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Airway oxidative stress and inflammation markers in exhaled breath from children are linked with exposure to black carbon

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ABSTRACT

Background: The current study aimed at assessing the associations between black carbon (BC) exposure and markers for airway inflammation and oxidative stress in primary school children in a Western European urban area.
Methods: In 130 children aged 6–12 years old, the fraction of exhaled nitric oxide (FeNO), exhaled breath condensate (EBC) pH, 8-isoprostane and interleukin (IL)-1β were measured in two seasons. BC concentrations on the sampling day (2-h average, 8:00–10:00 AM) and on the day before (24-h average) were assessed using measurements at a central monitoring site. Land use regression (LUR) models were applied to estimate weekly average BC exposure integrated for the time spent at home and at school, and seasonal average BC exposure at the home address. Associations between exposure and biomarkers were tested using linear mixed effect regression models. Next to single exposure models, models combining different BC exposure metrics were used.
Results: In single exposure models, an interquartile range (IQR) increase in 2-h BC (3.10 μg/m^3) was linked with a 5.9% (95% CI: 0.1 to 12.0%) increase in 8-isoprostane. FeNO increased by 16.7% (95% CI: 2.2 to 33.2%) per IQR increase in 24-h average BC (4.50 μg/m^3) and by 12.1% (95% CI: 2.5 to 22.8%) per IQR increase in weekly BC (1.73 μg/m^3). IL-1β was associated with weekly and seasonal (IQR = 1.70 μg/m^3) BC with respective changes of 38.4% (95% CI: 9.0 to 75.4%) and 61.8% (95% CI: 3.5 to 153.9%) per IQR increase in BC. An IQR increase in weekly BC was linked with a lowering in EBC pH of 0.05 (95% CI: −0.10 to −0.01). All associations were observed independent of sex, age, allergy status, parental education level and meteorological conditions on the sampling day. Most of the associations remained when different BC exposure metrics were combined in multiple exposure models, after additional correction for sampling period or after exclusion of children with airway allergies. In additional analyses, FeNO was linked with 24-h PM_{10} levels, but the effect size was smaller than for BC. 8-Isoprostane was not linked with either 2-h or 24-h concentrations of $PM_{2.5}$ or PM_{10}.
Conclusion: BC exposure on the morning of sampling was associated with airway oxidative stress while 24-h and weekly exposures were linked with airway inflammation.

1. Introduction

Particulate matter (PM) is a heterogeneous mixture highly variable in particle size and content. Generally, the small-sized combustion-derived particles (<$PM_{2.5}$) are thought to be more harmful to health than PM from other sources (Janssen et al., 2012). Combustion-derived particles such as black carbon (BC, mainly in size range <2.5 μm) have a larger surface area per unit mass and a greater capacity to reach deep into the airways (Seaton et al., 1995). Moreover, since these particles are enriched in organic carbon content and pro-oxidative polycyclic aromatic hydrocarbons (PAHs) they have a high oxidative potential (Miyata and van Eeden, 2011). Janssen et al. (2011) showed that BC – formed by incomplete combustion of fossil fuels, biofuels, and biomass – is a valuable additional air quality indicator to evaluate the health risks of air pollution dominated primarily by combustion particles. The study showed that

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the estimated increase in life expectancy associated with a hypothetical traffic abatement measure was four to nine times higher for BC compared with an equivalent change in $PM_{2.5}$ mass (Janssen et al., 2011).

In epidemiological studies, short-term exposure to BC has been associated with respiratory symptoms in asthmatic (Patel et al., 2010; Spira-Cohen et al., 2011) and non-asthmatic (Patel et al., 2010) children. BC exposure was furthermore linked with airway inflammation markers in asthmatics (McCreanor et al., 2007; Patel et al., 2013) and non-asthmatics (Patel et al., 2013). The health effects of PM exposure are likely mediated by oxidative stress and the activation of inflammatory cells (Knol et al., 2009; Miller et al., 2012). Moreover, diesel exhaust particles (DEPs), largely consisting of BC, are thought to induce and enhance allergic inflammation (Inoue and Takano, 2011).

