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Mitochondrial DNA content in blood and carbon load in airway macrophages. A panel study in elderly subjects

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Manuscript Details

Manuscript number	ENVINT_2018_99
Title	Mitochondrial DNA content in blood and carbon load in airway macrophages. A panel study in elderly subjects
Article type	Research Paper

Abstract

Background: Mitochondria are sensitive to air pollutants due to their lack of repair capacity. Changes in mitochondrial DNA copy number (mtDNAcn) or content is a proxy of mitochondrial damage and has been associated with recent exposure to air pollution. Inhaled particulate matter (PM) is phagocytosed by airway macrophages (AMs), and black carbon of the phagocytosed PM measured in AM (AM BC) reflects personal pollution exposure. Objectives: The present study investigated the relation between the internal marker AM BC and ambient NO2 concentration and examined the associations of mtDNAcn with NO2 and AM BC. Methods: A panel of 20 healthy retired participants (10 couples) living in Belgium underwent repeated assessments of health and air pollution exposure at 11 time points over one year. We increased exposure contrast temporarily by moving participants for 10 days to Milan, Italy (high exposure) and to Vindeln, Sweden (low exposure). Personal exposure to NO2 was measured during 5 consecutive days prior to each assessment time point. The amount of BC was assessed by image analysis in AMs retrieved from induced sputum collected at 7 time points. Blood mtDNAcn was determined by qPCR at each time point. Associations between AM BC and NO2, and of mtDNAcn with NO2 and AM BC were estimated using linear mixed effect models adjusted for covariates and potential confounders. Results: Mean concentrations of 5-day average NO2 were higher in Milan (64 µg/m3) and lower in Vindeln (4 µg/m3) than Belgium (26 µg/m3). Each 10 µg/m3 increment in NO2 exposure during the last 5 days was associated with 0.07 μm^2 (95% CI: 0.001 to 0.012) increase in median area of AM BC. A 10 µg/m3 increase in NO2 was associated with 3.9% (95% CI: 2.2 to 5.5%) decrease in mtDNAcn. Consistently, each 1 µm2 increment in median area of AM BC was associated with 24.8% (95% CI: 6.8 to 39.3%) decrease in mtDNAcn. Conclusion: In this quasi-experimental setting involving moving persons to places with high and low ambient air pollution, we found changes in AM BC according to ambient air pollution levels measured during the previous 5 days. Both higher ambient NO2 and the internal lung BC load, paralleled mitochondrial compromises as exemplified by lower mtDNA content.

Keywords	Mitochondrial DNA copy number; black carbon; airway macrophages; nitrogen dioxide
Taxonomy	Biomarkers, Mitochondrial DNA, Air Pollution, Exposure Assessment

Corresponding Author	Tim Nawrot
Corresponding Author's Institution	Hasselt University
Order of Authors	Yang Bai, Lidia Casas, Hans Scheers, Bram Janssen, Benoit Nemery, Tim Nawrot
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Dear Editor,

Please, find attached a manuscript entitled *'Mitochondrial DNA content in blood and carbon load in airway macrophages. A longitudinal study in elderly subjects'*, which we would like to submit for publication in *Environment International*.

Mitochondria have been shown to be sensitive to environmental insults, which are considered to play a central role on the axis of oxidative stress, inflammation, and cellular energy production. During a one-year follow-up period, we studied, in a quasi-experimental design, subacute changes in blood mitochondrial DNA (mtDNA) content of healthy old volunteers with contrasting exposures by moving to high (Milan) and low (Northern Sweden) polluted European spots. The blood mtDNA content was inversely associated with the internal exposure marker, carbon load in airway macrophages. Moreover, the changes of airway carbon load was in response to 5-day ambient NO_2 concentrations.

Our findings demonstrate that changes in personal exposure parallels mitochondrial function and that higher exposure compromises the function of the mitochondria within 5 days. Therefore, we believe that our manuscript merits publication in a leading scientific journal, such as *Environment International*.

For your information, we have uploaded as supplemental file a manuscript on the same participants, which describes the recruitment in more detail and made reference to in our paper.

We hope you find our manuscript interesting and we look forward hearing from you.

Sincerely yours,

Tim Nawrot Yang Bai Benoit Nemery
On behalf of all authors

Highlights

- Personal exposure to air pollution was assessed by external and internal markers.
- Repeated measures over 1-year and changing places to contrast exposures
- Carbon load in airway macrophages was associated with ambient NO_2 over a 5-day period.
- Decreased blood mitochondrial DNA content in response to higher airway carbon load.

Mitochondrial DNA content in blood and carbon load in airway macrophages. A panel study in elderly subjects

Yang Bai [a]

Email: yang.bai@kuleuven.be

Lidia Casas [a]

Email: lidia.casasruiz@kuleuven.be

Hans Scheers [a]

Email: hansscheers@hotmail.com

Bram G. Janssen [b]

Email: bram.janssen@uhasselt.be

Benoit Nemery [a]

Email: ben.nemery@kuleuven.be

Tim S. Nawrot [a, b] *

* Corresponding author

Tel. +32-11-268382

E-mail: tim.nawrot@uhasselt.be

[a] Center for Environment and Health, Department of Public Health and Primary Care, KU Leuven, Herestraat 49, 3000, Leuven, Belgium

[b] Centre for Environmental Sciences, Hasselt University, Campus Diepenbeek, Agoralaan Gebouw D, 3590 Diepenbeek, Belgium

Abstract

Background: Mitochondria are sensitive to air pollutants due to their lack of repair capacity. Changes in mitochondrial DNA copy number (mtDNAcn) or content is a proxy of mitochondrial damage and has been associated with recent exposure to air pollution. Inhaled particulate matter (PM) is phagocytosed by airway macrophages (AMs), and black carbon of the phagocytosed PM measured in AM (AM BC) reflects personal pollution exposure.

Objectives: The present study investigated the relation between the internal marker AM BC and ambient NO_2 concentration and examined the associations of mtDNAcn with NO_2 and AM BC.

Methods: A panel of 20 healthy retired participants (10 couples) living in Belgium underwent repeated assessments of health and air pollution exposure at 11 time points over one year. We increased exposure contrast temporarily by moving participants for 10 days to Milan, Italy (high exposure) and to Vindeln, Sweden (low exposure). Personal exposure to NO_2 was measured during 5 consecutive days prior to each assessment time point. The amount of BC was assessed by image analysis in AMs retrieved from induced sputum collected at 7 time points. Blood mtDNAcn was determined by qPCR at each time point. Associations between AM BC and NO_2, and of mtDNAcn with NO_2 and AM BC were estimated using linear mixed effect models adjusted for covariates and potential confounders.

Results: Mean concentrations of 5-day average NO_2 were higher in Milan (64 $\mu g/m^3$) and lower in Vindeln (4 $\mu g/m^3$) than Belgium (26 $\mu g/m^3$). Each 10 $\mu g/m^3$ increment in NO_2 exposure during the last 5 days was associated with 0.07 μm^2 (95% CI: 0.001 to 0.012) increase in median area of AM BC. A 10 $\mu g/m^3$ increase in NO_2 was associated with 3.9% (95% CI: 2.2 to 5.5%) decrease in mtDNAcn. Consistently, each 1 μm^2 increment in median area of AM BC was associated with 24.8% (95% CI: 6.8 to 39.3%) decrease in mtDNAcn.

Conclusion: In this quasi-experimental setting involving moving persons to places with high and low ambient air pollution, we found changes in AM BC according to ambient air pollution levels measured

during the previous 5 days. Both higher ambient NO_2 and the internal lung BC load, paralleled mitochondrial compromises as exemplified by lower mtDNA content.

Keywords: Mitochondrial DNA copy number, black carbon, airway macrophages, nitrogen dioxide

1. Introduction

Combustion-derived black carbon (BC), which serves as a surrogate for traffic-related particles, has been identified as a major risk factor for air pollution-triggered adverse health outcomes, particularly in vulnerable populations including the elderly (Brook et al., 2010; Ostro et al., 2015; Samoli et al., 2016). Recent exposure to BC is likely linked to inflammation through the generation of reactive oxygen species (ROS) and oxidative stress (Hou et al., 2013; Lin et al., 2015; Zhong et al., 2016). The abnormal signaling triggers an adaptive response through an overproduction of mitochondria, a major source of ROS (Malik and Czajka, 2013; Michel et al., 2012). The excess ROS can, in turn, damage the mitochondrial DNA (mtDNA) resulting in chronic inflammation (Malik and Czajka, 2013). The number of mitochondria in a cell varies from hundreds to a few thousands, each of which carries 2 to 10 copies of mtDNA (Malik and Czajka, 2013; Wei and Lee, 2002). The mtDNA copy number (mtDNAcn), measured as a ratio of mtDNA to nuclear DNA, is correlated with the size and number of mitochondria, which can change due to environmental stressors (Lee and Wei, 2005). Blood or tissue mtDNAcn has been shown to correlate with exposure to ambient particulate matter (PM) (Hou et al., 2010) and BC (Hou et al., 2013; Zhong et al., 2016), both in occupational settings (Hou et al., 2013, 2010) and due to prenatal exposure (Janssen et al., 2012; Rosa et al., 2017). These findings suggest that mtDNAcn, reflecting mitochondrial dysfunction, may serve as a marker to represent a biological effect along the pathway of PM-induced health effects.

