td>COPD</td>
<td>11.2 ± 10.9</td>
<td>0.8 - 46.6</td>
<td>24</td>
</tr>
<tr>
<td>Rest</td>
<td>7.3 ± 9.4</td>
<td>0.8 - 65.4</td>
<td>24</td>
</tr>
</tbody>
</table>

Levels of NOx that were below the detection limit (DL) were set to 0.5 × the DL. Thus, all samples are included in the analysis.

* Differences statistically significant (p < 0.0001), t-test on slope linear regression analysis.
† Differences not significantly different, t-test on slope linear regression analysis.
This study also shows that measurement of EBC NOx in the
before such effects become marked enough to change air

Disease status Lag % change EBC NOx (95% CI) Inhaled steroid use (daily dose in micrograms) Lag % change EBC NOx (95% CI)

Asthma 0 6.6 (–7.7 to 23.2) Naive 0 27.6* (5.8 to 53.8)
COPD 0 12.2 (–11.6 to 42.4) ≤999 0 11.4 (–20.2 to 55.6)
NS 0 25.4 (–36.9 to 149.2) ≥999 0 4.3 (–14.8 to 27.8)

Asthma 1 10.5 (–6.1 to 30.1) Naive 1 34.1* (11.0 to 62.0)
COPD 1 20.9 (–7.6 to 58.1) ≤999 1 21.0 (–18.1 to 78.7)
NS 1 91.4# (–3.0 to 277.7) ≥999 1 12.5 (–13.6 to 46.5)

Asthma 2 1.1 (–13.3 to 18.0) Naive 2 14.6 (–5.9 to 39.5)
COPD 2 13.9 (–10.6 to 45.3) ≤999 2 –11.0 (–38.7 to 29.4)
NS 2 16.8 (–36.0 to 113.4) ≥999 2 18.0 (–5.0 to 46.5)

Disease classification (NS: non-specific lung disease) and inhaled steroid use (naive: no inhaled steroid). Associations are expressed as the percentage change of NOx in EBC for increments of 10 μg/m³. All models adjusted for city, season and temperature on day of the test. Lag 0 is effect of air pollution of the 24-h noon—noon period ending on the day of EBC collection.

#p<0.10; *p<0.05.

EBC, exhaled breath condensate.

DISCUSSION

This study has shown an association between measured exposure to ambient coarse particles at central sites and EBC NOx, a marker of oxidative stress. This was seen consistently across all four cities. There was no effect associated with PM_{2.5}, but effects from PM_{10} fell between those seen with the coarse and PM_{2.5} fractions. As we have already shown no association of lung function with any measure of PM in this study,^{13} this suggests that EBC NOx may reflect an effect of PM exposure before such effects become marked enough to change airflow. This study also shows that measurement of EBC NOx in the field has promise as a tool for assessing the impact of air pollution exposure in epidemiological studies, particularly following populations over time.

Coarse particle effect?

We have shown that, when considering the whole group, regardless of centre or diagnosis, PM_{coarse} at the central site was the strongest predictor for EBC NOx. The effect was shown across all cities, which strengthens the belief that this is a consistent rather than a chance finding. The consistency across the three evaluated lags also suggests a non-chance finding. The association was further robust to various adjustments. If this is truly a causal association, it might be expected that there would be an association between EBC NOx and more personal PM exposure measures, such as indoor PM or PM levels outside each home (whatever the size fraction). We did find a significant association with home outdoor coarse particles, though not stronger than with central site PM. We did not find any association with coarse particles in indoor air, where people spend a large fraction of their time. This suggests that the observed association with central site coarse particles is either a chance finding or that there is something different about the coarse particles at the central site either in terms of toxicity or that the

Sensitivity analysis

Effect estimates were similar when city-specific temperature and season effects were included. The estimate for coarse particle (central site, lag 1) was 19.4% (95% CI 4.6 to 36.8) and 18.9% (95% CI 4.0 to 35.9) when season and temperature were specified as city-specific, respectively. Model fit (Akaike’s Information criterion) was worse for models with city-specific terms. Models that included cubic trend terms instead of the season indicators also showed similar effects. For coarse particles, central site lag 1, the effect estimate was 18.8% (95% CI 4.8 to 34.7).

