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Abstract

Background: Few studies have been performed on pulmonary effects of air pollution in the elderly, a vulnerable population with low reserve capacity, and mechanisms and susceptibility factors for potential effects are unclear.

Objectives: We evaluated the lag structure of air pollutant associations with lung function and potential effect modification by DNA methylation ($<$ or \geq median) at 26 individual CpG sites in nine candidate genes in a well-characterized cohort of elderly men.

Methods: We measured forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), and blood DNA methylation one to four times between 1999-2009 in 776 men from the Normative Aging Study. Air pollution was measured at fixed monitors 4 hours to 28 days before lung function tests. We used linear mixed effects models to estimate the main effects of air pollutants and effect modification by DNA methylation.

Results: An interquartile range (IQR) increase in sub-chronic exposure (3- to 28-days cumulated), but not in acute exposure (during the previous 4 h, or the current or previous day), to black carbon, total and non-traffic particles with aerodynamic diameter $< 2.5 \mu\text{m}$, carbon monoxide, and nitrogen dioxide was associated with a 1 to 5% decrease in FVC and FEV_1 ($p < 0.05$). Slope estimates were greater for FVC than FEV_1 and increased with cumulative exposure. The estimates slopes for air pollutants (28-days cumulated) were higher in participants with low ($<$ median) methylation in *TLR-2* at position 2 and position 5 and high (\geq median) methylation in *GCR*.

Conclusions: Sub-chronic exposure to traffic-related pollutants was associated with significantly reduced lung function in the elderly; non-traffic pollutants (particles, ozone) had weaker

associations. Epigenetic mechanisms related to inflammation and immunity may influence these associations.

Introduction

By 2030, there will be 72.1 million people aged 65 years or older, representing 19% of the U.S. population according to the Department of Health and Human Services (United States Department of Health and Human Services 2014). The aging process reduces physiological capacity, which makes elderly more susceptible to many health threats. There is compelling evidence that short- and long-term exposure to ambient air pollution, especially due to traffic, adversely affect lung function (Brunekreef et al. 1995). However, most of the studies have focused on children. While research examining the effects of air pollution on forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁) in elderly is warranted (Sandstrom et al. 2003), to our knowledge, they have not yet been investigated.

The underlying mechanisms linking air pollution and lung function are not fully characterized. Lung function has been shown to be strongly heritable, part of which is controlled by inflammatory genes (Sunyer et al. 2008). Consistent with this observation, many studies have reported associations between lung function and polymorphisms within genes coding for inflammatory, oxidative stress and innate immunity mediators such as *CRP* (Sunyer et al. 2008), *IL-6* (He et al. 2009), *iNOS* (Islam et al. 2009) or *TLRs* (Budulac et al. 2012). Other studies have reported stronger associations between air pollution and pulmonary outcomes in subjects with such polymorphisms (Kerkhof et al. 2010; Yang et al. 2008). There is increasing evidence that epigenetic mechanisms may interact with genetic variation to influence disease pathogenesis and the inheritance of disease traits.

Methylation in CpG-rich regions within gene promoters is commonly associated with repressed gene expression, as it may impede the binding of transcription factors (Hashimshony et al. 2003).

Studies in humans have reported associations between blood DNA methylation and cardiovascular diseases (Stenvinkel et al. 2007), respiratory health (Lepeule et al. 2012), and survival (Baccarelli et al. 2010).

We hypothesized that short-term exposure to traffic-related air pollutants would be associated with lung function decrease in a cohort of elderly men. Subsequently, we examined whether methylation level within or near the promoter region of selected genes related to inflammation, immunity, endothelial function, and oxidative stress may be a susceptibility factor for lung function impairment.

Methods

Study population and pulmonary health

Our study included 776 elderly men living in the Boston area, enrolled in the Normative Aging Study cohort (Bell et al. 1966). Participants provided written informed consent and the study was approved by the institutional review boards of all participating institutions. Subjects completed one to four clinical examinations between 1999-2009. Each visit took place in the morning after an overnight fast and smoking abstinence. At each visit, medication use (corticosteroids, sympathomimetic α and β , anticholinergics), pulmonary disorders, and smoking history were collected using the American Thoracic Society questionnaire (Ferris 1978).

Spirometric tests were performed following a strict protocol in accordance with American Thoracic Society guidelines, as previously reported (Sparrow et al. 1987). Spirometry was assessed in the standing position with a noseclip using a 10-litre water-filled survey-recording spirometer and an Eagle II minicomputer (Warren E. Collins, Braintree, Massachusetts). Values were adjusted by body temperature and pressure. A minimum of three acceptable spirograms was

obtained, of which at least two were reproducible within 5% for both FVC and FEV₁. Each technician underwent training prior to taking measurements for this study.