Li *et al.* (2003) illustrated that the uptake of environmental ultrafine particles in phagocytes could induce major structural damage in mitochondria and, therefore, might contribute to oxidative stress. Fossil fuel exhaust is the primary source of ultrafine carbonaceous particles that form environmental PM. Carbonaceous PM can be inhaled and deposited along the respiratory tract in a size-dependent manner (Saxena et al., 2008). These particles are phagocytosed by airway macrophages (AMs) and retained in the cytoplasm, which can be visualized with microscopy (Bai et al., 2015). In adults, the area of phagocytosed black carbon in AM (AM BC) reflects the past PM exposure. However, the

relevant exposure window that influences the carbon in AM is not established. Both long-term (Belli et al., 2016; Jacobs et al., 2010) and short-term (Belli et al., 2016; Nwokoro et al., 2012) exposure windows have been reported so far.

We conducted a panel study with semi-controlled exposure to both high and low levels of air pollution that differed widely from the subject’s residence. With this design, we sought to examine how the AM BC reflects the change in ambient air pollution and to investigate whether blood mitochondrial DNA content is associated with air pollution exposures.

2. Methods

2.1. Study design

As described in detail in another article (Scheers et al. submitted for publication, see supplementary material for review). We designed a panel study including a quasi-experimental design with successive “medium-high-medium-low-medium” air pollution levels. To achieve such exposure contrast both temporally and spatially, we included 20 healthy elderly (10 couples) who lived in Flanders, Belgium, representing an intermediate level of pollution (annual average PM_{10}: 20 – 30 $\mu m/m^3$). This study ran from September 2013 to September 2014, during which two 10-day group trips were organized, one from October 6 to 17, 2013 to Milan, Italy, representing high exposure (annual average PM_{10}: 40 – 50 $\mu m/m^3$) and the other one from June 1 to 12, 2014 to Vindeln, Sweden, representing low exposure (annual average PM_{10} < 10 $\mu m/m^3$) (EEA 2012) (Figure 1). During the trips, the study subjects were encouraged to do outdoor touristic activities in the urban area in Milan and in the rural nature in Vindeln.

During the whole study, we collected data over 11 measurement time points for multiple health endpoints and exposures, with sputum induction being performed on 7 time points (Figure 1). All the clinical measurements were performed at the University Hospital in Leuven, the Ospedale Maggiore in Milan, or Umeå University in Umeå (50 km from Vindeln).

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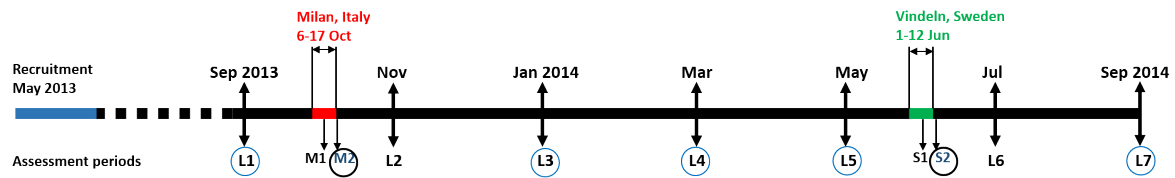

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305 100 **Figure 1.** Timeline of the study. Health assessments were performed in Leuven (L1 to L7), in Milan
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307 101 (M1 and M2), and in Vindeln (S1 and S2). All variables were measured in 20 subjects on 11 time
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309 102 points, except for sputum induction, which was performed on 7 time points.
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312 103 ◯ Sputum induction was performed together with other measurements.
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315 104 ◯ Sputum induction was performed in Leuven within three days after return from the trip.
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321 106 **2.2. Subjects**
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323 107 We recruited a convenience sample of healthy elderly man-woman couples. Enrolment required
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325 108 each candidate to attend an interview including a questionnaire on general health and
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327 109 sociodemographic characteristics, and a physical examination to ensure that the candidate
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329 110 participants were in good general health. Inclusion criteria included an age range from about 60 to
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331 111 about 75, retired or available to travel during the study period, fluent in Dutch, being non-smokers
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333 112 (or having quit at least one year), and with each partner fulfilling the inclusion criteria. Exclusion
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335 113 criteria were a history of serious cardiovascular disease or cancer, and other diseases that could
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337 114 interfere with the health measurement, as well as mobility problems or unstable mental health that
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339 115 would prevent the subject from full participation. Eventually, we selected 10 male-female healthy
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341 116 retired couples aged 58 to 76 years old at recruitment. All subjects were given detailed oral and
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343 117 written information on the study and gave written informed consent. This study was approved by the
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345 118 Ethical Committee of KU Leuven (S55482).
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2.3. AM Carbon quantification

2.3.1. Induced sputum

Spirometry was performed according to standard guidelines (Miller et al., 2005) using an EasyOne spirometer (ndd Medical Technologies, Inc., MA, USA). Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were recorded. Subjects with post-bronchodilator FEV_1 ≥ 80% underwent sputum induction according to a standard protocol (Pizzichini et al., 1996). Nebulized saline (3, 4, and 5%) was administered through De Vilbiss nebuliser (Ultra-Neb 2000 model 200HI) in 3 sequential 7-minute inhalation periods. Lung function was measured before each inhalation period for the detection of clinically significant bronchoconstriction. Induced sputum was processed within 2 hours after induction. Briefly, the sputum plugs were selected and weighed. A volume of Hanks' balanced salt solution containing 0.1% dithiothreitol (Sigma, St Louis, MO, USA) and 3% bovine serum albumin (Sigma) of four times the weight was added. Portions were agitated with a vortex, placed on a bench rocker for 5 minutes, filtered through a 70 µm Falcon cell strainer, and centrifuged at 1500 rpm for 10 minutes. The sputum supernatant was removed and stored at -80 ˚C for cytokine analysis (not reported here). The cell pellet was resuspended in 1000 µl phosphate-buffered saline. A total nonsquamous cell count was performed in a hemocytometer and expressed as millions per milliliter of selected induced sputum. The proportion of salivary squamous cells was noted and cell viability was determined by trypan blue exclusion method. Cytospins were prepared by cytocentrifuging (Shandon Scientific, Techgen, Zellik, Belgium) 15,000 cells onto glass slides and stained with Diff-Quik (Medion Diagnostics, Düdingen, Germany).

2.3.2. Image analysis

Twenty subjects attended for sputum induction in all sessions, except session M2 at which 14 subjects attended. Due to inappropriate storage (n = 10) and failure to produce adequate sputum (n = 28), we obtained 96 samples in total. Among the 96 samples, only 63 contained a sufficient number of AMs (≥ 50) for assessing carbon load.

The area of carbon in AM was determined as previously described (Jacobs et al., 2010). Briefly, digital images of 50 randomly selected AM from each cytospin slide were obtained at ×100 magnification. Color images were converted to 32-bit black and white images using ImageJ (National Institutes of Health, USA). Automatic "threshold" command and freehand selection were combined to select the black particles that were within the cell. The software generated a number of pixels which were converted to an area in micrometers squared (for our analysis: 146 pixels = 10 µm at ×100 magnification). The median area (μm^2) from 50 AM in each sputum sample was calculated and used for the statistical analyses.

2.4. Mitochondrial DNA content

Genomic DNA was isolated from buffy coat of venous blood stored in EDTA tubes using the QIAamp® DNA minikit (Qiagen GmbH, Hilden, Germany). The yield (ng/µL) and purity ratios (A260/280 and A260/230) of the extracted DNA were determined with the NanoDrop spectrophotometer (2000c, Thermo Scientific). The mtDNA content was determined using a quantitative real-time PCR (qPCR) assay by taking the ratio of two mitochondrial gene copy numbers (MTF3212/R3319 and MT-ND1) to two single-copy nuclear reference genes (RPLP0 and ACTB) as previously described (Janssen et al., 2012). Base software (Biogazelle, Zwijnaarde, BE) was used to normalize data and correct for run-to-run differences.

2.5. Environmental pollution data

Personal exposure to environmental NO_2 was monitored using Radiello diffusive samplers (Sigma-Aldrich, Bellefonte, PA, USA). Sampling period was defined as 5 days prior to each health assessment day in Leuven and to the second health assessment day in Milan and Vindeln. The subjects wore the clip-on device moving around during the day, while at night, the sampler was placed next to the bed. After each sampling period, the samplers were collected and sent to the laboratory of the Fondazione Salvatore Maugeri (Padova, Italy) for calculating the exposure to NO_2. NO_2 exposure was expressed as the average concentration ($\mu g/m^3$) over 5 days (Gerboles et al. 2000).

Meteorological data including daily mean temperature and relative humidity during sampling periods were obtained from the local meteorological websites for Belgium (Meteo België. 2016), Milan (Il Meteo. 2016), and Umeå (Weather Underground. 2016).