Table 4 Associations between coarse particles at the central sites of same day and two previous days and EBC NOx in subgroups defined by disease classification and inhaled steroid use for all cities combined

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Location</th>
<th>% change EBC NOx (95% CI)</th>
<th>% change EBC NOx (95% CI)</th>
<th>% change EBC NOx (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM_{2.5}</td>
<td>Central</td>
<td>–0.7 (–9.7 to 9.2)</td>
<td>1.0 (–8.0 to 10.8)</td>
<td>–0.9 (–11.3 to 10.7)</td>
</tr>
<tr>
<td></td>
<td>Outdoor</td>
<td>–0.8 (–9.3 to 8.5)</td>
<td>0.5 (–8.5 to 10.5)</td>
<td>2.0 (–7.5 to 12.6)</td>
</tr>
<tr>
<td></td>
<td>Indoor</td>
<td>0.8 (–8.1 to 10.6)</td>
<td>–2.7 (–12.3 to 8.1)</td>
<td>–4.6 (–18.8 to 12.1)</td>
</tr>
<tr>
<td>PM_{10}</td>
<td>Central</td>
<td>2.2 (–4.7 to 9.5)</td>
<td>4.7 (–2.8 to 12.6)</td>
<td>6.2 (–1.7 to 14.7)</td>
</tr>
<tr>
<td></td>
<td>Outdoor</td>
<td>1.7 (–4.4 to 8.1)</td>
<td>2.9 (–3.9 to 10.3)</td>
<td>5.0 (–1.8 to 12.2)</td>
</tr>
<tr>
<td></td>
<td>Indoor</td>
<td>0.9 (–6.8 to 9.4)</td>
<td>–0.4 (–8.6 to 8.6)</td>
<td>–8.3 (–18.1 to 2.6)</td>
</tr>
<tr>
<td>Coarse</td>
<td>Central</td>
<td>11.7# (–0.8 to 25.8)</td>
<td>20.4* (6.1 to 36.6)</td>
<td>11.5# (–0.9 to 25.5)</td>
</tr>
<tr>
<td></td>
<td>Outdoor</td>
<td>5.8 (–4.9 to 17.6)</td>
<td>6.9 (–6.0 to 21.6)</td>
<td>13.3* (5.0 to 27.8)</td>
</tr>
<tr>
<td></td>
<td>Indoor</td>
<td>–0.3 (–18.6 to 22.1)</td>
<td>7.1 (–9.2 to 26.3)</td>
<td>–16.0# (–30.7 to 1.8)</td>
</tr>
<tr>
<td>PNC</td>
<td>Central</td>
<td>–4.3 (–17.7 to 11.1)</td>
<td>–5.1 (–17.9 to 9.8)</td>
<td>–14.0# (–26.6 to 0.8)</td>
</tr>
<tr>
<td></td>
<td>Outdoor</td>
<td>2.9 (–8.6 to 15.7)</td>
<td>–4.3 (–16.6 to 9.8)</td>
<td>–6.1 (–17.7 to 7.1)</td>
</tr>
<tr>
<td></td>
<td>Indoor</td>
<td>–1.8 (–8.4 to 5.4)</td>
<td>3.6 (–5.7 to 13.8)</td>
<td>–6.2 (–14.1 to 2.4)</td>
</tr>
</tbody>
</table>

Associations are expressed as the percentage change of NOx in EBC for increments of 10 μg/m³ for PM_{2.5}, PM_{10} and PM_{coarse}. 10 000 p/cc for PNC. All models adjusted for city, season and temperature on day of the test. Lag 0 is the effect of air pollution for the 24-h noon—noon period ending on the day of EBC collection.

#p<0.10; *p<0.05.

EBC, exhaled breath condensate.
mix centrally reflects better overall exposure for these individuals. Various studies have documented differences in chemical composition between particles generated indoors and outdoor (Brunekreef et al22 and references therein). This emphasises the need to collect information on the chemical composition and surface characteristics of indoor and outdoor coarse particles. It is likely that particles measured indoors were to an appreciable extent from indoor sources, though we did not formally separate particles from indoor and outdoor origin. Central site outdoor measurements may also better reflect total individual exposure because they characterise the whole area where the subject moves during the day including those particles that penetrate indoors. We did not find a difference in coarse particle association with EBC NOx between subjects spending <3 h away from home (the median) and those who spent more. However, we did not have information on where subjects spent their time out of home (indoors, outdoors). The consistency of the finding across all cities of the association with coarse particles and the apparent size gradation of effect by particle size at the central site may, however, lend support to this not being a chance finding, even though the potential mechanisms remain elusive. Another explanation for the lack of an indoor coarse particle effect is the smaller contrast in indoor coarse particles (table 2).

If this is a true association, it coheres with the recognised association of the coarse fraction with health impacts24 and in this context is likely to reflect not just vehicle emissions. Data from the UK32 suggest that the main components of coarse particles are non-exhaust particles from road traffic (eg, brake and tyre wear particles) sodium chloride and soil-derived particles. The average sampling height was 5.4 m above the ground surface, hence a suspended road dust contribution is likely. It has been suggested that the coarse fraction is associated more with respiratory than cardiac end points, and these data would cohere with that suggestion.

