

ORIGINAL ARTICLE

Carbon in Airway Macrophages and Lung Function in Children

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ABSTRACT

BACKGROUND

Epidemiologic studies indirectly suggest that the inhalation of carbonaceous particulate matter impairs lung function in children. Using the carbon content of airway macrophages as a marker of individual exposure to particulate matter derived from fossil fuel, we sought direct evidence of this association.

METHODS

Airway macrophages were obtained from healthy children through sputum induction, and the area of airway macrophages occupied by carbon was measured. Lung function was measured with the use of spirometry. We modeled the exposure to primary particulate matter (PM) that is less than 10 μm in aerodynamic diameter (PM_{10}) at or near each child's home address. Linear regression was used to evaluate associations between carbon content of alveolar macrophages and variables that may affect individual exposure. To determine whether lung function that is reduced for other reasons is associated with an increase in the carbon content of airway macrophages, we also studied children with severe asthma.

RESULTS

We were able to assess the carbon content of airway macrophages in 64 of 114 healthy children (56 percent). Each increase in primary PM_{10} of 1.0 μg per cubic meter was associated with an increase of 0.10 μm^2 (95 percent confidence interval, 0.01 to 0.18) in the carbon content of airway macrophages, and each increase of 1.0 μm^2 in carbon content was associated with a reduction of 17 percent (95 percent confidence interval, 5.6 to 28.4 percent) in forced expiratory volume in one second, of 12.9 percent (95 percent confidence interval, 0.9 to 24.8 percent) in forced vital capacity, and of 34.7 percent (95 percent confidence interval, 11.3 to 58.1 percent) in the forced expiratory flow between 25 and 75 percent of the forced vital capacity. The carbon content of airway macrophages was lower in children with asthma than in healthy children.

CONCLUSIONS

There is a dose-dependent inverse association between the carbon content of airway macrophages and lung function in children. We found no evidence that reduced lung function itself causes an increase in carbon content.

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BLACK CARBON IS A MAJOR COMPONENT of inhalable particulate matter (particulate matter <10 μm in aerodynamic diameter [PM_{10}]) directly emitted from the combustion of fossil fuels.¹ Black carbon consists of a carbon core enriched with trace metals and organic compounds, and it is thought to mediate many of the adverse health effects reported in epidemiologic studies to be associated with PM_{10} .² Children are especially vulnerable to the adverse effects of PM_{10} ,² with the cumulative effects on the growth of lung function of particular concern. For example, Gauderman et al.³ studied air pollution data from monitoring stations in 12 California communities and reported that increased exposure to elemental carbon and gases from fuel combustion and PM_{10} was associated with impaired growth of lung function.

Airway macrophages are the primary phagocyte for inhaled PM, and in animal models, the amount of carbon-pigmented material in airway macrophages has been shown to reflect both the inhaled dose⁴ and the total particulate burden in the lung.⁵ In adults, the amount of carbonaceous particles extracted from the lung at autopsy reflects the long-term exposure to PM_{10} ,⁶ and PM in airway macrophages reflects exposure to inhalable PM in occupational settings.⁷ In this study, we sought to determine the association between the carbon content of airway macrophages and lung function in a group of healthy children and the association between carbon content and variables that may affect individual exposure — such as exercise,⁸ body-mass index,⁹ sex,¹⁰ and levels of PM_{10} derived from vehicle traffic at or near the home address.¹¹ To address the possibility of reverse causation — namely, that reduced lung function causes an increase in the carbon content of airway macrophages — we also assessed the carbon content of airway macrophages in children who showed evidence of the chronically reduced lung function that is associated with long-standing severe asthma.

METHODS

The study was conducted in Leicester, a medium-sized city in the United Kingdom with annual mean levels of total PM_{10} within U.K. air-quality recommendations.¹² The study protocol was approved by the institutional review board of the Leicestershire Research Ethics Committee. The parents of

the healthy children gave written informed consent, and the children gave written assent. Airway macrophages from children with asthma, which had been obtained as part of normal clinical management, were analyzed with the approval of the institutional review board.