The aim of the current study was to assess the effects of BC exposure during different time windows on airway oxidative stress and inflammation markers in a general population of primary school children in a Western European urban area. In Antwerp, the current study region, BC levels are expected to be at the higher end of the distribution of annual average concentrations in developed countries (EEA, 2011; U.S.EPA, 2012) because of the dense road network with heavy traffic and residential heating (Dons et al., 2014). BC exposure was estimated using air quality measurements at a central monitoring site for shorter time windows (2-h and 24-h average), and land use regression (LUR) modeling for longer term exposure (weekly and seasonal average). In recent years, LUR has gained attention and proved to be a useful technique for assessing medium or long term outdoor air pollution exposure (Brauer et al., 2003; Dons et al., 2013; Hoek et al., 2008; Ryan and LeMasters, 2007). In the children, we measured the fraction of exhaled nitric oxide (FeNO) as a marker of lower airway inflammation (Ricciardolo et al., 2004). Additional inflammation markers such as pH and interleukin (IL)-1β were analyzed in exhaled breath condensate (EBC). EBC is a non-invasive way to study the respiratory system and it contains non-volatile compounds that originate from the airway lining fluid (Effros et al., 2012). The acidification of the airways, reflected by a lowering of EBC pH, is associated with airway inflammation (Kuban and Foret, 2013). IL-1β is a pro-inflammatory cytokine involved in host defense and local and systemic inflammation (Dinarello, 2009). In the airways, IL-1β is produced by epithelial cells (Hirota et al., 2012; Sousa et al., 1996) and macrophages (Ishii et al., 2004). Oxidative stress in EBC was assessed by means of 8-isoprostane, a lipid peroxidation product of arachidonic acid reflecting oxidation of cell membrane phospholipids (Kuban and Foret, 2013).

The main focus in this paper is on BC exposure and the link with airway inflammation and oxidative stress markers. We studied whether those associations were also observed with other exposure parameters such as $PM_{2.5}$ and PM_{10} and if these associations were influenced by airway allergy.

2. Materials and methods

2.1. Study population

Children aged 6–12 years old were recruited from two primary schools (circa 2 km apart) in Antwerp, a city of approximately 500,000 inhabitants located in the northern part of Belgium (Europe). Invitations were sent to 744 children of which 242 were willing to participate. Subjects were eligible for participation when they had attended the current school for at least one year, had no plans to change school or home residence within the next year, had lived for at least one year in the Antwerp agglomeration, were not exposed to tobacco smoke inside their house (although children with smoking parents were not excluded) and were willing to donate biological samples. After the exclusion of 68 children for not meeting these criteria, 130 subjects were selected so that the population was equally distributed over both schools, grades, age and sex.

Both the biomonitoring and simultaneous air quality measurements were performed in two seasons (May–June and November–December 2011), each campaign lasting six weeks. In the first period, 130 children participated. In the second period six children dropped out of the study because they changed schools or the permission to participate was withdrawn. Parents were asked to fill in a questionnaire providing information on the child's allergy and asthma status, parental education and parental smoking behavior. Biomonitoring was performed at the schools on ten different days in the first period and on ten different days in the second period. All children were sampled between 9:00–13:00 h.

The study was approved by the ethical committee of the University of Antwerp and subjects' parents gave written informed consent to participate in the study.

2.2. Air quality assessment

2.2.1. Continuous BC, $PM_{2.5}$ and PM_{10} measurements at a central monitoring site

As part of the official monitoring network, BC, $PM_{2.5}$ and PM_{10} measurements were available at a central site in the city of Antwerp (monitoring station 42R801). BC was measured by a multi-angle absorption photometer (without size-selective inlet), $PM_{2.5}$ and PM_{10} were measured by a beta attenuation monitor (ESM FH62I-R). The monitoring site was located approximately 2 and 3 km away from the two schools which the children attended. The average distance (±SD) from the children's homes to the central monitor was 2.8 (±2.2) km. Overall, 90% of the children's home residences were located within a 4.6 km buffer around the monitoring station. 2-h BC exposure of the children was defined as the average BC concentration between 8:00 and 10:00 h at the day of biomonitoring. Exposure during morning rush hour is important since children are then traveling to school and pollution levels are elevated. The 24-h average on the day before sampling and the weekly average before sampling were also calculated.

During the study, a micro-aethalometer (type AE51, Aethlabs, San Francisco, CA, USA) was temporarily installed at the central monitoring site; the same devices were used to measure BC on several home locations. The correlation between BC measurements by MAAP and by the micro-aethalometer at the central site was very high ($R^2 = 0.78$ in period 1, $R^2 = 0.89$ in period 2).

2.2.2. BC measurements at home and LUR modeling

Weeklong BC measurements, using micro-aethalometers (type AE51, Aethlabs), were performed simultaneously at several home locations of children participating in the biomonitoring. In five consecutive weeks, 42 different locations were monitored. This was repeated in the second period, measuring at exactly the same locations. The central monitoring site served as a reference location. Based on these measurements, independent LUR models were built for the warmer (period 1) and the colder (period 2) season, but the explained variance was similar ($R^2_{warm} = 0.70$; $R^2_{cold} = 0.69$). The performance of the models was evaluated by calculating R^2 and RMSE from leave-one-out cross-validation (LOOCV $R^2_{warm} = 0.39$; $R^2_{cold} = 0.51$; impacted by one and two influential observations respectively). A detailed description of the LUR models can be found in Dons et al. (2014).