2.6. Statistical analysis

The mtDNAcn was natural log-transformed to better approximate a normal distribution. For comparisons of means, and proportions we applied Student's t-test, Mann-Whitney test, and the chi-square-statistic. The associations of AM BC with NO_2, and of mtDNAcn with NO_2 and AM BC were analyzed using linear mixed-effect models with random intercept for each subject to account for the repeated measures design of the study. Previous research showed that AM BC is positively associated with white blood cells (WBC) (Jacobs et al., 2010). Besides, the mtDNAcn might be affected by the contents of WBC and platelets (Knez et al., 2015). To investigate the associations between mtDNAcn and exposures, we adjusted the models for age, sex, and WBC. In sensitivity analyses, first we added the platelet-lymphocyte ratio to the mtDNAcn models to account for potential changes in blood composition, and second we excluded all subjects reporting having a cold at the moment of blood sampling. For the association between AM BC and NO_2, we included all 20 subjects. For the associations between mtDNAcn and exposures, we excluded one observation with an outlier mtDNAcn, and one subject was excluded from all time points because he started using corticosteroids during the follow-up. Since the dependent variable (mtDNAcn) was natural-log transformed, the resulting regression coefficients and their 95% confidence intervals (CI) were transformed to $[\exp(\beta)-1]\times100$. This transformation allows interpreting the coefficient as the percentage of increase in mtDNAcn.

All statistical analyses were performed using IBM SPSS version 24 (Armonk, NY, USA) or SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

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534 196 ## 3. Results
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536 197 ### 3.1. Characteristics of participants
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539 198 Ten male-female couples, 20 subjects in total, started the study in September 2013 and completed
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541 199 the study in September 2014, without dropout nor missed measurement period. Their baseline
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543 200 characteristics are shown in Table 1.
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546 201 **Table 1** Description of the study population at baseline (N = 20) †
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Characteristics	All subjects N = 20	Men N = 10	Women N = 10	P-value ‡
Age, years	65 (58 – 76)	68 (58 – 76)	64 (59 – 70)	0.29
BMI, kg/m^2	24.3 (18.9 – 29.4)	25.2 (18.9 – 29.4)	23.5 (19.2 – 29.1)	0.73
Smoking status, n (%)				0.66 [#]
Never/former	10/10	4/6	6/4	
AM BC at L1 (µm^2)*	0.346 (0.314)	0.348 (0.368)	0.340 (0.113)	0.64 [§]

549–563
564 202 AM BC, carbon load in airway macrophages.
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566 203 † All values are median (range) except for * mean (SD).
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569 204 ‡ P-value for Student t-test comparing males to females except for [#] Fisher exact test and [§] Mann-
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576 207 ### 3.2. 5-day average NO_2
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579 208 Personal 5-day average NO_2 levels are presented in Figure 2. We obtained the highest and lowest
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581 209 levels of NO_2 in Milan and Vindeln, respectively, being significantly different ($p < 0.001$) from the
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583 210 exposure at their residence in Belgium. We observed minor variations (coefficient of variation ranged
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585 211 from 18 to 38%) in NO_2 among the Leuven measurements (Figure 2).
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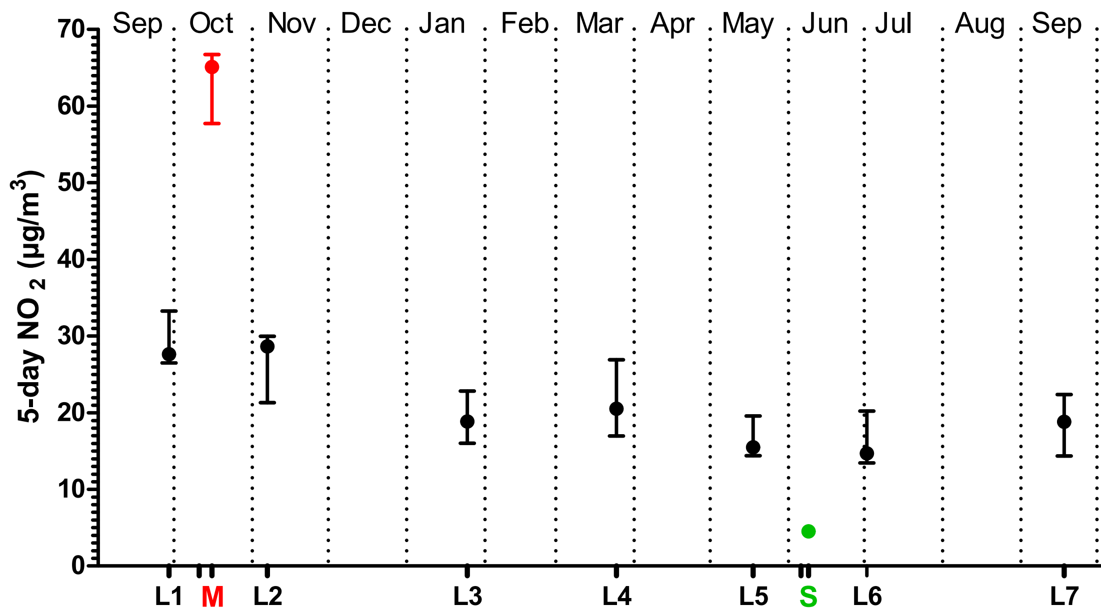

Figure 2 Median (IQR) of 5-day average NO_2 concentrations (n = 6 – 10, depending on the period). L1 to L7 were measured in Leuven, Belgium. M and S were measured in Milan, Italy and Vindeln, Sweden, respectively.

3.3. Carbon load in AMs

The individual success rate of sputum induction varied from 0 to 100%, yielding a mean (SD) success rate 72.9 (26.1) % for individuals. In comparison, the success rate at each time point varied from 62.5% to 87.5%, yielding a mean (SD) success rate 76.0 (9.0) %.

AM BC varied greatly (coefficient of variation ranged from 40 to 117%) throughout the study period (Figure 3). AM BC was 0.54 (95% CI: 0.15 to 0.93) μm^2 higher immediately after the trip to Milan (M2) than the first measurement in Leuven (L1) and remained somewhat higher but not significantly at L3, 12 weeks later. Immediately after the trip to Sweden (S2), AM BC was unchanged compared to the previous time point (L5) and underwent a minor nonsignificant decrease 12 weeks later (L7). Comparing AM BC measured in Leuven, none of the following measurements (L3 – 7) statistically differed from L1. No statistically significant differences in AM BC were detected between any two

Leuven measurements, except for L3 and L7, when AM BC at L7 was 0.48 (95% CI: 0.13 to 0.84) μm^2 lower than L3 (Figure 3).

We found significant associations between the indices of external exposure (5-day NO_2) and internal exposure (AM BC): each 10 $\mu g/m^3$ increase in 5-day average NO_2 was associated with an increase in AM BC of 0.07 (95% CI: 0.001 to 0.012) μm^2.

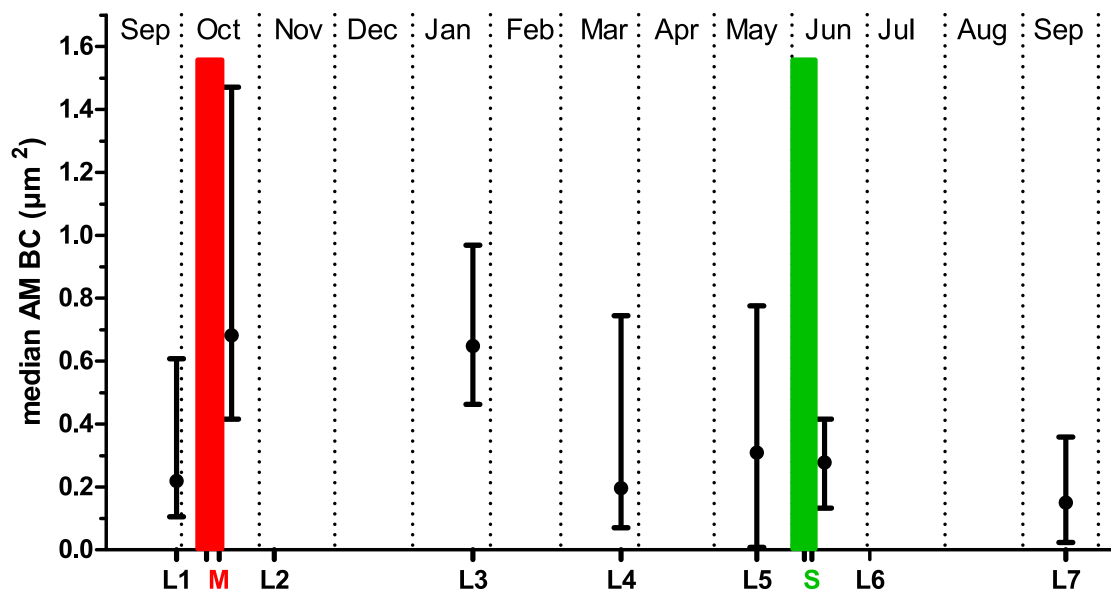

Figure 3 Median with IQR area of carbon load in airway macrophages on each average day of measurement. Bars and dots represent the IQR and median values, respectively. The red bar and green bar represent the period staying in Milan (Italy) and Vindeln (Sweden), respectively. L1 to L7 were measured in Leuven, Belgium (no measurements for L2 and L6). M and S were measured in Leuven within 3 days after returning from Milan and Vindeln, respectively (n = 5 – 14, depending on the period).