SUBJECTS

Healthy children were recruited from local schools. Children were included if they were 8 to 15 years of age, were living in Leicestershire, and were living in the same house they had lived in for at least one year before the study began and if their parents reported that they had normal levels of activity. We excluded children who had any chronic respiratory condition, symptoms consistent with a respiratory infection over the previous three months, passive exposure to tobacco smoke at home, or exposure to unusual sources of combustion particles (such as from incense sticks); children who were smokers; or children who lived in a house heated by coal combustion. To ensure that the carbon content of airway macrophages reflected exposures in Leicester, we also excluded children who had spent more than 5 days outside the city in the previous 3 months (the half-life of particles in airway macrophages after a single instilled dose is 3.9 months¹³). Since the PM exposure model we used is not valid for buildings more than two stories high, we excluded children whose home was located above the second floor. For safety reasons, children with a post-bronchodilator forced expiratory volume in one second (FEV_1) of less than 80 percent of the predicted value were also excluded.

Sputum induction was performed on various days from November 2002 through December 2003 at the Leicester Royal Infirmary. On the study day, children and parents together completed a questionnaire that requested the child's age, sex, number of siblings, race, and home address. The child's body-mass index was calculated from the height and weight on that day. The child also completed a separate questionnaire, from which metabolic and vigorous activity scores were determined^{14,15} (additional methods are listed in the Supplementary Appendix, available with the full text of this article at www.nejm.org). Exposure to environmental tobacco smoke was assessed with the collection of saliva samples from each child, which were tested for cotinine according to a gas-liquid chromatographic method with a detec-

tion limit of 0.1 ng per milliliter in a commercial laboratory (ABS Laboratory).¹⁶ In the cotinine assay, a level of 14.3 ng per milliliter or less rules out active smoking,¹⁷ and a level of 1.2 ng per milliliter is the 90th percentile value for children who live with no adult smokers.¹⁸

Children with asthma were identified retrospectively from those referred for sputum induction (to assess airway eosinophilia) from a clinic for severe asthma from January 2002 through October 2005. All children with asthma had chronic persistent wheeze and cough while taking inhaled beclomethasone dipropionate at a dosage of 800 μg or more per day and an inhaled long-acting β_2 -agonist twice daily. Demographic, treatment, and spirometric data on the day of sputum induction were obtained from a retrospective review of the clinical records. Activity data were not obtained. In this group, in contrast to the healthy group, an FEV₁ of less than 80 percent of the predicted value did not preclude sputum induction.

LUNG FUNCTION

Lung function was recorded no more than 20 minutes before sputum induction with the use of a Vitalograph 2120 spirometer and Vitalograph 2120 Spirotrac IV software. This system was compliant with the recommendations of the American Thoracic Society and the European Respiratory Society for lung function in children.^{19,20} Spirometry was conducted according to the recommendations of the American Thoracic Society.¹⁹ FEV₁, forced vital capacity (FVC), forced expiratory flow between 25 and 75 percent of the FVC (FEF₂₅₋₇₅) and the FEV₁:FVC ratio were used as the primary variables of lung function. Each plot of the flow rate against volume during an FVC maneuver (flow volume curve) was visually examined for each child, and if the final expiratory phase was stopped owing to a Valsalva maneuver or hesitation but the first portion of the curve was acceptable, only the FEV₁ was calculated.

Lung function was measured both before and 15 minutes after a dose of 200 μg of albuterol was given by means of a Volumatic metered-dose inhaler with a spacer (Allen and Hanburys). Pre- and post-bronchodilator lung function was expressed as the percentage of the predicted normal value — according to reference equations that adjusted for height, weight, sex, and race^{21,22} — and as a z score based on a reference population from the

United Kingdom, which was adjusted for height and sex but not race.²³

INDUCED SPUTUM

Sputum induction was conducted in healthy children and children with asthma according to a standard method.²⁴ Nebulized saline (4.5 percent) was administered through an ultrasonic nebulizer (Sonix 2000, Clement Clarke International) in three sequential five-minute inhalation periods. FEV₁ was measured throughout the inhalation periods for the detection of clinically significant bronchoconstriction. Induced sputum was processed according to a standard technique.²⁵ Dithiothreitol (Sigma) was used as a mucolytic agent, and airway cells were removed. The airway cells were cytocentrifuged (Cytospin, Shandon Scientific) onto glass slides according to a standard method,²⁶ and stained with Diff-Quik (Dade Behring). The induced-sputum leukocyte differential was measured in 300 nonsquamous nucleated cells per child.