Methacholine challenge tests were conducted between 1984-2000 using procedures adapted from Chatham et al. (1982). We used data from the most recent test available for each subject at that visit. Participants with ischemic heart disease or baseline FEV₁ < 60% of the predicted value were excluded, and some elected not to participate. Methacholine inhalations were administered at incremental doses corresponding to 0, 0.330, 1.98, 8.58, 16.8, and 49.8 µmol. Participants whose FEV₁ declined by 20% in response to any of the doses at or before 8.58 µmol were classified as having airway hyperresponsiveness. Participants whose FEV₁ did not decline by 20% in response to any of the administered doses, and participants who demonstrated a 20% decline in FEV₁ at higher methacholine dosages (16.8 or 49.8 µmol) only were categorized as having no airway hyperresponsiveness.

DNA Methylation

DNA was extracted from stored frozen buffy coat of 7 ml whole blood, using QiAmp DNA blood kits (QIAGEN). DNA methylation was quantitated using bisulfite-polymerase chain reaction and pyrosequencing (Tost and Gut 2007) within or nearby the promoter regions of a total of nine genes: *Carnitine O-acetyltransferase (CRAT)*, *coagulation factor-3 (F3)*, *glucocorticoid receptor (GCR)*, *intercellular adhesion molecule (ICAM-1)*, *interferon-gamma (IFN-γ)*, *interleukin-6 (IL-6)*, *inducible nitric oxide synthase (iNOS)*, *8-oxoguanine DNA glycosylase 1 (OGG1)*, and *toll-like receptor-2 (TLR-2)*. Primers and conditions were previously described (Lepeule et al. 2012). These genes were selected because they are expressed in leukocytes (AceView 2013) and are related to cardio-respiratory health, inflammation and oxidative stress mechanisms (Lepeule et al. 2012; Poole et al. 2011; Sin and Man 2008). The

promoter regions were located using Genomatix Software (Genomatix Software Inc, Ann Arbor, MI, USA) (Supplemental Material, Table S1). For each gene, we measured between 1-5 CpG sites (positions) located within or outside of CpG islands. DNA methylation analysis was repeated on each sample and results were averaged to reduce assay variability. We used non-CpG cytosine residues as built-in controls to verify bisulfite conversion. The degree of methylation was expressed for each DNA locus as % methylated cytosines over the sum of methylated and unmethylated cytosines. Because of assay failure and limited amounts of DNA available from each subject, DNA methylation analysis was successful on varying numbers of subjects for each sequence. All samples were analyzed consecutively by one laboratory technician.

Environmental measurements

Exposure to urban background pollution was estimated using fixed monitoring stations. Ambient concentrations of black carbon (BC) and of particles with aerodynamic diameter less than 2.5 μm ($\text{PM}_{2.5}$) were measured hourly at Harvard Supersite, positioned at the top of a building located < 1 km from the medical center, where the participants' visits took place. BC concentrations were measured using an aethalometer (Magee Scientific Inc., Berkeley, CA, USA) and $\text{PM}_{2.5}$ mass concentrations were measured using a Tapered Element Oscillating Microbalance (TEOMs model 1400A; Rupprecht and Patashnick Co., East Greenbush, NY, USA). TEOM measurements were corrected for loss of semi-volatile particles during sampling, using a collocated gravimetric sampler. About 10% of hourly missing measurements for BC and $\text{PM}_{2.5}$ were imputed through a linear regression, where each pollutant was regressed against season, long-term time trend, day of the week, mean temperature, relative humidity, barometric pressure, extinction coefficient, and previous and following day measurements of the pollutant (Zanobetti and Schwartz 2007).

We regressed hourly PM_{2.5} concentrations against BC (mainly contributed by traffic sources in Boston) and used the residuals as a surrogate measure of non-traffic PM_{2.5} (Schwartz et al. 2005). Hourly carbon monoxide (CO), ozone (O₃), and nitrogen dioxide (NO₂) concentrations were measured by local state monitors (4 monitors for CO and O₃, 5 for NO₂) and averaged over all monitors. The median distance of the participant homes was 20.8 km to the BC and PM_{2.5} monitoring site, and 20.2, 22.3, and 21.4 respectively for the CO, O₃, and NO₂ monitors. We obtained temperature and relative humidity data from the United States Surface Airways and Airways Solar Radiation hourly data (National Environmental Satellite D, and Information Service 2003).

Daily averages were calculated when at least 75% of the hourly values for a given day were available. For each pollutant concentration, we considered a range of short-term and subchronic exposure windows preceding each subject's examination, including: 4 hours, 24 hours (lag 0), previous day (lag 1), and 3, 7, 14, and 28-day moving averages. Since all visits were scheduled for the morning, exposure windows were calculated from 8am the day of the visit.