Previous studies on EBC markers

There are few epidemiological studies that have linked air pollution with markers of oxidative stress in EBC, most of them in children.36-39 Significant associations with various markers such as malondialdehyde and pH have been reported, but no study evaluated NOx and coarse particles.

There are a wide variety of inflammatory mediators that may be measured to monitor oxidative stress in airways disease. EBC NOx (total nitrate + nitrite) is suggested as a reliable marker due to its stability and close correlation with other common markers of oxidative stress, such as hydrogen peroxide, which is more challenging to measure due to its rapid breakdown and 8-isoprostanate.8-9 The measurement of more complex lipid- or protein-based molecules such as eicosanoids or cytokines is more challenging due to the low concentrations of these markers in EBC and the inconsistency in commercial assays. EBC NOx is a marker of oxidative/nitrosative stress, which correlates moderately with exhaled NO.10 EBC NOx measurements have been employed as a more stable end product of NO metabolism in the airways.

EBC NOx is an indicator of oxidative stress in the absence of changes in lung function previously reported11 and that use of EBC NOx should be considered in further population-based studies.

Differences of EBC between cities

The higher levels of EBC NOx in the Athens subjects are difficult to explain. Higher long-term exposure or different particle composition could be a factor, but an alternative explanation lies in differences in the selection of patients or other lifestyle factors. An analysis including lifestyle factors available from the baseline questionnaire (smoking status, pack-years smoked, educational level, age, sex), disease status, medication use and traffic density near the home did not explain the difference between the cities.

Strengths and limitations

The RUPIOH study is the largest field-based study of its kind to employ EBC as a method to assess oxidative stress in the context of indoor and outdoor air pollution, involving 153 subjects in four European cities with repeated measures of EBC over time. This method of EBC collection, developed for ease of use in the field, proved cheap and portable, suitable for use in a patient’s home, was easily adopted by different research groups and was well accepted. However, 18% of the total number of samples were contaminated with saliva, which is unacceptable high, but could be reduced by greater vigilance in the field. EBC NOx was detected in 98.4% of all samples with a range of values indicating that it has potential as a marker of oxidative stress in these types of study.

Questions have been raised about the measurement of NOx species in EBC. The full effect of EBC sample contamination with NOx species in the general laboratory environment (eg, glassware) or the collection device (eg, silicon/Teflon® or glass inner surface) has yet to be established. The ATS/ERS taskforce on EBC published recommendations to minimise the potential for such contamination, suggesting that all surfaces coming into contact with EBC during collection or analysis should be rinsed thoroughly with distilled/deionised water, as ambient NOx oxidises rapidly on surfaces increasing the chance of contamination.32 33 In this study, particular care was taken to minimise the likelihood of such contamination occurring, both during the collection and analysis of EBC. All collection tubes and non-rebreathing valves underwent a final rinse in distilled water prior to use, and only sterile items were used in the storage and analysis of samples, for example, cryovials and pipette tips. With regard to the collection device, we selected Teflon® collection tubes due to the materials known non-reactive properties, while being made entirely of carbon and fluorine, negating the chance of NOx species leaching into the sample during collection. EBC NOx is a stable test. Storage at 4-6°C for up to 24 h has not been shown to affect the concentration in any way, giving a greater flexibility for the storage of domiciliary samples.34 It is unlikely that different degrees of misclassification of exposure have played a role in finding associations for specific particle metrics, as the lowest misclassification was found for PM2.5.11

CONCLUSIONS

These data suggest that EBC NOx may be useful as an indicator of pollutant-associated oxidative stress in an epidemiological setting. NOx was found in the majority of EBC samples, but levels did not differ significantly between the asthma and COPD subgroups. Coarse particle concentration at the central site was the best predictor of EBC NOx, although the lack of association with more personal measures of particle exposure (particularly the indoor exposure) makes interpretation difficult. EBC NOx should be further investigated as a marker of oxidative stress in subjects with airways disease in response to air pollution exposure.

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Contributors SM, AHM and JGA were responsible for development of the exhaled breath condensate methodology, including the laboratory analysis of the samples. SM and JGA provided the first draft text of the paper. RMH, KK, NP, JB, HtB, KH, JGA and GH contributed to the conduct and design of the local studies. AA, SM and GH contributed to the statistical analysis. All authors commented on the draft text and agree with the text. Primary responsibility for the study is with JGA, SM, AHM and JGA. JGA provided the first draft text of the paper. RMH, KK, NK, JP, HtB, KH, JGA and GH contributed to the development of the exhaled breath condensate collecting system. Thesis (PhD). SM, JGA and GH were responsible for development of the exhaled breath condensate collecting system. Thesis (PhD).

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Association between exhaled breath condensate nitrate + nitrite levels with ambient coarse particle exposure in subjects with airways disease

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