CARBON CONTENT OF AIRWAY MACROPHAGES

Airway macrophages were visualized by light microscopy. The area occupied by black material (carbon) in each macrophage was assessed as previously described²⁷ by an investigator who was blinded to lung function. Scion Image software was used to calculate the carbon content of airway macrophages, which was defined a priori as the median area occupied by carbon (μm^2) in 100 randomly selected cells per child.

MODELED PRIMARY PM₁₀

The annual mean level of primary PM₁₀ — that is, the component of PM₁₀ emitted directly from local sources of combustion — was calculated for each child's home address using the Airviro dispersion model (Swedish Meteorological and Hydrological Institute),²⁸ as previously reported.¹¹ Specifically, we modeled the hourly emissions of primary PM₁₀ from road traffic using Airviro and a database containing estimates for hourly traffic flows, speeds, and vehicular mixes (e.g., cars vs. trucks). The dispersion of primary PM₁₀ was calculated with the use of the actual wind speed and direction measured for the time period.¹¹

STATISTICAL ANALYSIS

Data were summarized as means \pm SD or medians and ranges. The baseline characteristics of the

Table 1. Characteristics of Children with Sufficient Numbers of Airway Macrophages for Analysis.*

Characteristic	Healthy Children (N=64)	Children with Asthma (N=9)	P Value
Age — yr	11.5±2.3	13.6±2.0	0.009
Male sex — no. (%)	35 (55)	5 (56)	0.96
Race — no. (%)†			0.68
White	40 (62)	5 (56)	
Nonwhite	24 (38)	4 (44)	
Weight — kg	45.5±13.4	49.8±13.6	0.36
Height — cm	149.8±13.2	153.7±8.7	0.37
Body-mass index	19.9±3.8	21.01±5.2	0.44
FEV ₁ — % of predicted value	100.1±11.1	71.8±13.0	<0.001
FVC — % of predicted value	103.0±11.2	92.5±15.4	0.02
Eosinophils in sputum — %			<0.001
Median	0.25	31.17	
Range	0–35	4–75	
Carbon content of airway macrophages — μm^2			<0.001
Median	0.41	0.00	
Range	0.03–1.14	0.00–0.12	
Modeled annual mean primary PM ₁₀ — $\mu\text{g}/\text{m}^3$ ‡			0.02
Median	1.21	1.81	
Range	0.10–2.71	0.17–2.13	

* Plus-minus values are means \pm SD. The body-mass index is the weight in kilograms divided by the square of the height in meters.

† Race was self-reported.

‡ PM₁₀ was modeled at or near the home address.