Residential addresses of the children were geocoded and seasonal BC concentrations were calculated for each study subject using the LUR models. Seasonal LUR-estimates for home and school locations were recalculated into daily concentrations by using the temporal trend observed at the central monitoring site. Average BC exposure during seven days before medical examination was assessed, taking into account exposures at home and at school and the time spent in each of these microenvironments.

2.3. Biomonitoring

2.3.1. FeNO

FeNO was analyzed using a portable NIOX MINO® (Aerocrine, Solna, Sweden) according to the manufacturer's protocol, including inhaling through the mouthpiece and exhaling for 5 s at a flow of 50 mL/sec. Two measurements were performed. When these differed more than 10% a third measurement was carried out. The two or three measurements were averaged to obtain a final FeNO value.

2.3.2. Markers in EBC

EBC was collected using an RTube™ sampling device (Respiratory Research, Inc., Austin, TX, USA). The RTube™ was mounted with an aluminum sleeve that was cooled on dry ice for at least 10 min before collection. Subjects were asked to breath tidally through a mouthpiece connected to the tube for 15 min, yielding approximately 1 mL of EBC. No food was taken 1-h prior to collection. After collection the EBC was immediately divided in aliquots of 250 μL, using 1.5 mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Samples were kept on dry ice and stored at −80 °C until further analysis. The pH was analyzed exactly 5 min after EBC collection in the 1.5 mL tube using a microelectrode (SI Analytics GmbH, Mainz, Germany). IL-1β was analyzed using a Meso Scale Discovery (MSD) Ultra-Sensitive Kit (Meso Scale Discovery, Rockville, MD, USA) which had a detection limit of 50 fg/mL. Samples below the detection limit (21% of all samples) were given a value of 25 fg/mL. Plates were read using a SECTOR® Imager 6000 instrument (Meso Scale Discovery). 8-Isoprostane was measured using a Cayman Chemical 8-isoprostane enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). All samples were above the detection limit of the assay, which was 2.7 pg/mL.

2.3.3. Assessment of airway allergies

Allergen-specific nasal immunoglobulin E (IgE) was measured using the Rhinostick test as previously described (Marcucci and Sensi, 1989). Earlier, it was shown that the Rhinostick test had a higher specificity and sensitivity for the diagnosis of nasal allergies than the skin prick test (Marcucci et al., 2004), making it an ideal tool to non-invasively assess nasal allergies in epidemiological settings. Briefly, a plastic stick containing five allergen covalently coated paper disks (grass pollen, mix of tree pollen, cat epithelium, mold or house dust mite) plus a negative control disk with chicken albumin (Sigma Aldrich, Milan, Italy) was inserted into the nostril for 15 min. After sampling, sticks were stored in a buffer (NaCl 0.9%, Tween 0.1% and NaN_3 0.05%) at 4 °C. IgE levels were analyzed using a previously described ELISA method (Marcucci and Sensi, 1989).

In addition, information on allergy was obtained through the questionnaire ['Does your child have any of the following allergies (cat–house dust mite–mold–tree–grass) diagnosed by a medical doctor?'].

Children were categorized as having airway allergies when they had a positive Rhinostick test or when an airway allergy was indicated in the questionnaire.

2.4. Statistical analysis

Statistical analysis was performed using SPSS Statistics 20 (IBM, Armonk, NY, USA). Except for EBC pH, all biomarker data were log10 transformed to obtain a normal distribution. Sex, age, parents' education level (based on high school not finished (low), high school finished (middle) and higher education college or university (high) — the highest level of education of the mother or the father was considered), airway allergy (yes/no) and meteorological data (temperature and relative humidity on the day of sampling at the central monitor) were included as covariates in all exposure–effect models, since these are either known to be associated with the biomarkers or showed a significant association in simple regression analysis.

Associations between FeNO, EBC pH, EBC IL-1β and EBC 8-isoprostane and seasonal and weekly LUR estimates and 24-h and 2-h BC concentrations at the central monitor were assessed using multiple linear mixed effect regression models for repeated measures. Moreover, exposure–response associations were tested using multiple exposure models combining seasonal and 2-h, seasonal and weekly, and weekly and 2-h BC concentrations. Effect estimates were calculated as unit or percentage change in biomarker levels associated with an interquartile range (IQR) increase in BC concentration. In additional analyses, 2-h and 24-h concentrations of $PM_{2.5}$ and PM_{10} at the central monitor were studied instead of BC.

A series of sensitivity analyses were conducted. All models were additionally adjusted for sampling period. We furthermore replaced weekly LUR data by weekly BC concentrations at the central monitor. Since biomarker levels may be influenced by airway allergies, all exposure–effect associations were also studied with and without the inclusion of the covariate airway allergy in the models. In addition, all associations were tested with the exclusion of subjects with airway allergy.