Statistical analysis

We studied the effects of air pollutants on lung function using a mixed linear model. Separate models were run for each pollutant, exposure window, and lung function measurement:

$$Y_{it} = \beta_0 + u_i + \beta_1 \text{Air pollutant}_{it} + \beta_2 X_{2it} + \dots + \beta_p X_{pit} + \varepsilon_{it} \quad [1]$$

where: Y_{it} was the log-transformed lung function measurement for participant i at visit t , β_0 was the intercept, u_i was the random effect, β_1 was the effect of the air pollutant on lung function measurement, X_{2it} to X_{pit} were the $p-1$ covariates, and ε_{it} was the within-participant error. We selected the following adjustment covariates *a priori* and added a quadratic term whenever it was significant: age (linear and quadratic), ln(height) (linear and quadratic) and standardized weight

(linear), race, education level, smoking status, cumulative smoking in pack-years, season of the medical exam (using sine and cosine of time), day of the week, visit number, temperature and relative humidity (matched on air pollutant exposure window), physician-diagnosed chronic lung conditions (asthma, emphysema, chronic bronchitis), methacholine responsiveness, medication use. As participants with chronic lung conditions are expected to be sicker than average, we explored potential modification of air pollution effects (28-days moving average) by emphysema, chronic bronchitis, methacholine responsiveness (as an objective indicator of asthma) and COPD (defined as GOLD stage II ($FEV_1/FVC < 70\%$ and $FEV_1 < 80\%$ predicted) or higher).

Several sensitivity analyses were performed. First, we adjusted models for cardiovascular diseases (coronary heart diseases, stroke), diabetes, and hypertension. Next, we excluded participants with physician-diagnosed asthma, emphysema, or chronic bronchitis, and participants with methacholine responsiveness. Finally, to adjust for the fact that healthier men are more likely to come back for subsequent visits, we applied inverse probability weighting (Hernan et al. 2006) using logistic regression to calculate the probability of having a subsequent visit given age, education level, body mass index, smoking status, pack-years, hypertension, cholesterol, diabetes, FEV_1 , asthma, emphysema, chronic bronchitis, methacholine responsiveness and air pollutant concentration at previous visit. When estimating interactions between DNA methylation and pollutants, we repeated analyses excluding subjects with chronic lung conditions or taking lung related drugs (corticosteroids, sympathomimetic α and β , anticholinergics).

We then investigated whether DNA methylation in selected genes influences susceptibility by modeling interactions between 28-day moving average air pollutant concentrations and

methylation at individual CpG sites (26 total sites over 9 genes), with methylation dichotomized as high or low at each site based on the median of the distribution (Table 1). In addition, we estimated interactions with high or low methylation based on the average over all CpG sites within each gene. Since methylation of long interspersed nucleotide elements (LINE-1) and Alu repetitive elements has been associated with lung function (Lange et al. 2012), we also modeled interaction of LINE-1 and Alu methylation (dichotomized as high or low based on the median of the distribution) with air pollutant concentrations as a secondary analysis. LINE-1 and Alu methylation each were measured in three replicates and averaged for statistical analysis.

Air pollution, DNA methylation and lung function were all time-varying variables. Models were also adjusted for the percentage of neutrophils, lymphocytes, basophils, eosinophils, and monocytes measured at each visit. Possible mediation of the effects of air pollution on lung function through DNA methylation was tested separately by including DNA methylation variables in the model for the main effects [Equation 1].

P-values < 0.05 were considered statistically significant. All statistical analyses were conducted with SAS version 9.2 (SAS Institute, Cary, NC).

Results

Descriptive results

Participants were mainly white, well educated, and former smokers (Table 2). At first visit, 88% of them were 65 years old or above. Spearman correlation between FVC and FEV₁ was 0.90. Air pollutant concentrations were relatively low in terms of particles with an average of 0.9 ± 0.4 $\mu\text{g}/\text{m}^3$ for BC and 11 ± 7 $\mu\text{g}/\text{m}^3$ for total PM_{2.5} (Table 3). BC concentrations explained 34% of the PM_{2.5} variability, suggesting that the remaining part was explained by non-traffic sources.

The correlation between PM_{2.5} residuals and CO was low ($r = 0.12$). Since CO is a marker of traffic pollution, this low correlation confirms that BC accounts for most of the variability due to traffic PM_{2.5}, and that the PM_{2.5} residuals are a marker of non-traffic particles.

Air pollution and lung function

An interquartile range increase in both total and non-traffic PM_{2.5} concentration from the day before lung function measurement (lag 1) to 28-days cumulated exposure was associated with significantly lower FVC by 0.5 to 2.5% (Figure 1). For BC, only cumulated exposures of 14-, 21- and 28-days were significantly associated with FVC, which was 2 to 5% lower in association with an interquartile range increase. Significant associations with FEV₁ were mainly limited to 28-day moving averages for BC, total PM_{2.5} and non-traffic PM_{2.5}. As for gases, an interquartile range increase in CO or NO₂ concentration from 4- to 28-day moving averages was associated with a significant decrease in FVC by 1 to 5%; We estimated similar results for FEV₁, but restricted to 14- to 28-days moving averages for NO₂. For all pollutants, estimated effect sizes increased with longer averaging times. BC and NO₂ had the largest estimated effects on lung function parameters. Associations with IQR increases in 28-day moving average exposures were significantly stronger in participants with emphysema (for BC, PM_{2.5}, and NO₂ with FVC, and for ozone and FEV₁) and in participants with chronic bronchitis (for ozone and non-traffic PM_{2.5} with both outcomes, and for PM_{2.5} with FEV₁) (see Supplemental Material, Table S2).