3.4. Blood mtDNA copy number

Compared with baseline levels in Belgium the blood mtDNAcn decreased significantly during the stay in Milan (M1 *versus* L1, -23.7%; 95% CI: -40.8 to -12.0%, Figure 4). After the return to Belgium the

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244 mtDNAcn (L2 to L5) was restored partially but was still lower than L1. Moving to a lower exposure
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245 area in Sweden was accompanied by a minor non-significant increase in mtDNAcn (S1 and S2), but a
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246 further increment in mtDNAcn was observed upon return in Belgium after 10 days in Sweden,
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247 resulting in higher levels [L6, 19.4% (95% CI: 4.1 to 34.8%); L7, 16.7% (95% CI: 1.4 to 32.1%)] than the
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248 baseline L1 (Figure 4).
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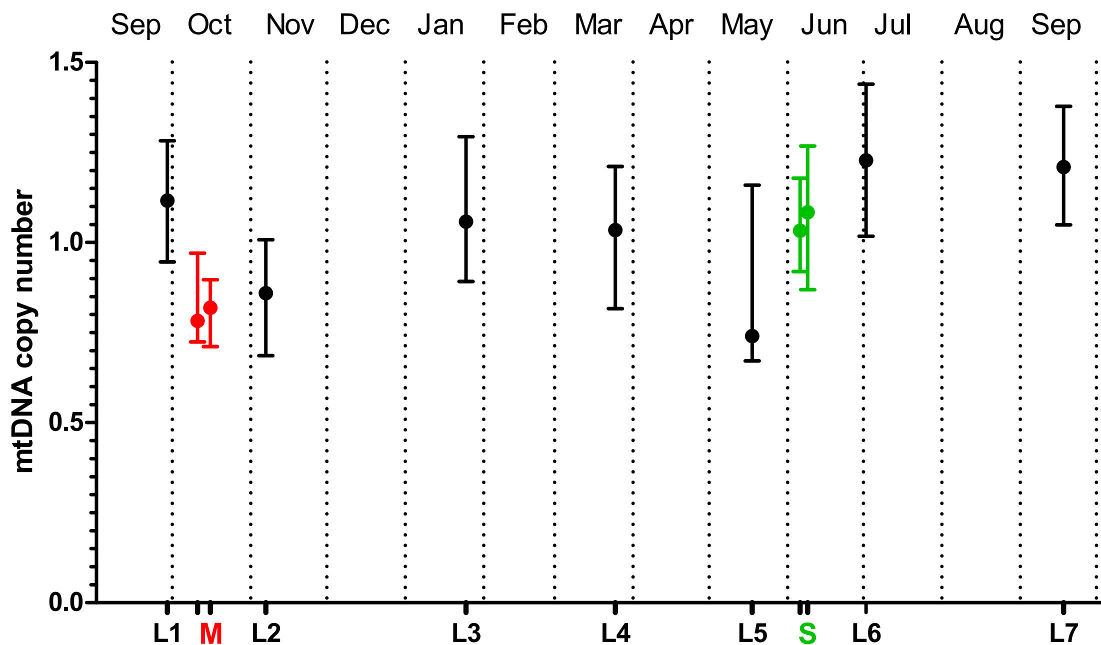

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250 **Figure 4** mtDNA copy number in blood on each average day of measurement. Bars and dots
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251 represent the IQR and median values, respectively. L1 to L7 were measured in Leuven, Belgium. M
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252 was measured in Milan, Italy. S was measured in Vindeln, Sweden.
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254 **3.5. Blood mitochondrial DNA content in association with external and internal exposure**
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255 The associations between mtDNAcn and both external and internal exposures to air pollution are
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256 presented in Table 3. Results shown are those obtained by models adjusted for temperature, sex,
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257 age, and WBC.
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258 Blood mtDNAcn was inversely associated with both 5-day average NO_2 and AM BC. For example,
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259 mtDNAcn was 3.9% (95% CI: 2.2 to 5.5%) lower for each 10 µg/m^3 increment in 5-day average NO_2,
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and 24.8% (95% CI: 6.8 to 39.3%) lower for each 1 μm^2 increase in AM BC, indicating a reduction in mtDNAcn with increasing air pollution exposure (Table 2).

To test the robustness of our results, we further adjusted for platelet/leukocytes ratios. This additional adjustment did not substantially change estimates between the original model (Table 2). Furthermore, excluding the observations of persons reporting having a cold did not alter the reported associations (Table 2).

Table 2 Adjusted # relative changes (%) with their 95% CI in mtDNA for a 10 $\mu g/m^3$ increase in 5-day cumulative NO_2 and for a 1 μm^2 increase in median area of AM BC.

	Number of observations	Adj I #	Adj II#	Adj I# excluding individuals with cold§
NO_2†	204	-3.9 (-5.5, -2.2)***	-3.7 (-5.3, -2.1)***	-3.3 (-5.0, -1.5)**
AM BC	54	-24.8(-39.3, -6.8)*	-22.3 (-36.7, -4.5)*	-22.7 (-37.6, -4.3)*

AM BC, carbon load in airway macrophages.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Adj I: adjusted for sex, age, and white blood cells; Adj II: adjusted for Adj I and platelet/lymphocyte and platelet/neutrophil.

† Models additionally adjusted for temperature.

§ Number of observations for NO_2 was 177 and for AM BC was 51.

4. Discussion

Changes induced by air pollution include oxidative stress, inflammation, and altered cellular energy production. Mitochondria have been shown to be sensitive to environmental insults and are considered to play a central role on the axis of oxidative stress, inflammation and cellular energy

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829 279 production. During a 1-year follow-up period, we studied subacute changes in blood mtDNA content
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831 280 of healthy older volunteers semi-experimentally exposed to contrasting exposures by moving to high
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833 281 and low polluted spots. The airway carbon load changed rapidly after a brief increase in pollutant
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835 282 exposure and was inversely associated with blood mtDNA content.
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838 283 **4.1. AM BC as an internal exposure marker**
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841 284 The present study builds on prior epidemiologic studies that have revealed the relation between AM
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843 285 BC and particulate pollutants. Increased AM BC area was reported to be associated with residentially
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845 286 modeled annual average PM_{10} (Kulkarni et al., 2006) and 6-month average PM_{10} (Jacobs et al. 2010).
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847 287 However, in another study that compared AM BC content in London cyclists and non-cyclist,
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849 288 Nwokoro *et al.* (2012) found that increased AM BC in cyclists was only associated with ambient BC
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851 289 during commuting time, reflective of recent past exposure. A recent study added new findings to the
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853 290 reflection of exposure timing of AM BC, which indicated AM BC content was associated with not only
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855 291 3-month but also 1-week monitored indoor $PM_{2.5}$ (Belli et al., 2016). These inconsistent results
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857 292 suggest that the time window of exposure reflected by AM BC remains ill-defined.
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860 293 Here, we observed an immediate increase in AM carbon load after the trip to Milan and possibly a
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862 294 delayed decrease in AM carbon load after the trip to Sweden. These results suggest that: 1) clearing
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864 295 particles may take more time than uptake of particles; 2) the two mechanisms, clearance and uptake,
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866 296 interact thus resulting in a delay in responding to environmental change. However, AM BC content
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868 297 measured in Leuven at later time points did not statistically differ from the measurement at L1
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870 298 (Figure 3). These results are compatible with an independent panel study that we performed among
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872 299 healthy young subjects from various countries (Bai et al., submitted for publication). In that study, we
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874 300 found that AM BC reflects the average PM_{10} exposure of the past year, and that AM BC decays with
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876 301 an initial half-life of about 53 days when moving from a high pollution level to a moderate pollution
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878 302 level, whereas in the Belgian residents, we observed a steady status of AM BC. Taken together, it
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seems that AM BC is rapidly sensitive (a few days) to even a briefly increased exposure and is only slowly sensitive (a few weeks) to decreased exposure.

4.2. Associations between exposures and mtDNAcn

In our quasi-experimental design the blood mtDNA content, a measure of mitochondrial function, paralleled the AM carbon load. These findings in elderly are in agreement with those in two birth cohorts indicating that higher prenatal exposure to NO_2 (Clemente et al., 2015) or particulate air pollution (Janssen et al., 2012) during the last trimester of pregnancy was associated with lower placental mtDNAcn.

On the contrary, in a study of 675 elderly individuals, every standard deviation (SD) increase in 5-day BC moving average was found to be associated with 0.12 SD increase in blood mitochondrial DNA content (Zhong et al., 2016). In a study of 166 elderly, monthly averaged residential exposure to $PM_{2.5}$ was associated with higher mtDNAcn while annual average residential exposure to $PM_{2.5}$ was associated with lower mtDNAcn (Pieters et al., 2015). Taken together, the above mentioned findings suggest that exposure windows and concentrations, and studied tissues, are important to regulate the PM-associated formation of ROS and inflammation. Hou and coworkers showed that finer particles, EC (Hou et al., 2013) and PM_1 (Hou et al., 2010), resulted in greater changes in mtDNAcn than larger particles. Along similar lines, our study indicated that AM BC was associated with a greater effect in mtDNAcn than external NO_2 (IQR change in exposure being associated with -15.0% vs -7.2% change in mtDNAcn, respectively).