3. Results

3.1. BC, $PM_{2.5}$ and PM_{10} concentrations

An overview of the pollutants measured at the central monitor during the 2-h and 24-h periods is given in Table 1. In Fig. 1, the 2-h concentrations for BC are displayed. BC exposure at the central monitor during seven days before medical examination was on average (±SD, min–max) 1.66 (±0.22, 1.34–2.03) μg/m^3 in period 1 and 4.82 (±2.26, 1.96–8.72) μg/m^3 in period 2.

The integrated BC exposure at school and at home during seven days before medical examination, estimated using LUR modeling, was on average (±SD, min–max) 1.48 (±0.27, 1.04–2.57) μg/m^3 in period 1 and 3.16 (±1.57, 1.02–8.59) μg/m^3 in period 2 (Fig. 2). Average seasonal BC concentrations at the home address of the children, assessed using LUR modeling, were 1.51 (±0.22, 0.91–2.42) μg/m^3 and 3.28 (±0.56, 2.42–5.41) μg/m^3 in periods 1 and 2, respectively (Fig. 2).

The Pearson correlation coefficients between 2-h BC concentrations and 24-h concentrations at the central monitor, and weekly and seasonal BC levels estimated using LUR were 0.80, 0.72 and 0.48 respectively. The correlation coefficients between 24-h BC concentrations at the central monitor and weekly and seasonal BC levels assessed via LUR were 0.87 and 0.50. The correlation coefficient between weekly and seasonal LUR BC concentrations was 0.63. At the central monitor, the correlation coefficients between BC concentrations and $PM_{2.5}$ and PM_{10} levels were 0.83 and 0.78 for 2-h exposures, and 0.93 and 0.89 for 24-h exposures, respectively. The correlation coefficient between weekly BC levels assessed via LUR and weekly BC levels at the central monitor was 0.95.

3.2. Study population and biomarkers

The characteristics of the study population are described in Table 2. The study population consisted of 130 children between 6 and 12 years old, with an equal distribution between boys and girls. Sixteen (13.0%) children had a positive Rhinostick test. In addition, four more children were identified as having airway allergies based on questionnaire data ['Does your child have any of the following allergies (cat–HDM–mold–tree–grass) diagnosed by a medical doctor?']. In total, 20 (15.4%) children were classified as having airway allergies. Biomarker levels for each period are displayed in Table 3. In period 1 ten children had a FeNO value above 35 ppb, the limit for likely eosinophilic airway inflammation (Dweik et al., 2011). In period 2, seven of these children were also above this limit. Except for two subjects, all these children were diagnosed with airway allergies, explaining the higher values. FeNO data were missing for five subjects in period 1 and for two subjects in period 2 due to the inability of the children to perform the breathing

Table 1
Pollution levels at the central monitoring site.

	2-h average				24-h average			
	Period 1		Period 2		Period 1		Period 2	
	Mean ± SD	Min–max	Mean ± SD	Min–max	Mean ± SD	Min–max	Mean ± SD	Min–max
BC (μg/m^3)	2.78 ± 1.06	1.15–4.76	6.64 ± 3.99	2.23–15.9	2.06 ± 0.88	0.69–3.48	5.06 ± 2.91	0.82–8.75
$PM_{2.5}$ (μg/m^3)	15.4 ± 9.7	3.5–33.8	25.3 ± 19.4	3.0–65.8	12.4 ± 3.4	7.0–18.0	23.7 ± 13.7	8.0–45.0
PM_{10} (μg/m^3)	32.0 ± 13.8	5.3–53.5	42.1 ± 25.2	10.0–98.8	23.9 ± 8.1	11.0–38.0	40.8 ± 21.0	17.0–73.0

maneuver. IL-1β in EBC was not analyzed in four and two participants in periods 1 and 2 respectively because not enough sample was available. EBC 8-isoprostane was missing for one person in period 1 due to low sample volume.

3.3. Associations between biomarkers and BC

The associations between biomarkers and the different BC metrics using single exposure models are displayed in Table 4. Table 5 shows the results of multiple exposure models combining different BC exposure metrics. FeNO was significantly associated with 24-h average BC concentrations at the central monitor in the single exposure model, showing an increase of 16.7% (95% CI: 2.2 to 33.2%) per IQR increase in BC. This association was also observed in the multiple exposure model with seasonal BC concentrations assessed using LUR. An IQR increase in weekly BC exposure estimated using LUR was associated with 12.1% (95% CI: 2.5 to 22.8%) higher levels of FeNO. This effect was consistently observed for multiple exposure models combining weekly exposure with either 2-h concentrations at the central monitor or seasonal LUR concentrations. In the single exposure models, IL-1β was significantly associated with weekly and seasonal average BC concentrations assessed via LUR with respective changes of 38.4% (95% CI: 9.0 to 75.4%) and 61.8% (95% CI: 3.5 to 153.9%). Both these associations remained similar in multiple exposure models with 2-h BC concentrations at the central monitor. When weekly and seasonal exposures were analyzed in combination, only the association with weekly BC concentrations remained. 2-h BC levels at the central monitor were associated with a 5.9% (95% CI: 0.1 to 12.0%) increase in 8-isoprostane in single exposure models. In multiple exposure models with weekly and seasonal BC levels estimated via LUR this association was also observed. An IQR increase in weekly BC levels assessed using LUR was linked with a lowering in EBC pH of 0.05 (95% CI: −0.10 to −0.01) in both the single exposure model and the multiple exposure model with seasonal BC. In the multiple exposure model with 2-h BC levels at the central monitor this association was no longer observed.