Further adjusting for cardiovascular diseases (40% of participants), diabetes (17%), and hypertension (82%) did not change the results (not shown). When participants with asthma, emphysema, chronic bronchitis, or methacholine responsiveness (or missing) were excluded ($n = 254$), model estimates were generally consistent with the main analysis, although p-values were larger. Ozone exposure over lag 1 and 3- to 5-day moving averages was associated with

significantly lower FVC and FEV₁. When further controlling for potential survival bias using inverse probability weighting, results varied slightly (see Supplemental Material, Figure S1). Associations of BC, CO, and NO₂ with lung function were stable or stronger. Associations of O₃, total PM_{2.5}, and non-traffic PM_{2.5} with FVC and FEV₁ were significant for 28-day cumulative exposures only.

Effect modification by DNA methylation

Correlations were relatively high for CpG sites in three of four genes with CpG sites measured at two positions (*CRAT*, *IFN-γ* and *IL-6*), and for one of three pairs of CpG sites in *ICAM-1* ($0.78 \leq r \leq 0.81$, see Supplemental Material, Table S3). For the three genes with 4-5 sites evaluated, there was no evidence of strong correlation between CpG sites within the same gene ($r \leq 0.55$). When further adjusting for DNA methylation levels as potential mediators, associations between lung function and air pollutants (28-days moving average) stayed in the same direction; p-values were slightly larger and sometimes became not significant for some genes such as *OGGI* (not shown).

Associations between IQR increases in 28-day average air pollutant concentrations and the lung function measures were significantly different between participants with high versus low DNA methylation status at several CpG sites (results for interactions that were significant for at least one outcome are shown in Figure 2, complete results are shown in Supplemental Material, Figure S2).

Associations of BC, total PM_{2.5} and non-traffic PM_{2.5} with FVC were significantly stronger among participants with higher methylation at the CpG site measured in *GCR* (Figure 2) and these interactions remained significant when participants with chronic lung conditions or taking

lung related drugs were excluded from the analysis (data not shown). Associations also were significantly stronger among those with higher methylation at one of five CpG sites measured in *F3* (for BC and FVC) and for those with higher methylation at one of two CpG sites measured in *IL-6* (for CO and FVC). Associations of total and non-traffic PM_{2.5} with FVC were significantly stronger among participants with lower methylation at one of the five CpG sites in *TLR-2* (position 2), while the association between NO₂ and FEV₁ was significantly stronger among participants with higher CpG methylation at position 5 in *TLR-2*. We did not observe any statistically significant modification of air pollution effects by LINE-1 or Alu methylation (results not shown).

Discussion

In the elderly population of males examined in the present study, acute exposure to air pollutants (4h, lag 0, lag 1) was generally not significantly associated with lung function, but sub-chronic exposures to all tested pollutants from 3- to 28-days moving averages were significantly associated with lower FVC and FEV₁ (1 to 5% lower per interquartile range increase in air pollution concentrations). Associations with 28-day moving average exposures were stronger in participants with lower methylation levels in one of five CpG sites evaluated in *TLR-2* (position 2), and stronger among participants with higher methylation in *GCR* (one CpG site evaluated), *TLR-2* (position 5), *F3* (position 1), and *IL-6* (position 2). To our knowledge, this is the first study to report associations of short-medium term air pollutant exposures with FVC and FEV₁ in an elderly population, and the first to report evidence of epigene-environment interactions on lung function measures.

A study performed in elderly reported a significant negative association between the peak expiratory flow and PM_{2.5} and PM₁₀ on the same day and up to 4 lagged days (Lee et al. 2007). Epidemiological studies have produced heterogeneous results regarding the lag structure and very few have explored exposure windows up to one month. In adults and school children, lower FVC and FEV₁ were associated with increases in CO, NO₂, PM₁₀, and O₃ from the examination day up to 3 days before (Chang et al. 2012; Hoek et al. 1993; Moshhammer et al. 2006; Schindler et al. 2001). In contrast, other studies in adults have reported no associations of current and previous-day exposures to NO₂, O₃ or particles with FEV₁ or FVC (Peacock et al. 2011; Steinvil et al. 2009; Trenga et al. 2006), and/or delayed associations within 3 to 7 days (Barraza-Villarreal et al. 2008; de Hartog et al. 2009; Hoek and Brunekreef 1993; Steinvil et al. 2009). In terms of effect estimates, Schindler et al. (2001) reported a decrease by 0.4 to 0.8% in FVC and FEV₁ associated with a 10 µg/m³ increase in the 4-days cumulated exposure to NO₂ in never-smoker adults, which is consistent with the 1.1% decrease in FVC we estimated in association with a 15 µg/m³ increase in the 4-day NO₂ moving average. However, such comparisons are usually made difficult by different modeling choices across studies.