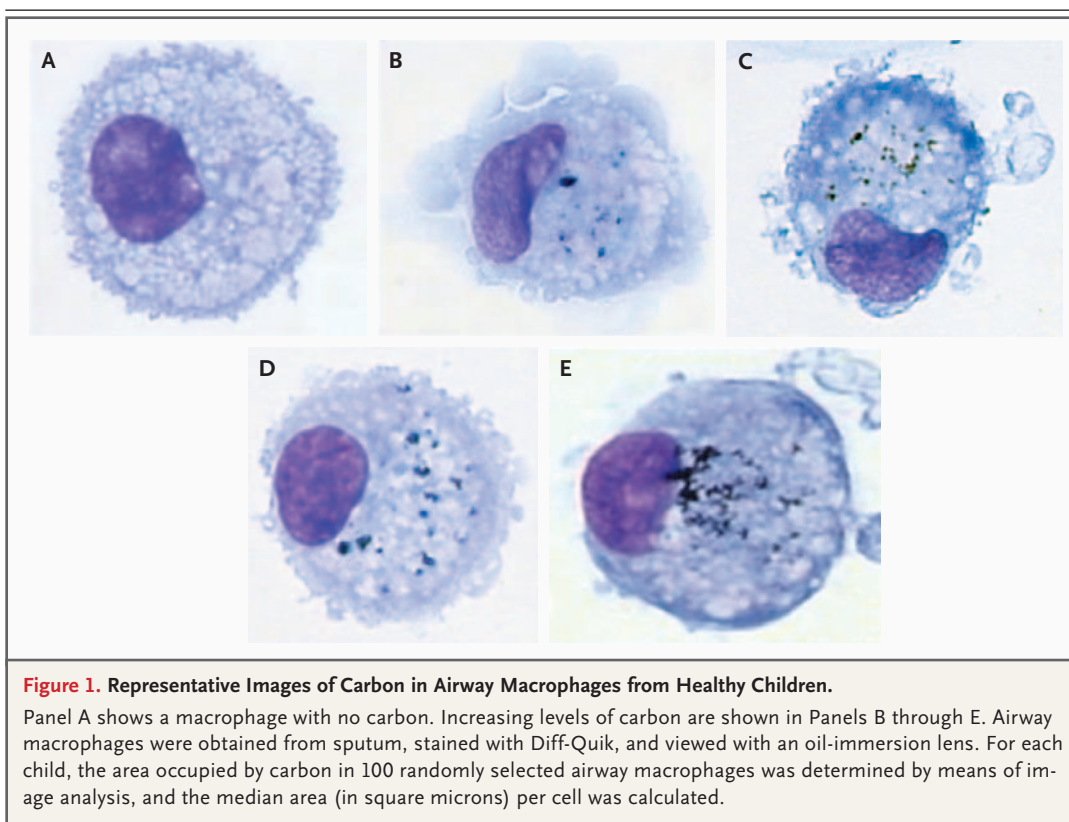
healthy group and the group with asthma were compared with the use of an unpaired t-test for the means, the chi-square test for the proportions, and the Mann–Whitney test for skewed data. Normal linear models were used to explore the associations between variables. Q-Q plots of the residuals, the residuals versus the fitted values, and the residuals versus leverage were used to test the assumptions of all linear models. We also used nonparametric tests in the place of linear regression to confirm the results. In the multiple regression models, confounders were included if they were significant at a 0.05 level or they altered the coefficient of the main variable by more than 10 percent in cases in which the main association was significant. The potential confounding variables considered were age, sex, race, weight, height, birth order, number of siblings, exercise measures, and cotinine levels in saliva. A P value of less than 0.05 was considered to indicate statistical significance, and reported P values are two-sided. Analyses were carried out with the use of SAS version 8.2 for Windows, R version 2.2.0

(www.r-project.org), and SPSS version 12.0.1 for Windows.

RESULTS

Sufficient numbers of airway macrophages for an assessment of the area occupied by carbon were obtained from 64 of the 114 healthy children (56 percent) from whom sputum was induced (Table 1 and Fig. 1). There were no significant differences in the demographic variables between children in whom sufficient numbers of airway macrophages were obtained and children in whom insufficient numbers were obtained (Table 1 in the Supplementary Appendix). Cotinine levels in saliva from all children were below the level that indicates active smoking, and 62 of the 64 children had cotinine levels below the 90th percentile for households of nonsmokers.¹⁸

In healthy children, there was no association between the carbon content of airway macrophages and age, weight, height, body-mass index, activity scores, or cotinine level in saliva, according