Furthermore, the associations between biomarkers and 2-h and 24-h concentrations of $PM_{2.5}$ and PM_{10} at the central monitor were tested. In addition to 24-h BC levels, FeNO was also significantly associated with 24-h concentrations of PM_{10} at the central monitor, but the effect size was smaller than that for BC. The estimated effect sizes of 2-h $PM_{2.5}$ and PM_{10} exposures for 8-isoprostane in EBC were similar in magnitude as for BC, but were however not significant. 8-Isoprostane was not linked with 24-h concentrations of $PM_{2.5}$ or PM_{10}.

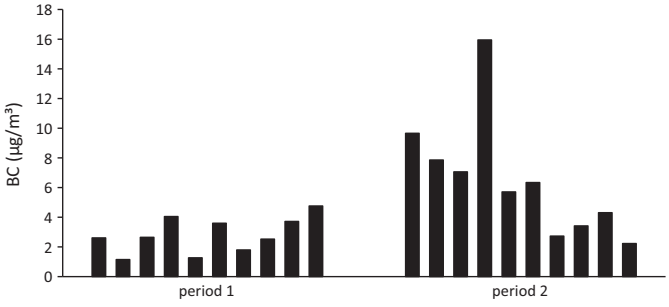

Fig. 1. BC concentrations at the central monitoring station on the days of biomonitoring (8:00–10:00 h average) in both periods.

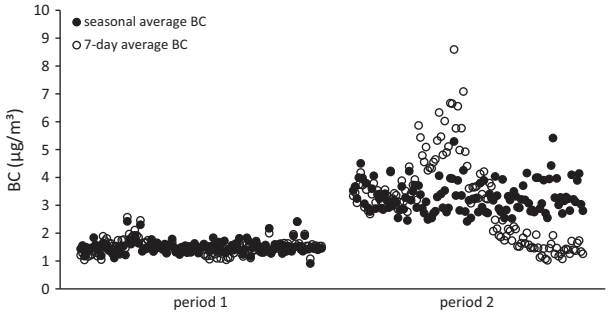

Fig. 2. Seasonal and weekly BC concentrations for all children in both periods assessed using LUR. The data in the x-axis are chronically ordered based on the day of medical examination.

3.4. Sensitivity analyses

Several sensitivity analyses were conducted. When the covariate period was added to the models, all observed associations remained similar except for two associations with IL-1β. The effect estimate of weekly BC concentrations estimated using LUR combined with 2-h BC exposure at the central monitor on IL-1β lowered by 15% and was no longer significant. The observed effect estimates of seasonal BC exposure assessed via LUR on IL-1β disappeared when the models were adjusted for period. When weekly LUR data were replaced by weekly average BC exposures at the central monitoring site, the estimated biomarker changes remained the same for a 1.73 μg/m³ increase in BC. Furthermore, excluding the variable airway allergies from the models did not affect the observed results. When the analyses were performed with the exclusion of children with airway allergy, the associations were similar to those in Tables 4 and 5.

Table 2
Population characteristics (N = 130). Results are displayed as % (N) or mean ± SD.

Male sex	50 (65)
Age (years)	8.9 ± 1.4
BMI (kg/m^2)[a]	16.5 ± 2.5
Underweight	13.8 (18)
Normal weight	74.6 (97)
Overweight	8.5 (11)
Obese	3.1 (4)
Airway allergy[b]	15.4 (20)
Positive RS test	13.0 (16)
Doctor-diagnosed airway allergies	6.9 (9)
Doctor-diagnosed asthma	11.5 (15)
Education parents[c]	
Low	1.5 (2)
Middle	21.5 (28)
High	75.5 (98)

[a] Corrected for sex and age based on 'Flanders 2004 children's growth curves'.
[b] Included when the child had a positive Rhinostick allergy test or when doctor-diagnosed airway allergies were indicated in the questionnaire.
[c] The highest level of education of the mother or the father was considered. Education level was based on high school not finished (low), high school finished (middle) and higher education college or university (high).

Table 3
Biomarker measurements in period 1 and period 2.