The discrepancy in the results of mtDNA content, as to direction and effect-size, can be explained by the dynamic nature of mtDNA. Mitochondrial DNA fluctuates under the influence of age, ethnicity, tissue investigated, but most importantly depends on oxidative stress level, cell antioxidant capacity, type of environmental factor, and dose of exposure (Castegna et al., 2015; Shaughnessy et al., 2014). The current hypothesis is that mild oxidative stress may stimulate mtDNA copy number synthesis and abundance as a compensatory mechanism, while escalating oxidative stress levels may result in

decreased or no synthesis due to severe oxidative damage in cells (Lee and Wei, 2005). Taken this hypothesis into account, we suggest that a cumulative exposure to high concentrations of NO_2 and BC leads to clearance of cells with highly damaged or dysfunctional mitochondria. Similarly, the relative mtDNA content was increased in the lung tissues of light smokers but significantly decreased in heavy smokers (Lee et al., 1998).

4.3. Strengths and limitations

The major strength of our study is its design. We took the advantage of the geographical variation in air pollution in different regions in Europe and deliberately exposed the participants to a wide range of air pollution levels. This design gave us the opportunity to examine the exposure-response relationship over a wide exposure range. In addition, we measured personal exposure to NO_2 using clip-on devices thus allowing a positive relation to be detected between AM BC content and personal measured NO_2. This finding is in agreement with the relation between AM BC and external ambient BC concentration reported in prior studies (Bai et al., 2015; Nwokoro et al., 2012). Our study contributes to accumulating evidence to show the feasibility of using AM BC as an internal marker for personal exposure assessment.

This study also has limitations. Firstly, the sample size (n = 20) was small. Although we performed 11 times health measurements, some observations were excluded from analysis because some measurements, for example induced sputum, were not obtained at all time points, mainly due to technical limitations. On the other hand, we obtained a unique dataset including 1-year follow-up of volunteers with on average 11 measurements of mtDNA content and 7 measurements of AM BC per volunteer. Secondly, although the use of personal diffusive samplers provided information on individual NO_2 exposure, the concentrations of NO_2 were averaged over 5 days and we could not differentiate daily concentrations. Therefore, it is not possible to study whether the observed effects were caused by the most recent exposure or by cumulative past exposure.

5. Conclusion

In a panel of 20 elderly subjects, we showed that average past 5-day average NO_2 exposure was positively associated with BC content in airway macrophages. By use of these personal markers of exposure, within a semi-experimental setting, we showed that blood mtDNA content was inversely associated with external 5-day average NO_2 exposure and internal AM BC content. These findings suggest that 1) internal AM BC is an effective exposure marker to study the PM-effects relations, and 2) blood mtDNA content is a proxy to indicate mitochondrial damage induced by recent environmental exposures.

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Competing interests

The authors declare that they have no competing interests.

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SUPPLEMENTARY FILE

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The supplementary file contains confidential contents only available for the reference of the editors and reviewers.

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Changing places to study acute and subacute effects of air pollution on cardiovascular health

Hans Scheers Ph.D.[1], Lidia Casas Ph.D., M.D.[1], Tim S. Nawrot Ph.D.[1,2], and Benoit Nemery Ph.D., M.D.[1]

[1]Environmental Health Unit, Department of Public Health and Primary Care, KU Leuven, Belgium

[2]Centre for Environmental Sciences, UHasselt, Belgium

Corresponding author:

Benoit Nemery

KU Leuven, Department of Public Health and Primary Care

Herestraat 49, O&N I, PB 706

3000 Leuven

Belgium

Tel. +32 16 330801

e-mail : ben.nemery@kuleuven.be

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Key Points

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Question: What is the influence of moving persons to varying levels of ambient air pollution on arterial carotid stiffness and other indicators of cardiovascular health?

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Findings: In a panel study with 10 male-female couples of healthy elderly volunteers, we found significant associations between 7-days exposure to air pollution and arterial stiffness, e.g. a 4.4% decrease in compliance for a 10 µg/m^3 increment in PM_{10}.

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Meaning: Our experiment shows that short to medium-term exposure to elevated or decreased levels of air pollution affects arterial stiffness in elderly people.

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Abstract

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Importance: Exposure to air pollution is associated with cardiovascular disease. Health outcomes associated with temporal changes in exposure may inform on health benefits of permanent decreases of air pollution levels.

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Objective: To evaluate acute and subacute effects of deliberate exposure to varying levels of ambient air pollution on several indicators of cardiovascular health.

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Design: In a panel study, we repeatedly measured cardiovascular health endpoints and personal exposure to air pollution over one year in 20 persons at home and during two ten-day periods in locations with higher and lower exposure levels.

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Setting: Between September 2013 and September 2014 participants underwent measurements on seven occasions in Leuven, Belgium (intermediate level of air pollution) and twice during each 10-day stay in Milan (Italy; high pollution) and Vindeln (Sweden; low pollution).

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Participants: Twenty nonsmoking healthy volunteers (10 male-female couples, aged 59-75 years).

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Exposure: Exposure to PM_{10}, $PM_{2.5}$, black carbon, and NO_2 was measured at the individual level.

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Main outcomes and measures: Blood pressure, carotid arterial stiffness,

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Results: Compared with Leuven (BE), exposure to pollutants was higher in Milan (IT) and lower in Vindeln (Se), with the highest contrast found for NO_2 (… µg/m^3 vs …µg/m^3 and … mg/m^3,

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respectively) We found strong associations between 7-days exposure to air pollution and arterial stiffness, e.g. a 4.7% (95% confidence interval (CI): -6.9;-2.5%; P<0.001) decrease in compliance for each 10 µg/m^3 increment in PM_{10} (adjusted for covariates). Young's elastic modulus and pulse wave velocity, both direct measures of stiffness, were positively associated with personal exposure to NO_2. No relations were found with plasma CRP and white blood cells.

Conclusions and relevance: Our intervention study demonstrates that short/medium term exposure to air pollution results in changes in carotid arterial stiffness among elderly population.

Key words (3-5): particulate matter; black carbon; epidemiology; carotid arterial stiffness

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Introduction

Ambient air pollution is an important cause of respiratory and cardiovascular morbidity and mortality.[1,2] It has been abundantly demonstrated that short-term exposure to air pollution (hours to a few days of exposure) can trigger acute events such as myocardial infarctions, [3,4] whereas long-term exposure (after several years of exposure) has been linked to both the onset of acute events and the development of chronic diseases.[5,6] In addition to epidemiological research, controlled-exposure studies in animals and humans have provided insight into possible physiological pathways underlying the relationship between inhalation of pollutants and cardiovascular and respiratory health. These pathways have been reviewed recently.[7-9]

In this study, we aimed to combine the advantages of epidemiological and experimental studies, by deliberately moving a panel of study volunteers for several days to locations with contrasting levels of air pollution. We quantified several health-related endpoints that have been identified as intermediate steps between exposure and disease: systemic oxidative stress and inflammation,[10,11] endothelial function,[10,12], arterial stiffness,[13] and coagulation.[14]

We hypothesized that a decrease or increase of air pollution exposure, compared to the participants' place of residence, during one to two weeks would be associated with detectable subacute and reversible changes in biomarkers of cardiovascular health.

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Methods

Study design and participants

We conducted a panel study during one year in healthy elderly volunteers and measured multiple health endpoints and personal exposure to air pollution in locations with widely differing ambient air pollution levels. From September 2013 to September 2014, we collected data over 11 measurement periods: every five to ten weeks in Leuven, Belgium (seven episodes); twice during a 10-day stay in Milan, Italy (one halfway and one at the end of the stay); twice during a similar 10-day stay in Vindeln (a rural area near Umeå, northern Sweden) (see **Figure 5**). These locations are representative for the highest (Milan, >50 µg/m^3) and lowest (Vindeln, <10 µg/m^3) yearly averages in PM_{10} that can be found in Europe, with intermediate values for Leuven (30 µg/m^3)[15-17]. To limit differences in temperature between the two study trips, we stayed in Milan in autumn (October 2013) and in Vindeln in summer (June 2014).[18] Clinical measurements were performed in adequate study rooms at the UZ Leuven, the Ospedale Maggiore in Milan, and Umeå University. We collected blood in EDTA and heparin tubes for blood cell counts and measurement of plasma C-reactive protein (CRP), respectively. At baseline, plasma levels of cholesterol and glucose were also determined in fasted blood samples. Plasma samples from heparin tubes were kept frozen at -80°C for subsequent analysis of plasma CRP, cholesterol and glucose levels at the UZ Leuven laboratory (Tina-quant CRP latex assay, Roche, Vilvoorde, Belgium).

Our study panel consisted of 20 healthy retired persons. We invited people attending lectures for retired people in Leuven, as well as friends and acquaintances of the parents of the doctoral researcher (HS) to participate in the study. After screening (by BN) of approximately 51 volunteers, we retained 10 male-female couples with both partners fulfilling the inclusion criteria for age (approx. 60-75 years), smoking habits (having never-smoked or having quit smoking at least one year

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before the start of the study), good general physical and mental health, willing and available to travel during the study period. We excluded persons with mobility problems; a history of cardiovascular disease (except uncomplicated hypertension), cancer, or other diseases that could interfere with the measurements or would represent a risk during travel. We included couples because this reduced the accommodation costs during the travel periods. All participants were given detailed oral and written information on the study and gave written informed consent. The study was approved by the Ethical Committee of KU Leuven (S55482).