Our results mainly suggested adverse effects of traffic pollutants represented by BC, CO, and NO₂ on lung function, while results for non-traffic PM_{2.5} and O₃ were less clear. In Boston, BC is a marker of traffic particles influenced by both local traffic, with a morning peak, and long range transported traffic particles (Park et al. 2007). NO₂, a lower-airway irritant, is mainly a secondary pollutant, which in Boston, mostly originates from traffic and regional sources including power plants and vehicular emissions (Rattigan et al. 2010). There is limited physiologic rationale for an association of CO with reduced lung function. In fact, CO is used therapeutically for acute respiratory distress syndrome. Rather we interpret its association as an indication of traffic co-

pollutants such as ultrafine particles, BC, or NO₂, for which it could serve as a surrogate. In contrast, controlled exposure to diesel exhaust, rich in BC and NO_x, has been shown to produce pulmonary inflammation (Salvi et al. 1999). Associations with non-traffic PM_{2.5} were attenuated when adjusted for potential survival bias, suggesting that non-traffic PM_{2.5} were not or only slightly associated with lung function. While some studies have reported evidence of short-term effects of O₃ on lung function (Chang et al. 2012), others failed to demonstrate an association (Barraza-Villarreal et al. 2008). A previous analysis in the Normative Aging Study covering the period 1995-2005 estimated significantly lower FEV₁ and FVC in association with O₃ increases (Alexeeff et al. 2007). The present analysis with longer follow-up was able to reproduce those results only when participants with chronic lung diseases were excluded. We do not have any biological explanation for this result, but it is in agreement with the results reported by Lagorio et al. (2006) who did not find any effect of O₃ on lung function in adults with pre-existing conditions such as asthma and COPD.

There is a growing body of evidence showing that innate immunity response, and particularly TLRs, are implied in pulmonary inflammation (Poole et al. 2011). TLRs recognize damage-associated molecular patterns and activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which initiate the production of numerous cytokines and host-defense molecules. *TLR-2* is primarily expressed in blood leukocytes and lung (Zarembek and Godowski 2002). Consistent with this, our previous results showed that lower methylation in *TLR-2* was associated with decreased lung function (Lepeule et al. 2012). *TLRs* may also be involved in the development of respiratory diseases (Smit et al. 2009) and in gene-environment interactions. For instance, a placebo controlled intervention study conducted in 916 children from the Netherlands reported that *TLR-2* variants influenced the susceptibility of developing asthma in response to

NO₂ and PM_{2.5} exposure (Kerkhof et al. 2010). By showing that effects of NO₂, total PM_{2.5}, and non-traffic PM_{2.5} on lung function varied significantly according to the methylation status in the promoter region of *TLR-2*, our study provides preliminary support for epigenetic modification of susceptibility to effects of air pollution on lung function. We found inconsistent results between position 2 and position 5 for *TLR-2*. This may be interpreted as evidence against a causal effect of *TLR-2* on susceptibility. Also, Herman and Baylin (2003) have reported that hypomethylated regions in gene promoters are flanked to either side by methylated cytosines. Position 5 in the *TLR2* sequence analyzed may represent a neighboring methylation site not directly involved in *TLR2* gene suppression, yet hypermethylated in active genes.

GCR is an anti-inflammatory gene strongly related to stress (Miller et al. 2008), inflammation (Smoak and Cidlowski 2004) and lung diseases, which encodes a glucocorticoid protein receptor expressed in the lungs (Aceview 2013). The main ligands of GCR are corticosteroids. Upon ligand binding, the activated GCR can switch on anti-inflammatory genes and switch off inflammatory genes that encode signaling molecules and inflammatory receptors, which are regulated by proinflammatory transcription factors such as NF-KB cells and activator protein. Poor glucocorticoid responses related to genetic polymorphisms in *GCR* have been suggested to increase COPD risk and severity (Schwabe et al. 2009). Our results indicated a stronger association between particulate air pollution (BC, PM_{2.5}, non-traffic PM_{2.5}) and reduced lung function in participants with higher methylation at a CpG site in the *GCR* promoter region.

F3 encodes coagulation factor III (i.e. tissue factor), a major player in hemostasis. Previous results suggest that the promoter sequence we studied could regulate the gene (Mackman et al. 2007). We found stronger associations between BC and FVC among individuals with higher methylation at one of five CpG sites measured in *F3*. Similarly, we found stronger associations

between CO and FVC among men with higher methylation at one of two CpG sites evaluated in *IL-6*. *IL-6* encodes a protein that may act as both pro- and anti- inflammatory cytokine and previous results in our cohort did not find any association of IL-6 with lung function (Litonjua et al. 2003).