	Period 1 (total N = 130)		Period 2 (total N = 124)	
	N (% of total)	Median (P_{25}–P_{75})	N (% of total)	Median (P_{25}–P_{75})
FeNO (ppb)	125 (96)	9.0 (7.0–13.8)	122 (98)	10.5 (8.3–15.6)
EBC pH	130 (100)	6.18 (6.00–6.34)	124 (100)	6.06 (5.95–6.21)
EBC IL-1β (fg/mL)	124 (95)	86 (25–184)	122 (98)	292 (107–550)
EBC 8-isoprostane (pg/mL)	129 (99)	20.2 (12.6–31.1)	124 (100)	17.8 (13.5–22.5)

4. Discussion

In this study, we investigated whether exposures to BC during different time windows in a Western European urban area were associated with markers of airway oxidative stress and inflammation in primary school children. FeNO and IL-1β in EBC showed positive associations with 24-h, weekly or seasonal BC exposure. BC exposure on the sampling day was also associated with increases in EBC 8-isoprostane. The associations between biomarkers and 2-h and 24-h concentrations of BC were stronger and had a larger effect estimate than those between biomarkers and 2-h and 24-h concentrations of $PM_{2.5}$ or PM_{10}. These observations are in line with a recent report by the World Health Organization, suggesting that BC is a better indicator of harmful particulate substances from combustion sources than undifferentiated PM mass (Janssen et al., 2012).

We found consistent and robust associations between FeNO and 24-h BC levels at the central monitor and weekly BC concentrations estimated via LUR. Earlier, studies investigating effects of BC on airway inflammation described associations with FeNO in healthy as well as allergic individuals. In non-atopic children, FeNO was linked with domestic BC levels (Cornell et al., 2012), and healthy schoolchildren showed elevated FeNO in relation to short-term 24-h ambient BC exposures (Lin et al., 2011). In asthmatic children, an association between FeNO and 48-h exposures to BC (Sarnat et al., 2010), and elemental carbon (EC) and organic carbon (OC) (Delfino et al., 2006) was described. Furthermore, FeNO was higher in asthmatic children exposed to higher levels of black smoke the day before sampling (Fischer et al., 2002). Black smoke exposures during several time windows of up to seven days before sampling were also associated with FeNO in schoolchildren (Steerenberg et al., 2001). In adults, short-term BC exposures were linked with FeNO in subjects with respiratory disease (Jansen et al., 2005) and healthy commuters exposed to air pollution in traffic (Zuurbier et al., 2011). NO is produced via oxidation of L-arginine, catalyzed by the enzyme NO synthase (NOS). NOS exists in three isoforms, all present in several airway cells. NOS1 and NOS3 are constitutively expressed, while NOS2 as an inducible isoform is influenced by several stimuli including oxidative stress and cytokines (Ricciardolo et al., 2004). NOS2 releases NO several hours after exposure, which may continue up to several days (Ricciardolo et al., 2004). In children, it was shown that NOS2 expression in epithelial cells was strongly associated with FeNO levels (Lane et al., 2004). The observed associations between BC exposure and FeNO could therefore suggest a role for epithelial-mediated airway inflammation in air pollution related health effects.

Recently, EBC acidification was found to be associated with short-term BC exposure up to five days before sampling in asthmatic and non-

Table 4
Associations between biomarkers and BC concentrations. Results from single exposure models.

	IQR (μg/m³)	FeNO	EBC pH	EBC IL-1β	8-Isoprostane
		Estimate (95% CI)[a]	Estimate (95% CI)[a]	Estimate (95% CI)[a]	Estimate (95% CI)[a]
2-h[b]	3.10	3.7 (−2.2; 10.0)	−0.02 (−0.05; 0.00)	11.1 (−6.0; 31.4)	5.9 (0.1; 12.0)[#]
24-h[b]	4.50	16.7 (2.2; 33.2)[#]	−0.05 (−0.11; 0.00)	35.7 (−4.1; 92.2)	−2.1 (−13.8; 11.2)
Week[c]	1.73	12.1 (2.5; 22.8)[#]	−0.05 (−0.09; −0.01)[#]	38.4 (9.0; 75.4)[§]	−0.4 (−8.5; 8.4)
Season[c]	1.70	3.0 (−14.0; 23.5)	0.00 (−0.07; 0.08)	61.8 (3.5; 153.9)[#]	2.7 (−12.2; 20.2)

[a] Estimated % (FeNO, IL-1β, 8-isoprostane) or unit (pH) change (95% CI) per IQR increase in pollutant concentration. Effect estimates are adjusted for sex, age, parents' education level, airway allergy and meteorological data (temperature and relative humidity on the day of sampling at the central monitor).
[b] Concentrations at the central monitor.
[c] Concentrations estimated using LUR.
[#] $p < 0.05$.
[§] $p < 0.01$.