Collection of environmental data

Participants lived in or close to Leuven or Mechelen (maximum distance between the residences was 45 km) and we estimated their daily residential exposure to PM_{10}, $PM_{2.5}$, black carbon (BC) and NO_2 using interpolated values in 4 by 4 km grids, based on the Belgian telemetric air quality network.[19]. In Milan, we used the online database of the Regional Agency for the Protection of the Environment in Lombardy (ARPA Lombardia) and averaged values from the different monitoring stations in Milan to estimate exposure to the same pollutants.[20] In Vindeln, we averaged data from the nearest measurement stations in Umeå, Skellefteå and Strömsund to estimate regional levels of PM_{10}, $PM_{2.5}$, and NO_2.[21] BC was not measured by any of these monitoring stations.

In addition, we sampled outdoor concentration of pollutants by using two portable laser-operated aerosol mass analysers: an Aerocet 53 (Met One Instruments Inc, Grants Pass, OR, USA) for PM_{10} and $PM_{2.5}$, and a microAeth Model AE51 (AethLabs, San Francisco, CA, USA) to measure BC concentration. Because our own BC results correlated well with those from central monitoring stations on the same

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day (in Leuven or Milan, N = 57 days, Pearson's r = 0.76, $p<0.001$), we used our own measurements for Vindeln to fill the gap in the BC dataset from the Swedish monitoring stations.

Finally, personal exposure to NO_2 was measured using Radiello diffusive samplers (Sigma-Aldrich, Bellefonte, PA, USA). Six to 10 study volunteers wore the clip-on device during six days prior to each health assessment day in Leuven or to the last health assessment day in Milan and Vindeln. After the sampling period, samplers were sent to the lab of the Fondazione Salvatore Maugeri (Padova, Italy) for quantification of average exposure to NO_2.

Daily temperature and relative humidity during the study period were obtained from local meteorological websites for Belgium[22] and Milan[23] and an international website for Umeå.[24]

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Cardiovascular measurements

We measured blood pressure and carotid arterial stiffness at each study moment including nine measurement occasions in Belgium, two in Milan and two in Sweden. Endothelial function was measured once during each trip (on day 9 or 10) and in Belgium only in control periods immediately before and after trips, resulting in six time points with endothelial function assessments (see **Figure 5**).

Blood pressure

Systolic (SBP) and diastolic blood pressure (DBP) were measured according to guidelines of the European Society of Hypertension,[25] with an automated device (Stabilograph, Stolberg, Germany). After the subject had rested for at least 5 minutes, blood pressure was measured five times

consecutively in sitting position. We used the average of the last two measurements for analyses, and we calculated pulse pressure (ΔP) as average SBP minus DBP, and mean arterial pressure as DBP + ΔP/3.

Carotid arterial stiffness & endothelial function

We measured carotid arterial stiffness by using an ultrasound device with automatic boundary detection software in RF-mode (MyLabOne, Esaote Benelux, Maastricht, The Netherlands) according to previously reported protocols.[26] Participants were at rest for 10 minutes in a supine position before starting the measurements. All measurements were performed by the same trained investigator (LC) by longitudinal scanning of a 1 cm segment of the right common carotid artery at 1 cm proximally to the dilatation of the carotid bulb visualizing the lumen-intima and media-adventitia interfaces of the far arterial wall. Carotid intima-media thickness (CIMT) was determined under three different angles; i.e. 90, 130 and 180 degrees.

We averaged diastolic artery diameter (D) and systolic increase in diameter (ΔD) over three consecutive ultrasound measurements, each spanning eight cardiac cycles. We subsequently used D and ΔD to calculate four parameters related to arterial stiffness, as described in two standard papers.[27,28] Carotid distensibility (DC) and compliance (CC) coefficients are inversely related to arterial stiffness, and pulse wave velocity (PWV) is a direct measure of arterial stiffness. Young's Elastic Modulus (YEM) combines measures of arterial wall elasticity with intima media thickness (IMT). Intra-observer coefficients of variation ranged from 5.2% to 10.1% for the different stiffness parameters, indicating good reproducibility of measurements.[13]

Reactive hyperemia index (RHI), which is a measure for endothelial function was assessed using the EndoPAT 2000 device (Itamar Medical, Israel). Measurements were performed according to the manufacturer's instructions. Briefly, the subjects rested in supine position for a minimum of 20

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minutes before measurements. Each recording consisted of 5 minutes of baseline measurement, 5 minutes of occlusion measurement, and 5 minutes postocclusion measurement (hyperemic period). Occlusion of the brachial artery was performed on the nondominant upper arm. The occlusion pressure was at least 60 mmHg above the systolic blood pressure (minimally 200 mmHg, and maximally 300 mmHg).

Covariates

Information on smoking status (never or former), medication use for hypertension, and having a cold was obtained by face-to-face interviews. Since physical activity, alcohol consumption, and perceived mental health were assumed to differ between the home situation and a 10-day trip abroad, we aimed to correct for these variables.

During seven days preceding each health assessment day, study subjects recorded their average physical activity duration (PAD), by wearing a SenseWear Pro Armband (BodyMedia, Inc., Pittsburgh, PA), a validated multisensory activity monitor combining a triaxial accelerometer with different sensors.[29] Weekly consumed grams of alcohol were calculated based on self-reported alcohol use, which was scored during one week at baseline, on trips abroad, and at the end of the study. Perceived mental health was assessed at the start of each health assessment. Participants filled in the Positive and Negative Affect Schedule (PANAS), which comprises a positive (PA) and a negative mood scale (NA) based on 10 items each on instantaneous mental condition. [30]

Data management and analysis

Data management and statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). We investigated associations between health parameters and exposure to air pollution by using

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linear mixed models, accounting for the repeated-measures design of the study. We evaluated different lag structures for the exposure variables: 'acute' effects of air pollution were estimated by using lag day 0 (exposure on the day of measurement), and 'subacute' effects by calculating the average of lag days 0 to 6 (referred to as 'av06'), corresponding to the duration of exposure with the Radiello NO_2 sampler. We performed sensitivity analyses with different lag structures for the subacute exposure (av02 and av04). Age at baseline, sex, date of the examination, ambient temperature, relative humidity, heart rate, mean arterial pressure, having a cold, medication use (BP), and smoking status were included in all models. We tested the assumption of normal distribution of the error terms by visual inspection of the Q-Q plots of residuals. For PWV, DC, CC, YEM, white blood cells (WBC) and differential WBC counts, this assumption was only met after log10-transformation. Therefore, results for these outcomes are presented as % change, whereas parameter estimates of all other analyses are unit changes.

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Results

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Ten male-female couples started the study in September 2013, and all participants completed the study in September 2014, without any dropout or missed measurement episode for any participant. **Table 2** summarizes the main characteristics of the study population at baseline. No differences were observed between males and females, except for body height and DBP, which were both higher in males than in females. Five female volunteers took medication for blood pressure during the whole study period, one male started taking medication after period L2 (figure 1).

Individual exposure levels to PM_{10}, $PM_{2.5}$, NO_2 and BC are presented in **Figure 6**. Personal exposure to NO_2 and ambient levels of BC were clearly highest in Milan and lowest in Vindeln with intermediate values for Leuven (Belgium). Average concentrations of PM_{10}, $PM_{2.5}$ and NO_2 (monitoring stations) did not differ between Leuven and Vindeln. Standard deviations (SD) were smaller in Milan and Sweden because the exposure window was more uniform in time and space than in Leuven. Plasma CRP levels were related with air pollution exposure in the crude models, but this association disappeared in the adjusted models, due to the influence of the covariate 'having a cold'.

The adjusted associations of blood pressure and carotid arterial stiffness with ambient concentrations of PM_{10}, $PM_{2.5}$, BC and NO_2 are presented in Table 3. Crude individual data and unadjusted coefficients can be found in the supplement. Changes in blood pressure variables were not related to changes in pollutant concentrations, regardless of the time window. We detected no short-term associations (lag0) between pollutant concentrations and indicators of arterial stiffness, except a 2.0% (95% CI -3.5;-0.4%) decrease in CC for a 10 $\mu g/m^3$ increase in PM_{10}, and a similar association with $PM_{2.5}$. In contrast, we found robust effects of subacute exposure (av06 lag structure) to air pollution on all measures of arterial stiffness. These associations were strongest for PM_{10} and

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$PM_{2.5}$ [e.g. a 4.7 (-6.9;-2.5%) decrease in CC for a 10 µg/m³ increment in PM_{10}]. Analyses with different lag structures (av04 and av02) produced very similar results (see supplement).

Endothelial function, by use of the EndoPAT, was positively associated with both 24h and 7 days averages of exposure to different pollutants, e.g. RHI was 0.36 (95% CI 0.19;0.54) points higher for a 10 µg/m³ increment in PM_{10} (av06), indicating an improvement in endothelial function with increasing air pollution exposure (Table 3). Similarly, when using a binary RHI outcome variable with 1.67 as the cut-off value, the risk for having endothelial dysfunction decreased with increasing pollutant concentrations (results not shown).

Discussion

In a quasi-experimental study, we deliberately exposed 20 study volunteers to the range of ambient pollution levels that can be found in Europe by moving them over Europe, and investigated the association between their exposure to air pollution and relevant intermediate cardiovascular endpoints. We found that changes in the vascular function of the carotid artery parallels personal exposure to one week ambient air pollution. Young's elastic modulus and pulse wave velocity, both direct measures of stiffness, were positively associated with personal exposure to NO2, while the distensibility and compliance coefficient, both measures of elasticity, were inversely associated with NO2.