Altogether, these results suggest that methylation in inflammation- and immunity-related genes might contribute to effect of air pollution. DNA methylation marks are mainly established during early life, and although thought to be a fairly stable measure, DNA methylation has been associated with aging (Madrigano et al. 2012). In our data, almost all associations between air pollutants and lung function remained after adjustment for DNA methylation, which suggests that pulmonary effects of air pollutants were not mediated by methylation. We checked the locations at which we measured DNA methylation on UCSC Genome Build hg19. We specifically designed the assays to exclude SNPs from the CpG sites analyzed and the sequences hybridized by the PCR and PSQ primers. However, because dense genotyping data are not available for this study, we cannot exclude effects from SNPs nearby or remotely located relative to the sequence analyzed. We generally observed stronger associations of air pollutants with FVC than with FEV₁, suggesting that air pollution may affect more smaller airways and therefore restrictive lung diseases. In elderly, particle clearance might be less efficient or impaired by other dysfunctions. The apparently small effects of air pollution on lung function should not be underestimated as they might be well-tolerated by healthy population, but become life threatening for elderly or ill subjects (Gouveia and Fletcher 2000). In addition, repeated occurrences of short-term decrements in lung function and accompanying inflammation may play some role in the development of long-term decrements.

We acknowledge several limitations of our study. First, as often in air pollution studies, we did not account for indoor exposure to air pollutants. Assuming that elderly have reduced outdoor activities, indoor sources might be a larger contributor to personal exposure than in other populations. Under the assumption that indoor and outdoor particles are identical, errors due to indoor exposures have been shown of Berkson type, which is expected to increase standard errors (Zeger et al. 2000). If this assumption of identical indoor and outdoor particles does not hold, the estimated effect would generally be biased downward. Another source of measurement error lies in the low spatial resolution of our exposure model, limited to a few monitoring stations for the entire study area. However, short-term effects studies tend to focus on temporal variation rather than spatial variation. Assuming that daily variation in air pollutant concentrations is homogeneous across the study area might have introduced random noise in exposure estimates, which would tend to underestimate the association with lung function. Therefore it is unlikely that any exposure measurement error would bias the effect away from the null, and this might explain the somewhat lack of statistical significance of our PM_{2.5}-related results. Second, study results were observed in a cohort of elderly men, which may limit the generalizability of our results to similar populations. Third, for the sake of feasibility, we dichotomized DNA methylation at each CpG site to examine interactions with pollutants concentration. Interactions were analyzed for multiple CpG sites, pollutants and outcomes, which urge caution regarding potential false-positive findings. Finally, although we adjusted for the percentage of white blood cell types, we did not have data available for lymphocyte subsets. Therefore, we cannot rule out the possibility that the interactions are with those subset prevalences and not with the methylation per se.

Conclusion

This study adds to the growing body of literature on short- and long-term effects of traffic-related air pollutants mainly studied in children and young adults, by showing that sub-chronic but not acute exposure is associated with lower lung function in the elderly. As for the mechanisms, alongside previous studies on genetic variation in inflammatory and immunity genes and respiratory impairment susceptibility, our results suggest that epigenetic mechanisms related to inflammation and immunity may also be implicated in these associations.

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Table 1. Blood DNA methylation levels (percentage of 5-methylcytosine) in 1515 visits, the Normative Aging Study, 1999-2009.

Gene and position	n	Mean DNA methylation \pm SD	p5	p50	p95
<i>CRAT</i>	1411				
pos1		1.7 \pm 0.6	1.0	1.7	2.6
pos2		4.7 \pm 1.6	2.4	4.6	7.7
mean		3.2 \pm 1.0	1.7	3.2	5.1
<i>F3</i>	1273				
pos1		1.5 \pm 1.8	0.0	1.2	4.2
pos2		1.6 \pm 1.9	0.0	1.2	4.6
pos3		3.0 \pm 2.2	0.0	2.5	6.9
pos4		1.4 \pm 1.6	0.0	1.1	3.9
pos5		4.5 \pm 2.6	0.0	4.2	8.5
mean		2.4 \pm 1.3	0.9	2.2	4.5
<i>GCR</i>	1283				
pos1		47 \pm 6	36	47	55
<i>ICAM</i>	1173				
pos1		5.8 \pm 2.3	2.9	5.5	10.3
pos2		3.5 \pm 2.2	1.7	3.0	9.2
pos3		3.8 \pm 2.0	1.7	3.3	7.3
mean		4.4 \pm 1.8	2.3	4.0	8.1
<i>IFN-γ</i>	1460				
pos1		82 \pm 6	72	84	90
pos2		87 \pm 5	80	88	93
mean		85 \pm 5	76	86	91
<i>IL-6</i>	1469				
pos1		47 \pm 12	27	47	66
pos2		40 \pm 11	22	40	58
mean		43 \pm 10	26	44	62
<i>iNOS</i>	1017				
pos1		53 \pm 8	39	53	66
pos2		83 \pm 9	65	85	97
mean		68 \pm 7	55	69	79
<i>OGG-1</i>	915				
pos1		2.1 \pm 2.6	0.0	1.2	7.4
pos2		2.9 \pm 2.6	0.0	2.3	8.3
pos3		2.2 \pm 2.0	0.0	1.9	6.1
pos4		2.0 \pm 2.2	0.0	1.5	6.5
mean		2.3 \pm 1.4	0.7	1.9	4.9

Gene and position	n	Mean DNA methylation \pm SD	p5	p50	p95
<i>TLR-2</i>	1172				
pos1		2.8 \pm 1.8	0.0	2.5	6.1
pos2		3.5 \pm 2.0	0.0	3.3	7.1
pos3		2.9 \pm 2.0	0.0	2.6	6.3
pos4		3.7 \pm 2.1	0.0	3.4	7.3
pos5		2.0 \pm 1.9	0.0	1.8	5.6
mean		3.0 \pm 1.3	1.2	2.7	5.4

Table 2. Characteristics of the 776 men, the Normative Aging Study, 1999-2009.