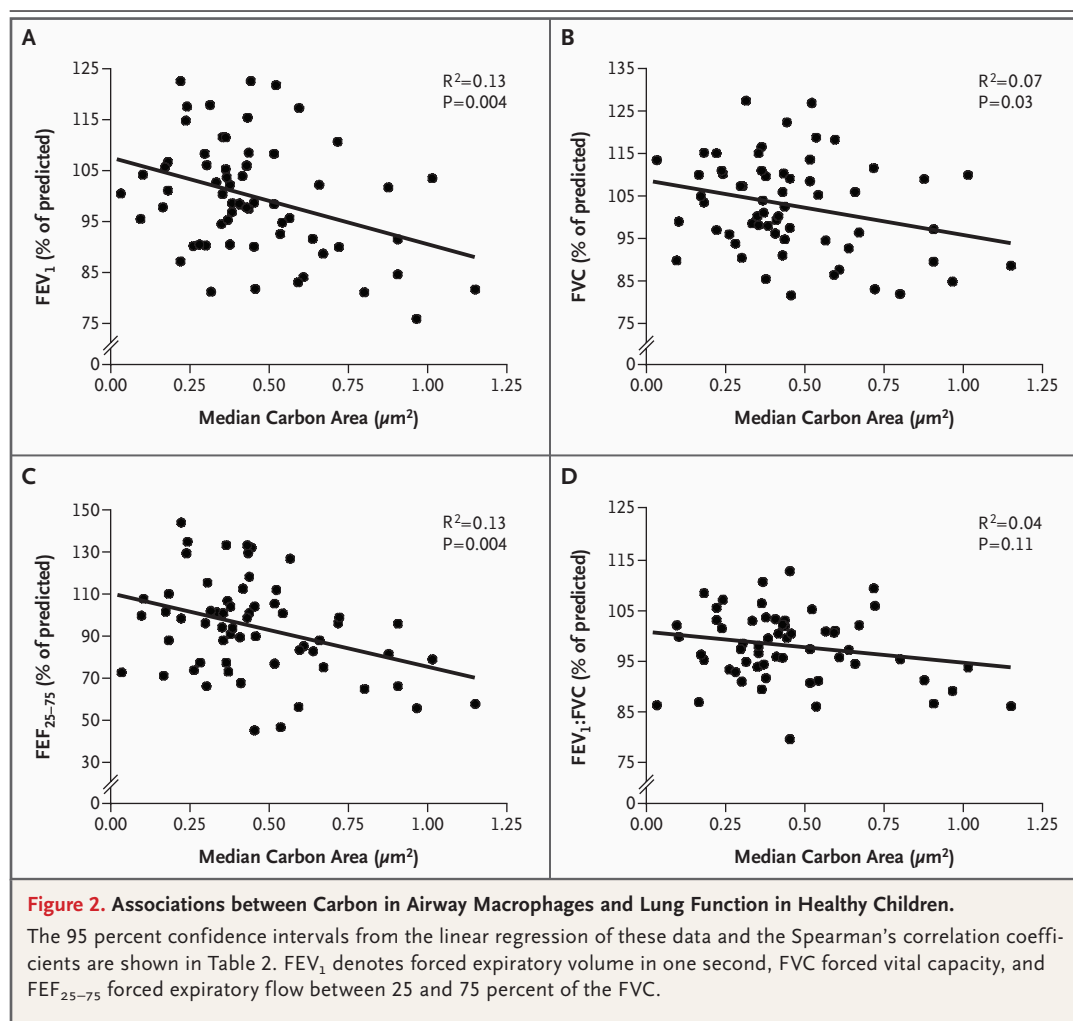
to parametric and nonparametric analyses (Table 2 in the Supplementary Appendix). There was no significant difference in carbon content between boys and girls ($P=0.59$). Race was the only variable to reach a significance level high enough to be considered for multiple regression ($P<0.001$).

There was an inverse, dose-dependent association between the carbon content of airway macrophages and lung function: each increase of $1.0 \mu\text{m}^2$ in carbon content was associated with a reduction of 17 percent (95 percent confidence interval, 5.6 to 28.4 percent) in the percentage of the predicted FEV_1 , of 12.9 percent (95 percent confidence interval, 0.9 to 24.8 percent) in FVC, and of 34.7 percent (95 percent confidence interval, 11.3 to 58.1 percent) in FEF_{25-75} (Fig. 2 and Table 2). There was no association between the carbon content of airway macrophages and the FEV_1 :FVC ratio. These inverse associations remained significant when lung function was expressed as a z score or measured 15 minutes after the administration of an inhaled bronchodilator (Table 2).