Arterial stiffness and reduced elasticity, as measured here by different parameters, were consistently associated with higher exposure to ambient air pollution. Young's elastic modulus and pulse wave velocity, both direct measures of stiffness[31], were positively associated with personal exposure, while

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2186 the distensibility and compliance coefficient, both measures of elasticity,[32] were negatively
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2188 associated with one week personal exposure contrast. The mechanisms responsible for the increase
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2190 in stiffness and air pollution remain to be elucidated but most likely increase in inflammation and
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2192 changes in cardiac autonomic function, as observed in studies on heart rate variability, can explain
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2194 the inverse association between arterial distensibility and air pollution exposure. Arterial stiffness is
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2196 an important determinant of increased blood pressure and pulse pressure, and therefore a risk factor
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2198 of events such as myocardial infarction and stroke.[27,33,34] Since acute effects of air pollution on
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2200 myocardial infarction and stroke have repeatedly been demonstrated,[1,2,4,35] our results provide a
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2202 plausible biological mechanism for this trigger effect. Similar associations between short-term air
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2204 pollution exposure and arterial stiffness were found in recent intervention and epidemiological
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2206 studies.[13,36-38] The small changes that we found are not clinically relevant for an individual, but the
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2208 entire population is exposed to air pollution, including more vulnerable individuals. Small average
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2210 effects may reflect substantial changes in the most susceptible portion of the population.[39-41]
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2212 Moreover, the effects were considerably larger for the 7-days averaged pollutant concentrations
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2214 than for one-day values, indicating that medium-term exposure increases the detrimental effect of
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2216 air pollution.
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2221 We found no evidence of systemic inflammation, quantified as concentrations plasma CRP. Either by
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2223 a release of inflammatory cytokines into the circulation, or by direct translocation of particles
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2225 through the lung-blood barrier into the circulation,[8] systemic inflammation is held responsible for
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2227 noxious processes such as endothelial dysfunction, development of atherosclerosis, reduced HRV,
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2229 coagulation, and thrombosis.[7-9] However, in general, controlled-exposure studies at relatively low
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2231 exposure levels in healthy humans, such as the present study, did not demonstrate robust
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2233 inflammatory responses.[7]
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We had intended to assess relations between blood cell parameters and air pollution exposure. Hematocrit was negatively associated with air pollution (data not shown). However, it proved impossible to make confident comparisons between counts of erythrocytes, leukocytes or platelets obtained in the three locations, because these analyses were made with different devices in the three laboratories, thus leading to systematic errors that we could not reliably correct. When only the measurements made in Leuven were considered, no significant associations were observed for hematologic parameters.

When we designed the study, we selected the study locations based on their annual PM averages. We expected to find ambient PM_{10} concentrations as low as 10 µg/m^3 in rural Sweden and as high as 50 µg/m^3 in Milan during several days in a row. However, PM concentrations obtained from central monitoring stations were highly variable during both stays, resulting in average one-week exposures higher than expected in Vindeln (av06 PM of 19.8 µg/m^3 in S1) and lower than expected in Milan (av06 PM of 30.6 µg/m^3 in M2) (Figure 6)..[15,16] Nevertheless, such differences between locations were bigger for BC and NO_2 concentrations obtained from both, monitoring stations and personal exposure. This may be explained by the fact that BC and NO2 aretypical traffic-related pollutants with much more spatial variation in ambient concentration than PM.[48]

Our longitudinal study includes 11 health assessment episodes during one year in a panel of 20 healthy elderly volunteers, without any missing measurements, drop-out or important changes in health status. Moreover, we used a large battery of objective health and exposure measurements, including personal exposure measures of NO_2. This strongly increased the statistical power of the analyses, allowing us to find subtle, but significant changes in cardiovascular health parameters related to changes in air pollution in only 20 subjects. Although our quasi-experimental design has

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clear benefits compared with a pure observational study, some limitations must be mentioned. A 10-day group travel abroad is very different from the common home situation in many aspects that can confound the association between biological endpoints and exposure to air pollution. Including PAD, steps, alcohol use, PA and NA in our models did not produce substantially different results. We still may have overlooked other, real confounders of the associations found. However, when we totally excluded a possible “trip effect” by analyzing only Leuven data or by just comparing Milan to Sweden results, the parameter estimates were still similar to those when we analyzed the whole dataset.

Contrary to our hypothesis, RHI was positively associated with pollutant concentration, and the risk of having endothelial dysfunction was lower with increasing air pollution. The effect was strongest for the 7-days averaged concentrations. This result was unexpected, since endothelial dysfunction, a marker of atherosclerotic processes,[41] has repeatedly been associated with increased air pollution exposure levels.[7,9,10,12]

Endothelial function was measured six times in this study, and the highest average and median value were recorded in Milan (session M2), which had also the highest levels of air pollution. Measurements in Milan took place between 16:00h and 20:00h, whereas those in Leuven were always between 8:00h and 12:00h, and those in Vindeln were spread over the whole day. There are indications that endothelial function sustains a circadian rhythm, with a lower RHI in the morning.[42] Moreover, the same authors question the suitability of EndoPAT to measure endothelial function in small panels, such as in our study[42] Whatever the case may be, when removing the M2 results from the analysis, no positive or negative association between any of the pollutants and endothelial function could be detected. Therefore, our results on endothelial function and air pollution exposure must be interpreted with care.

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Public health relevance

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The changes we found in carotid arterial stiffness and hematology, in relation to exposure to air pollution, were small and probably of little clinical relevance for the healthy individual study participants. However, since ambient air pollution is ubiquitous, the whole population is exposed, including more susceptible subgroups such as children, patients with preexisting diseases, and elderly.[49] As a consequence, small individual risks result in a large global burden. Moreover, the time window of exposure in our study was relatively short. Many people living in urban environments are continuously exposed to much higher levels of air pollution.[50] Long-term exposure to air pollution induces pathophysiological processes, eventually causing cardiovascular events and chronic diseases. Thus, it increases the risk for mortality to an even greater extent than the triggering effect of short-term exposures.[2,7]

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Overall, 3.7 million deaths and 3.1% of disability-adjusted life years (DALY) worldwide are attributed to air pollution, placing it in the top 10 of risk factors.[51] In our study, we found that decreases in air pollution exposure, compared to the 'normal' level of exposure, were associated with reduced arterial stiffness and improved elasticity. Our result is in line with follow-up analyses of the Harvard Six Cities cohort study, showing a reduction in mortality risk in association with a decrease in ambient PM concentration.[52,53] These observations demonstrate that measures leading to a reduction in exposure to air pollution are likely to have beneficial public health effects.

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Conclusion

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In a panel study of healthy elderly moved to different places to contrast exposure representative for different ambient air pollution levels typical for Europe, we found evidence for subacute effects of

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exposure to PM, BC and NO_2 on carotid stiffness. In this susceptible group, improved air quality results within 7 days in higher elasticity of the common carotid artery.

Acknowledgements

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Disclosures

None.

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Figure legends

*Figure 5. Timeline of the study. L1 to L7: health assessment periods in Leuven; M1-2: stay in Milan; S1-2: stay in Sweden. All variables mentioned in the text were measured in 20 study volunteers in all 11 periods, except for endothelial function (only L1, M2, L2, L5, S2, and L6, indicated with *) and plasma levels of cholesterol and glucose (only L1, baseline).*

Figure 6. Personal exposure to PM_{10}, $PM_{2.5}$, BC and NO_2 during the study period. All symbols and error bars represent means with SD obtained from values averaged over one week preceding the day of health assessment ('av06' lag structure). Circles indicate data from central monitoring stations, squares are own measurements (NO_2: Radiello device; BC: Aethlab device). N=20 for each data point, except Radiello NO_2 (N=6 to 18, depending on the period).

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Tables

Table 2. Baseline characteristics of the study participants.[a]

Characteristic	All participants (N=20)	Males (N=10)	Females (N=10)	P-value[b]
Age, y	65 (58-76)	68 (58-76)	64 (59-70)	0.29
Height, m	1.71 (1.58-1.96)	1.76 (1.69-1.96)	1.66 (1.58-1.71)	<0.001
Body-mass index, kg/m^2	24.3 (18.9-29.4)	25.2 (18.9-29.4)	23.5 (19.2-29.1)	0.73
Smoking status, No. (%)				
Former	10 (50%)	6 (60%)	4 (40%)	0.66
Never	10 (50%)	4 (40%)	6 (60%)	
Blood pressure, mm Hg				
Systolic	132 (109-165)	133 (113-165)	127 (109-155)	0.53
Diastolic	80 (65-105)	85 (67-105)	76 (65-89)	0.06
Plasma cholesterol, mg/dL[c]				
Total	206 (144-282)	206 (160-238)	207 (144-282)	0.72
LDL	133 (57-212)	133 (93-150)	130 (57-212)	0.91
Plasma glucose, mg/dL[c]	99 (86-131)	100 (88-131)	99 (86-112)	0.37
Medication for hypertension, No. (%)	6 (30%)[d]	1 (10%)[d]	5 (50%)	0.14

[a]All values are medians (range).
[b]P-value for t-test comparing males to females (except smoking status and medication use: Fisher exact test).
[c]Measured in fasted blood samples.
[d]One male study subject started taking medication during the course of the study (after period M2).