Table 5
Associations between biomarkers and BC concentrations. Results from multiple exposure models.

			FeNO		EBC pH		EBC IL-1β		8-isoprostane	
Combined BC exposures		IQR (μg/m³)	estimate (95% CI) [a]		estimate (95% CI) [a]		estimate (95% CI) [a]		estimate (95% CI) [a]	
2-h and week	2-h [b]	3.10	−0.3	(−6.8; 6.7)	−0.01	(−0.04; 0.03)	−4.3	(−22.1; 17.6)	9.4	(2.1; 17.2) [#]
	Week [c]	1.73	12.4	(1.1; 25.1) [#]	−0.04	(−0.09; 0.01)	43.6	(6.7; 93.0) [#]	−8.0	(−17.0; 2.1)
2-h and season	2-h [b]	3.10	3.7	(−2.2; 9.9)	−0.02	(−0.05; 0.00)	10.8	(−6.2; 30.9)	6.1	(0.3; 12.2) [#]
	Season [c]	1.70	2.6	(−14.3; 22.9)	0.01	(−0.07; 0.08)	61.2	(3.1; 151.9) [#]	4.4	(−10.8; 22.1)
24-h and season	24-h [b]	4.50	16.9	(2.4; 33.4) [#]	−0.05	(−0.11; 0.00)	33.2	(−5.9; 88.4)	−1.9	(−13.7; 11.5)
	Season [c]	1.70	4.4	(−12.7; 24.7)	0.01	(−0.07; 0.08)	58.7	(1.7; 148.0) [#]	2.5	(−12.5; 20.0)
week and season	Week [c]	1.73	15.2	(4.4; 27.0) [§]	−0.05	(−0.09; −0.01) [#]	31.5	(2.7; 68.5) [#]	−0.7	(−8.9; 8.2)
	Season [c]	1.70	−0.3	(−12.1; 13.2)	−0.02	(−0.08; 0.04)	38.6	(−12.3; 118.8)	3.0	(−12.2; 20.9)

[a] Estimated % (FeNO, IL-1β, 8-isoprostane) or unit (pH) change (95% CI) per IQR increase in pollutant concentration. Effect estimates are adjusted for sex, age, parents' education level, airway allergy and meteorological data (temperature and relative humidity on the day of sampling at the central monitor).
[b] Concentrations at the central monitor.
[c] Concentrations estimated using LUR. 24-h concentrations were not analyzed in combination with 2-h or weekly concentrations since the Pearson correlation coefficient between these exposure metrics was very high (0.80).
[#] $p < 0.05$.
[§] $p < 0.01$.

asthmatic children (Patel et al., 2013). It is known that EBC acidity is linked with airway disease, which is believed to reflect increased airway inflammation (Kuban and Foret, 2013). In our study, the acidification of EBC was associated with weekly BC exposure in some of the models.

Our data showed that IL-1β levels in EBC were associated with weekly and seasonal BC exposures assessed using LUR. Here, the associations with weekly BC exposure seemed to be the most robust when comparing the different multiple exposure models. To our knowledge, we are the first to describe these associations in relation to airways in children. Earlier, IL-1β was found to be higher in blood samples from children living in a highly polluted area compared to samples from children in a less polluted area (Calderon-Garciduenas et al., 2008). In adults, the blood levels of IL-1β were recently observed to be linked with highway proximity (Brugge et al., 2013), ambient PM_{10} exposure (Tsai et al., 2012) and controlled diesel exhaust exposure (Krishnan et al., 2013). *In vitro* studies demonstrated that IL-1β secretion was triggered in human macrophages after PM exposure (Bengalli et al., 2013; Ishii et al., 2004). Furthermore, it was recently shown that *ex vivo* human airway epithelium produced IL-1β in response to PM (Hirota et al., 2012). The molecular pathway behind the PM-induced IL-1β release from epithelial cells and macrophages is suggested to involve the nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) inflammasome (Bengalli et al., 2013; Hirota et al., 2012). The NLRP3 inflammasome is an intracellular danger-sensing protein complex consisting of NLRP3 protein and inactive caspase-1. After inflammasome activation, the autocleavage of caspase-1 causes conversion of immature pro-IL-1β to mature IL-1β (Latz et al., 2013). Recently, *in vitro* and *in vivo* work suggested that IL-1β is produced in an NLRP3-dependent manner in response to PM exposure (Hirota et al., 2012). In addition, oxidative stress seemed to be an important contributor to inflammasome activation in macrophages *in vitro* exposed to PM_{10}, since PM_{10}-induced IL-1β was largely inhibited when a ROS scavenger was added (Bengalli et al., 2013). Lastly, recent investigations indicated that low non-toxic concentrations of ultrafine BC induced oxidative stress through the production of ROS in cultures of rat lung epithelial cells (Buchner et al., 2013).