Participants characteristics at 1st visit	Statistics
Age, mean \pm SD, yr	72.3 \pm 6.8
Race, n (%)	
Black	14 (1.8)
White	754 (97.2)
Missing	8 (1.0)
Height, mean \pm SD, cm	173.5 \pm 7.0
Weight, mean \pm SD, kg	85.3 \pm 14.3
Education, n (%), yr	
<12	30 (3.9)
12	187 (24.1)
13-15	219 (28.2)
>15	333 (42.9)
Missing	7 (0.9)
Smoking status, n (%)	
Never	220 (28.3)
Current	33 (4.3)
Former	523 (67.4)
Packs years ^a , mean \pm SD	21.6 \pm 26.8
Asthma, n (%)	46 (5.9)
Chronic bronchitis, n (%)	53 (6.8)
Emphysema, n (%)	29 (3.7)
Methacholine responsiveness, n (%)	74 (9.5)
Missing	125 (16.1)
Corticosteroids, n (%)	53 (6.8)
Sympathomimetic (α , β), n (%)	56 (7.2)
Anticholinergic, n (%)	14 (1.8)
FVC, mean \pm SD, liters	3.3 \pm 0.7
FEV ₁ , mean \pm SD, liters 1 st sec	2.5 \pm 0.6
Visits characteristics (n = 1515)	
Season, n (%)	
Spring (March-May)	350 (23.1)
Summer (June-Aug)	430 (28.4)
Fall (Sept-Nov)	490 (32.3)
Winter (Dec-Feb)	245 (16.2)

Participants characteristics at 1st visit	Statistics
Day of the week, n (%)	
Tuesday	71 (4.7)
Wednesday	394 (26.0)
Thursday	824 (54.4)
Friday	226 (14.9)
Visit number, n (%)	
1	776 (51.2)
2	501 (33.1)
3	192 (12.7)
4	46 (3.0)

forced vital capacity = FVC, forced expiratory volume in 1 second = FEV₁.

^aAmong current or former smokers.

Table 3. Environmental characteristics 24 hours before lung function assessment for 1515 visits, the Normative Aging Study, 1999-2009.

Characteristic	mean \pm SD	5 th , 95 th Percentiles	IQR	Corr: BC	Corr: CO	Corr: NO ₂	Corr: O ₃	Corr: PM _{2.5}
Air pollutant ($\mu\text{g}/\text{m}^3$) ^a								
BC	0.9 \pm 0.4	0.5, 1.1	0.6					
CO	502 \pm 285	299, 660	362	0.42				
NO ₂	38 \pm 12	30, 45	15	0.59	0.62			
O ₃	47 \pm 24	28, 60	33	-0.21	-0.29	-0.31		
PM _{2.5}	11 \pm 7	6, 13	7	0.70	0.34	0.52	0.04	
PM _{2.5} non-traffic ^b	0 \pm 5	-3, 1	5	0.18	0.12	0.25	0.24	0.80
Weather								
Temperature ($^{\circ}\text{C}$)	13 \pm 9	7, 20	13					
Relative humidity (%)	68 \pm 16	56, 81	25					

Black carbon = BC, carbon monoxide = CO, interquartile range = IQR, nitrogen dioxide = NO₂, ozone = O₃, particles less than 2.5 μm = PM_{2.5}.

Corr: Spearman correlation coefficients. All p-values were < 0.05.

^aSample sizes were between 1499 and 1515, indicating very few missing data. ^bSince non-traffic PM_{2.5} values are the residuals of the regression of PM_{2.5} against BC, the average was 0.

Figure legends

Figure 1. Percent difference in forced vital capacity (FVC, black circles) and forced expiratory volume in 1 second (FEV₁, grey circles) (and 95% confidence intervals (bars)) associated with one interquartile range increase in air pollutant concentration, the Normative Aging Study, 1999-2009. Depending on the pollutant, the number of observations ranged from 1259 to 1275. Results were adjusted for age, race, height, weight, education level, smoking status, cumulative smoking, season of the medical exam, day of the week, visit number, temperature, relative humidity, asthma, chronic bronchitis, emphysema, methacholine responsiveness, corticosteroids, sympathomimetic alpha and beta, anticholinergics. The interquartile range ($\mu\text{g}/\text{m}^3$) was 0.6 for black carbon, 362 for carbon monoxide, 15 for nitrogen dioxide, 33 for ozone, 7 for total particles < 2.5 μm and 5 for non-traffic particles < 2.5 μm .