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Table 3. Adjusted[a,b] changes (95% CI) in blood pressure , markers of arterial stiffness associated with a 10 µg/m³ increase in PM_{10} or NO_2, a 5 µg/m³ increase in $PM_{2.5}$ or a 1 µg/m³ increase in BC.

Acute effects (lag0)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (personal sampler)**
Systolic BP, mm Hg [a]	-0.16 (-1.47;1.14)	0.11 (-0.57;0.78)	-0.02 (-0.99;0.94)	-1.02 (-2.11;0.06)	n/a
Diastolic BP, mm Hg [a]	-0.47 (-1.34;0.40)	-0.15 (-0.61;0.30)	-0.02 (-0.72;0.69)	-0.39 (-1.12;0.34)	n/a
Pulse pressure, mm Hg [a]	0.26 (-0.67;1.19)	0.25 (-0.23;0.73)	-0.04 (-0.73;0.65)	-0.66 (-1.44;0.12)	n/a
PWV, % [b]	0.7 (-0.1;1.6)	**0.4 (0.0;0.9)***	0.3 (-0.3;0.9)	0.3 (-0.5;1.0)	n/a
Distensibility of the carotid artery, % [b]	-1.5 (-3.2;0.3)	**-0.9 (-1.8;0.0)***	-0.7 (-2.0;0.6)	-0.6 (-2.1;0.9)	n/a
Compliance of the carotid artery, % [b]	**-2.0 (-3.5;-0.4)***	**-1.1 (-1.9;-0.3)***	-0.8 (-2.0;0.3)	-1.0 (-2.3;0.3)	n/a
Young elastic modulus, % [b]	1.2 (-0.8;3.2)	1.0 (0.0;2.0)	0.8 (-0.7;2.2)	0.5 (-1.2;2.1)	n/a
RHI [b]	**0.20 (0.10;0.30)****	**0.19 (0.06;0.32)***	**1.67 (0.76;2.57)****	**0.12 (0.03;0.21)***	n/a
Subacute effects (av06)	**PM_{10}**	**$PM_{2.5}$**	**BC**	**NO_2 (stations)**	**NO_2 (personal sampler)**
Systolic BP, mm Hg [a]	0.23 (-1.8;2.26)	0.25 (-0.66;1.15)	-0.12 (-1.57;1.34)	-1.28 (-2.53;-0.04)	-0.14 (-1.10;0.81)
Diastolic BP, mm Hg [a]	-0.90 (-2.23;0.43)	-0.24 (-0.85;0.37)	-0.17 (-1.17;0.82)	-0.78 (-1.65;0.10)	-0.28 (-0.95;0.39)
Pulse pressure, mm Hg [a]	1.11 (-0.36;2.59)	0.47 (-0.17;1.11)	0.03 (-1.00;1.06)	-0.55 (-1.44;0.34)	0.11 (-0.58;0.79)
PWV, % [b]	**2.0 (0.8;3.3)****	**0.9 (0.4;1.5)****	0.9 (-0.1;1.9)	0.7 (-0.1;1.6)	**0.6 (0.0;1.3)***
Distensibility of the carotid artery, % [b]	**-4.6 (-7;-2.2)****	**-2.1 (-3.3;-1.0)****	**-2.4 (-4.3;-0.4)***	**-1.8 (-3.4;-0.1)***	-1.3 (-2.5;0.0)
Compliance of the carotid artery, % [b]	**-4.7 (-6.9;-2.5)****	**-2.1 (-3.2;-1.1)****	**-2.5 (-4.3;-0.7)***	**-2.0 (-3.5;-0.5)***	**-1.4 (-2.6;-0.3)***
Young elastic modulus, % [b]	**3.8 (0.8;6.9)***	**1.9 (0.5;3.3)***	**2.3 (0.2;4.5)***	1.5 (-0.4;3.5)	1.4 (0.0;2.8)
RHI [b]	**0.36 (0.19;0.54)****	**0.20 (0.08;0.31)****	**0.27 (0.12;0.42)****	**0.19 (0.09;0.30)****	0.07 (-0.02;0.15)

For all results, N=218 (11 time points), except for RHI, where N = 118 (6 time points).
Statistically significant results are highlighted in bold. * P<0.05; ** P<0.01; *** P<0.001
n/a: not applicable as personal sampling was based on passive sampler integrating exposure during 6 days
[a]Adjusted for age at baseline, sex, HR, smoking status, having a cold, medication use for blood pressure, date, temperature, relative humidity.

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[b]Additionally adjusted for arterial pressure.

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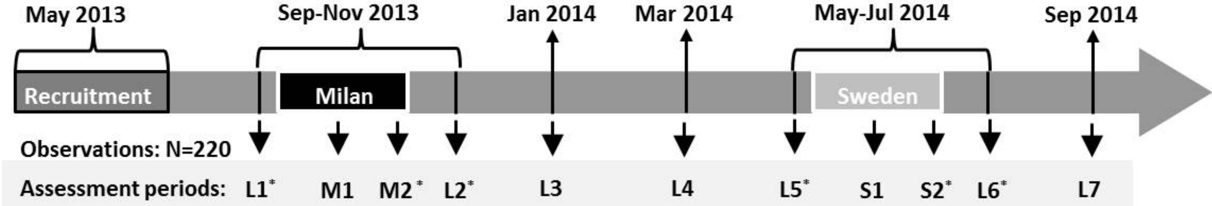

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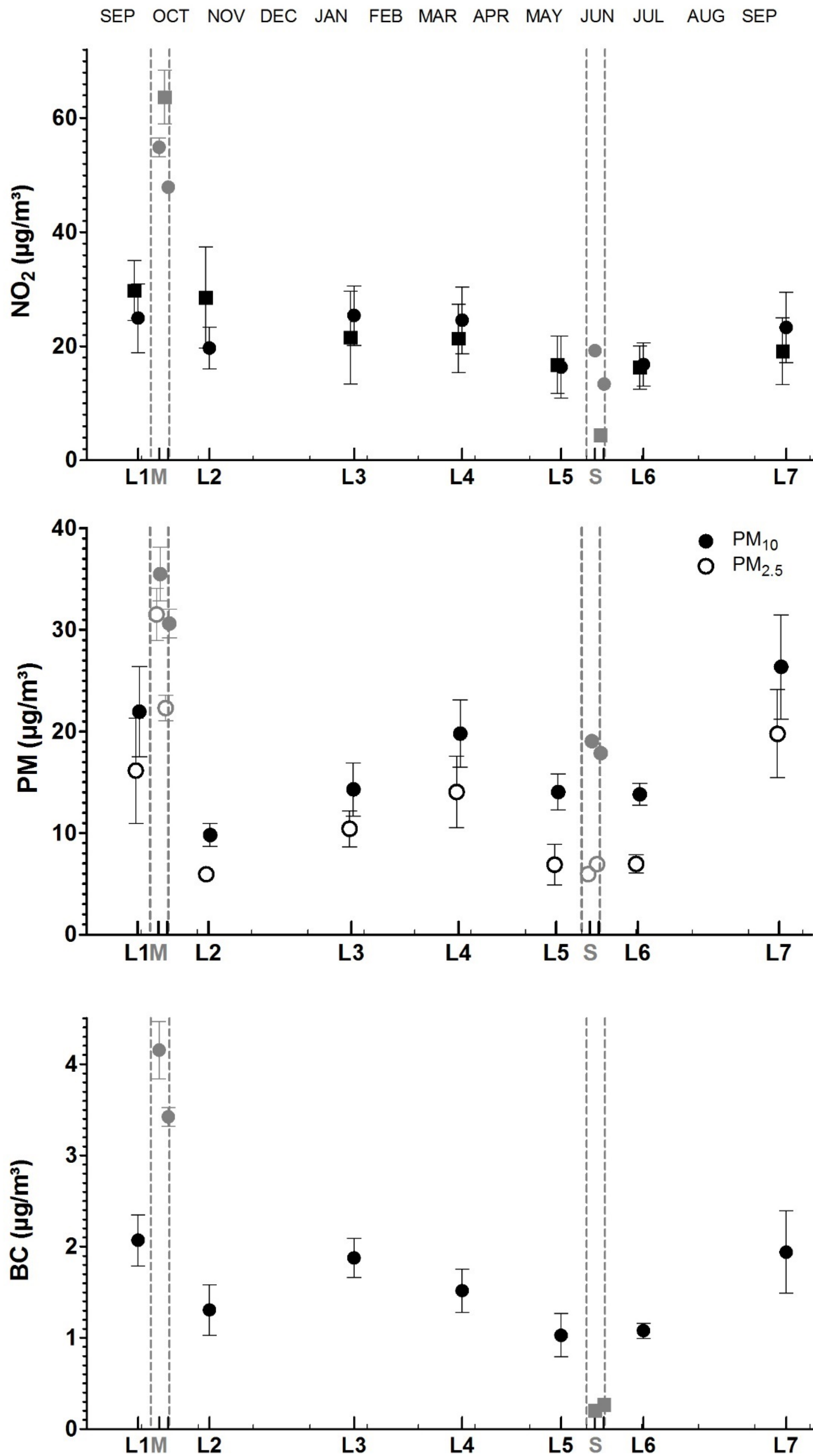

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