Overall, *in vitro* studies provide evidence that the release of signaling molecules or mediators is triggered in lung epithelial cells and macrophages in response to increased oxidative stress. Since oxidative stress is suggested as a key player in the health effects induced by PM exposure (Miller et al., 2012), it is possible that PM compounds activate these cells via oxidative stress in a similar way. In our study, airway oxidative stress assessed via 8-isoprostane in EBC was linked with BC exposure on the morning of sampling, but not with any of the longer exposure windows. It is known that 8-isoprostane is rapidly formed in a non-enzymatic way and has a half-life of less than 20 min (Morrow et al., 1990; Roberts and Morrow, 2000). In smoking subjects, 8-isoprostane was increased rapidly after acute exposure to cigarette smoke both in healthy controls (Montuschi et al., 2000) and in asthmatics (Papaioannou et al., 2010). Recently, 8-isoprostane in EBC from both asthmatic and healthy children was associated with BC exposure up to five days prior to sampling (Patel et al., 2013). Moreover, EC and OC exposures averaged over four days before sampling were linked with EBC 8-isoprostane in healthy adults (Huang et al., 2012). In contrast, a link between EBC 8-isoprostane and $PM_{2.5}$ in asthmatic children was not observed (Liu et al., 2009).

A strength of our study is that by using EBC we could specifically look at markers in the airways. EBC is easy to collect during tidal breathing and can be sampled outside a clinical setting. It is a non-invasive way to study the respiratory system and non-volatile compounds in EBC originate from the airway lining fluid (Effros et al., 2012). EBC is especially relevant in the context of the health impact of air pollution exposure, since the respiratory system is the first target organ for airborne pollutants. However EBC also has some drawbacks considering quantification of mediators in it, since there is not a standardized protocol for sampling and no correction for dilution. The fact that biomarker levels are often very low is furthermore challenging for classical analysis techniques (Davis et al., 2012).

Generally, allergic subjects are assumed to be more susceptible to the effects of air pollution (Kelly and Fussell, 2011). A limitation of our study is that no a priori selection criteria were defined with respect to allergy in study participants, resulting in a relatively small number of subjects suffering from airway allergies. The associations between airway inflammation markers and BC exposures were observed after correction for airway allergies, and the exclusion of airway allergies from the models did not affect the observed effects.

In multi-exposure models, the correlations between different exposure metrics are likely to contribute to bias in individual exposure effect estimates. Hence, our results of the models combining different BC exposure metrics must be interpreted with caution. Another limitation is that we cannot rule out effects of other pollutants on the analyzed biomarkers since air pollution is a complex mixture of many different compounds. In our study, we focused on BC and PM, but NO_2 is also often used as a parameter to estimate exposure to traffic-related air pollution. Moreover, meteorological conditions and seasonal differences could influence the association between biomarkers and exposure. Some exposure misclassification in the children could not be avoided. It has been shown that the use of wearable air pollution monitors in combination with time–activity patterns and estimates of physical activity can give more accurate measures of personal air pollution exposure (Dons et al., 2011; Int Panis, 2010; Steinle et al., 2013). However, we did estimate exposure using LUR in which weekly exposure estimates were integrated for the time spent at home and at school, but not transport. LUR is preferable over estimations based on fixed monitoring stations for medium to long term exposures (Hoffmann et al., 2012; Lin et al., 2011; Park et al., 2010) since it takes into account intra-urban variations in air pollution. The performance of the LUR model for BC was comparable to other studies building models for BC or alternative traffic markers (Henderson et al., 2007; Hochadel et al., 2006; Morgenstern et al., 2007).

Short-term exposure in repeated measures studies can be estimated relatively well using a central monitoring station since within-person day-to-day differences in exposure are linked to changes in background concentrations. However, the adequacy of the central monitoring measurements may depend on differences in personal time–activity patterns, though in schoolchildren this is assumed to be relatively limited. In addition, day-to-day differences are influenced by the distribution of sources. In our case, the central monitoring site was close to both schools, which is a strength of the study. This may explain why our results remained similar when central monitoring data were used instead of LUR data to estimate the effect of weekly BC concentrations on biomarker levels. Moreover, it might be that uncertainty in the LUR estimates is associated with exposure misclassification to a similar degree as the concentrations at the central monitor.

In conclusion, we found that 24-h, weekly and seasonal BC levels were associated with airway inflammation markers while BC levels on the morning of sampling were linked with oxidative stress. Our results might suggest that BC exposure is associated with the activation of cells involved in the first line defense mechanisms of the airways such as airway epithelial cells and macrophages. Our results are in accordance with existing literature describing associations between air pollution FeNO, EBC pH and 8-isoprostane. The current study furthermore provides epidemiological evidence for the involvement of IL-1β as an important factor in PM-induced health effects, as previously suggested by *in vitro* work.

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