Figure 2. Percent difference in forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV₁) (and 95% confidence intervals (bars)) associated with one interquartile range increase in air pollutant concentration (28-days moving average) according to the methylation level (methylation level < median (circles), methylation level \geq median (triangles)), the Normative Aging Study, 1999-2009. *F3* = coagulation factor-3, *GCR* = glucocorticoid receptor, *IL-6* = interleukin-6, *TLR-2* = toll-like receptor-2. Interactions are materialized in black when statistically significant ($p < 0.05$) and in grey otherwise. Depending on the pollutant and the gene, the number of observations ranged from 767 to 1208. Results were adjusted for age, race, height, weight, education level, smoking status, cumulative smoking, season of the medical exam, day of the week, visit number, temperature, relative humidity, asthma, chronic bronchitis,

emphysema, methacholine responsiveness, corticosteroids, sympathomimetic alpha and beta, anticholinergics, and % of white blood cells type. The interquartile range ($\mu\text{g}/\text{m}^3$) was 0.6 for black carbon, 362 for carbon monoxide (CO), 15 for nitrogen dioxide (NO_2), 33 for ozone (O_3), 7 for total particles $< 2.5 \mu\text{m}$ ($\text{PM}_{2.5}$) and 5 for non-traffic $\text{PM}_{2.5}$.

FIGURE1

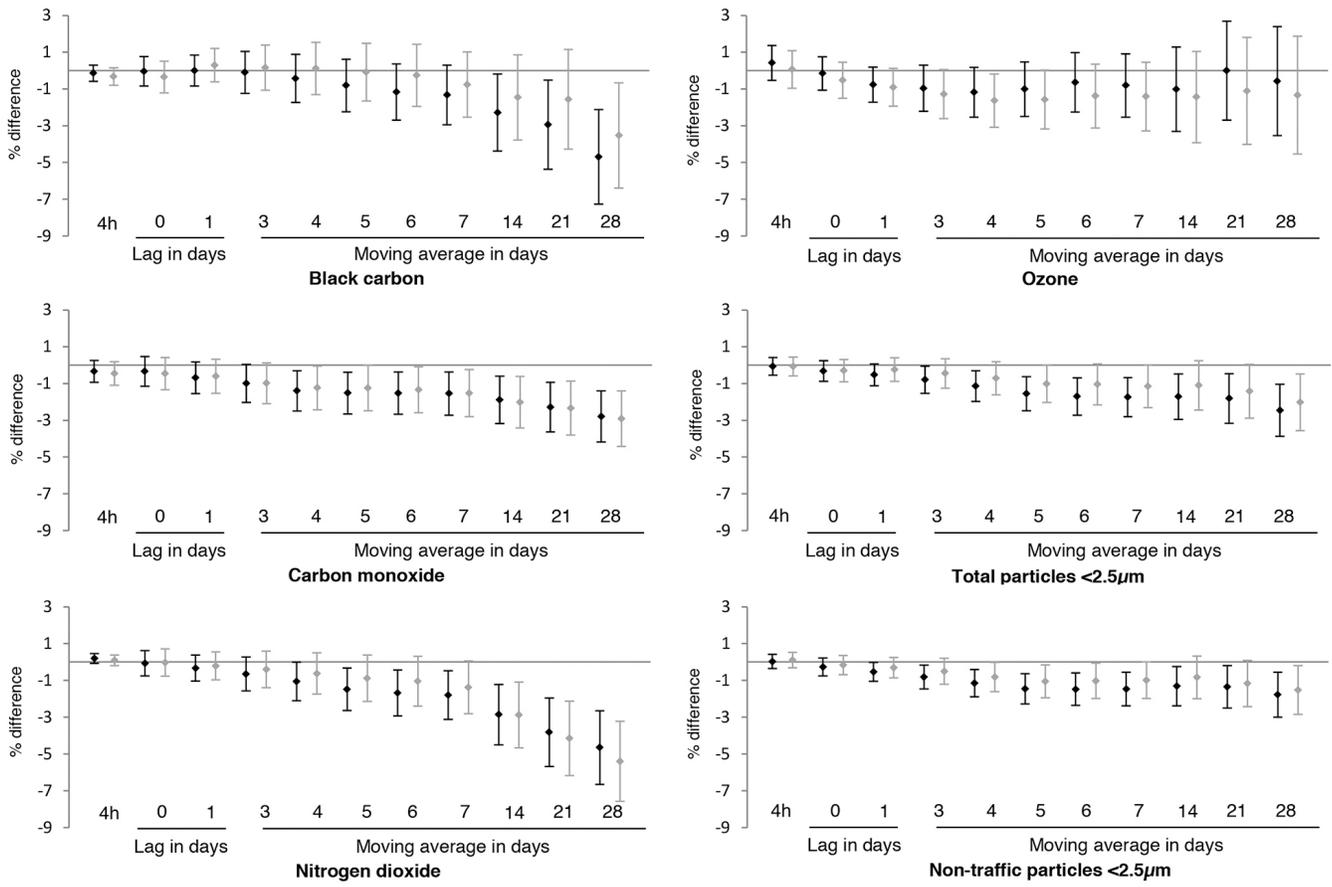


FIGURE 2