The annual mean level of primary PM_{10} at or near the home address was modeled for all healthy

children from whom sputum was induced (Table 1). Each increase of $1.0 \mu\text{g}$ per cubic meter in modeled primary PM_{10} was associated with a $0.10 \mu\text{m}^2$ (95 percent confidence interval, 0.01 to 0.18) increase in the carbon content of airway macrophages. Increased primary PM_{10} was inversely associated with the percentage of the predicted FEV_1 ($P=0.04$) and the FEF_{25-75} ($P=0.05$). In the multiple regression analysis, even after adjusting for the carbon content of PM_{10} , the carbon content of airway macrophages remained inversely associated with the percentage of the predicted values of FEV_1 , FVC, and FEF_{25-75} ($P=0.02$, $P=0.04$, and $P=0.02$, respectively), whereas PM_{10} was no longer significantly associated with lung function (Table 3). The stratification of exposure by race showed that nonwhite children had higher modeled primary PM_{10} at or near the home address than white children ($P<0.001$) (Fig. 3).

Sufficient airway macrophages were obtained from all nine of the nine children with asthma from whom sputum was induced (Table 1). Modeling indicated that, as compared with healthy children, children with asthma lived in areas with increased levels of primary PM_{10} ($P=0.02$), had a



reduced prebronchodilator FEV₁ ($P < 0.001$), and had an increased proportion of eosinophils in sputum ($P < 0.001$) (Table 1). There was a marked difference in the pattern of carbon loading in airway macrophages between children with asthma and healthy children. Specifically, carbon was not detected in the majority of airway macrophages from children with asthma, whereas the majority of airway macrophages from healthy children contained at least one area of carbon. Thus, the median area occupied by carbon, calculated from 100 cells per child, was 0 for 8 of the 9 children with asthma (Table 1).

DISCUSSION

We found an inverse, dose-dependent association between the carbon content of airway macrophages and FEV₁, FVC, and FEF₂₅₋₇₅ in healthy chil-

dren living in an area where the spatial variation in PM₁₀ is primarily due to emissions from road traffic. This inverse association is consistent with those reported in epidemiologic studies, which suggest a long-term effect of PM on lung function in children.

The most recent cross-sectional analysis of the cohort of 12 Southern California communities showed that a greater proportion of young adults with an FEV₁ of less than 80 percent of the predicted value than of those with a higher FEV₁ live in communities with high levels of elemental carbon and PM₁₀.³ Similarly, in their three-year study of eight European communities, Horak et al.²⁹ found reduced growth of FEV₁ and FEF₂₅₋₇₅ in children exposed to high levels of PM₁₀ in summer. An adverse effect of PM₁₀ on lung growth is a plausible explanation for the 3.8 percent difference in FEV₁ values reported in a cross-sectional

Table 2. Association between Lung Function and Carbon in Airway Macrophages from Healthy Children.*

Lung-Function Variable†	Linear Regression		Spearman's Rank Test	
	Coefficient (95% CI)‡	P Value	r	P Value
FEV ₁ (N=64)				
% of predicted value	-17.0 (-28.4 to -5.6)	0.004	-0.30	0.02
z score	-2.0 (-3.1 to -0.9)	<0.001	-0.40	0.001
FVC (N=61)				
% of predicted value	-12.9 (-24.8 to -0.9)	0.03	-0.24	0.05
z score	-2.3 (-3.4 to -1.1)	<0.001	-0.46	<0.001
FEF ₂₅₋₇₅ (N=61)				
% of predicted value	-34.7 (-58.1 to -11.3)	0.004	-0.30	0.02
z score	-1.2 (-2.2 to -0.2)	0.01	-0.31	0.02
FEV ₁ :FVC (N=60)				
% of predicted value	-6.1 (-13.7 to 1.5)	0.11	-0.12	0.34
z score	-0.2 (-1.0 to 0.6)	0.58	-0.09	0.47
Post-bronchodilator FEV ₁ (N=63)				
% of predicted value	-15.9 (-27.3 to -4.4)	0.007	-0.27	0.03
z score	-2.0 (-3.1 to -0.9)	0.001	-0.39	0.002

* Carbon in airway macrophages was defined as the median area (in square micrometers) occupied by carbon. CI denotes confidence interval, FEV₁ forced expiratory volume in one second, FVC forced vital capacity, and FEF₂₅₋₇₅ forced expiratory flow between 25 and 75 percent of the FVC.

† Percentages of the predicted values for lung function were adjusted for height, weight, sex and race; z score was adjusted for height and weight but not race. FVC and FEF₂₅₋₇₅ were not calculated for all children (see the Methods section). One child did not receive albuterol.

‡ The coefficient is of the change in lung function for each increase of 1.0 μm^2 in the carbon content of airway macrophages.

study of children living in areas with high levels and those living in areas with low levels of particulate pollution in Wuhan, China.³⁰ In the present study, we modeled primary PM₁₀ at or near the home address. Modeling allowed us to estimate small-scale spatial variations in the levels of traffic-derived PM₁₀. However, our modeling of the primary PM₁₀ did not account for exposures due to personal factors (such as time spent indoors and other activity patterns), did not estimate background exposures to PM₁₀ blown into the study area from other counties, and did not specifically model PM_{2.5} — the PM fraction that contains the highest proportion of elemental carbon.¹ Although the true relation of exposure to primary PM₁₀ and the carbon content of airway macrophages may therefore be stronger than that found in the present study, our group recently reported an independent association between modeled primary PM₁₀ at or near the home address and the prevalence and incidence of respiratory symptoms in a cohort of 4400 preschool children.¹¹

An alternative explanation for the inverse asso-

ciation between the carbon content of airway macrophages and lung function is that low-to-normal lung function increases the deposition of inhaled carbonaceous PM in the airway. We hypothesized that, if low lung function is a major cause of increased carbon content of airway macrophages, then high levels of carbon would be found in the airway macrophages of children with chronic asthma. We found lower levels of carbon in the airway macrophages of children with asthma, despite the higher levels of modeled primary PM₁₀, than in healthy children. This finding suggests that the phagocytosis of carbon particles by airway macrophages may be impaired in severe asthma. Indeed, Alexis et al.³¹ reported the significant impairment of phagocytosis of IgG-opsonized yeast by airway macrophages in a subgroup of adults with asthma who had eosinophilia in their induced sputum.

In experimental exposure studies, increased activity level, reduced age, and increased body-mass index have been shown to cause an increase in particle deposition in the lower airway.⁸⁻¹⁰ In

Table 3. Association between Lung Function, PM₁₀, and Carbon in Airway Macrophages in Healthy Children.*

Model	Variable	Coefficient (95% CI)†	P Value for Variable	R ² for Model	P Value for Model
FEV ₁ vs. PM ₁₀	PM ₁₀	-4.3 (-8.5 to -0.2)	0.04	0.06	0.04
FEV ₁ vs. (PM ₁₀ + AM carbon)				0.15	0.006
	PM ₁₀	-2.9 (-6.9 to 1.2)	0.17		
	AM carbon	-14.7 (-26.3 to -3.2)	0.02		
FVC vs. PM ₁₀	PM ₁₀	-1.2 (-5.6 to 3.2)	0.59	0.005	0.59
FVC vs. (PM ₁₀ + AM carbon)				0.07	0.10
	PM ₁₀	0.1 (-4.4 to 4.6)	0.96		
	AM carbon	-13.0 (-25.6 to -0.4)	0.04		
FEF ₂₅₋₇₅ vs. PM ₁₀	PM ₁₀	-8.6 (-17.3 to 0.1)	0.05	0.06	0.05
FEF ₂₅₋₇₅ vs. (PM ₁₀ + AM carbon)				0.15	0.008
	PM ₁₀	-5.5 (-14.2 to 3.1)	0.21		
	AM carbon	-30.4 (-54.6 to -6.1)	0.02		

* PM₁₀ was defined as the annual mean modeled particulate matter less than 10 μm in aerodynamic diameter modeled at or near the home address (in micrograms per cubic meters). AM carbon was defined as the median area (in square micrometers) occupied by carbon in airway macrophages. CI denotes confidence interval, FEV₁ forced expiratory volume in one second, FVC forced vital capacity, and FEF₂₅₋₇₅ forced expiratory flow between 25 and 75 percent of the FVC.

† The coefficient is of the change in lung function variable for each increase of 1.0 μm^2 in the carbon content in airway macrophages or increase of 1.0 μg per cubic meter in modeled annual mean primary PM₁₀ at or near the home address.

the present study, none of these variables were associated with carbon in airway macrophages. A larger study may therefore be needed to identify the independent effects of these variables under normal exposure conditions. However, we found that nonwhite children lived in areas with higher modeled primary PM₁₀ and had higher levels

of carbon in their airway macrophages than did white children. Similarly, a study in the United Kingdom that linked Airviro assessment of traffic-derived air pollution to race at or near the home address found higher exposures for nonwhite than white households.³²

There are limitations to our study. It is unclear whether the inverse association between the carbon content of airway macrophages and lung function represents a short-term or a long-term effect on lung function. Our results have, however, ruled out short-term reversible bronchoconstriction, since significant inverse associations remained after bronchodilator therapy. It is also unclear whether the carbon content of airway macrophages reflects long-term or short-term exposure to PM₁₀. However, some carbon will have been acquired several months before our analysis, since soot has been observed in airway macrophages at least 10 months after brief occupational exposures,⁷ and insoluble particles remain in airway macrophages for up to 3 months after instillation in experimental studies.¹³ The carbon content of airway macrophages may not reflect the content in more distal alveolar cells, since sputum induction samples macrophages from the larger airways.³³ In adults, however, the distribution of particles from the environment in bronchial mac-

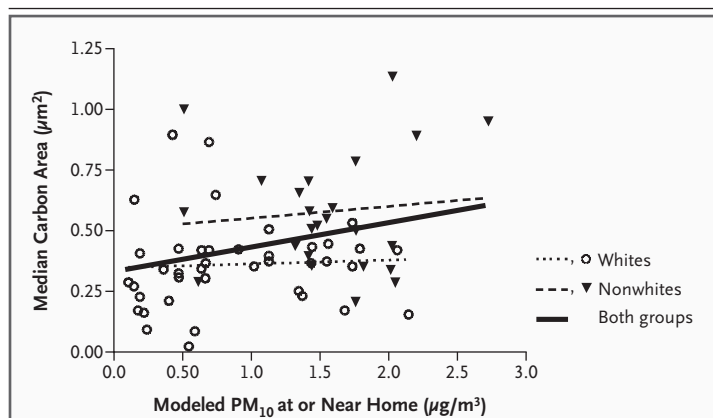


Figure 3. Relation between Modeled Annual Mean Primary PM₁₀ at or near the Home Address and Carbon in Airway Macrophages in Healthy Children.

The carbon content of airway macrophages was higher in nonwhite than in white healthy children (mean, 0.58 vs. 0.36 μm^2 ; $P < 0.001$). For all healthy children combined, R² was 0.081 ($P = 0.02$) and Spearman's correlation coefficient was 0.30 ($P = 0.01$). Separate regression lines are also given for white and nonwhite children ($P = 0.74$ and $P = 0.65$, respectively).

rophages is nearly identical to that in alveolar macrophages.^{13,34} Our limited success rate of 56 percent for sputum induction in healthy children may have introduced bias, although this rate is similar to that for healthy children reported in a previous study,³⁵ and there were no differences in any other measured variables between children from whom sputum was induced successfully and those from whom it was not.

We have not proved that the black material in airway macrophages is elemental carbon. In a previous study, our group found that particles in alveolar macrophages from healthy children were morphologically identical to aggregates of carbon nanoparticles from emissions of primary diesel exhaust,³⁶ and a preliminary analysis by means of electron energy-loss spectroscopy of the airway macrophages in the present study found no spectra indicating the presence of iron, titanium, silica, or sulfur (Geiser M: personal communication). It is therefore likely that a major proportion of the black-pigmented material is inhaled elemental carbon.

In conclusion, in this cross-sectional study, we found a dose-dependent, inverse association between the carbon content of airway macrophages and FEV₁, FEF₂₅₋₇₅, and FVC in healthy children. Since we directly assessed the carbon content of airway macrophages, our data strengthen the evidence for a causal association between the inhalation of carbonaceous particles and impaired lung function in children.

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Prof. Grigg reports being a member of the U.K. Government Expert Panel on Air Quality Standards. Dr. Rushton reports having been a member of the Industrial Injuries Advisory Council of the U.K. Department of Works and Pensions from 1994 to 2004 and being a member of the Committee on Toxicity of Chemicals in Food, Consumer Products, and the Environment of the U.K. Food Standard Agency. No other potential conflict of interest relevant to this article was reported.

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