Inhalation of house dust and ozone results in altered systemic levels of endothelial progenitor cells, oxidative stress and inflammation in elderly subjects.

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Running title: Concomitant exposure to ozone and house dust.

Abbreviations:xxxxxxxxxxxxxxxxxxxxxxxxx
Abstract:

Ambient air pollution including ozone and especially particulate matter (PM) represent important causes of cardiovascular disease (CVD). However, there is limited knowledge on indoor air PM with respect to CVD and the potential interactions between PM and ozone. In this a randomized cross-over study of controlled continuous 5.5 h exposure to either clean air, house dust at 250 µg/m³ (diameter<2.5 µm), ozone at 100 ppb or combined house dust and ozone among 23 healthy elderly subjects was conducted. The combined house dust and ozone caused a 48% (95% confidence interval: 24% to 65%; p=0.001) decrease in CD34⁺KDR⁺ late endothelial progenitor cells (EPC) in the blood shortly after exposure, whereas none of the single exposures resulted in a significant effect. The combined exposure also caused increased capacity for production of reactive oxygen species (ROS) in granulocytes and monocytes as well as an up-regulation of interleukin-8 levels in the blood, whereas ozone alone reduced gene expression of tumor necrosis factor-α and C-C motif chemokine ligand 2 while dust alone showed no effects. The exposure to combined dust and ozone also caused reduced levels of oxidized purines in DNA consistent with concomitant up-regulation of the repair enzyme oxoguanine DNA glycosylase-1. The reduction in late EPCs can be an indicator of cardiovascular risk caused by the combination of pulmonary oxidative stress induced by ozone and the inflammatory potential of the house dust. This data was corroborated with in vitro findings from exposed human macrophages and endothelial cells.

Introduction

A substantial body of literature has documented associations between exposure to particulate matter (PM) in ambient air and cardiovascular diseases (CVDs) with the elderly population particularly susceptible. However, there is little knowledge on the associations between exposure to house dust or
PM from indoor sources and cardiovascular effects in high-income countries. A recent report indicated that the annual burden of disease caused by indoor air pollution corresponds to a loss of 2 million life-years in Europe while; ventilation of indoor spaces is an effective way of reducing these risks. Sources of PM in the indoor air include combustion processes such as burning of candles, cooking and woodstoves. In addition, such indoor PM may interact with and adsorb biologically active aerosols containing microorganisms such as bacteria, viruses or fungi, or compounds derived from microorganisms in terms of endotoxins and glucans. A proportion of indoor levels of PM also originate from outdoor sources such as combustion-driven vehicles. Ozone concentrations tend to be lower indoors than outdoors, whereas the time-integrated exposure is similar for both outdoor and indoor settings due to the longer time spend indoors. The lower indoor concentrations of ozone is partly attributed to its reactivity; thus the concentration of secondary oxidation products of ozone tends to be high due to the large surface to air volume ratio.

Indoor exposure to PM2.5 has been associated with low microvascular function and increase in biomarkers of inflammation, whereas biological components in settled house dust have only been associated with elevated markers of inflammation. Systemic effects of PM involve oxidative stress and inflammation, although this is not a consistent finding in humans and animal models. Controlled human exposure studies with ozone concentrations above 75 ppb have shown elevated levels of biomarkers of oxidative stress and inflammation, supported by animal experiments, whereas epidemiological studies of ambient air levels have been inconclusive with regards to systemic endpoints. A recent study supported acute adverse effects of higher levels of ozone on inflammatory biomarkers and heart rate variability in healthy subjects, whereas heart rate variability and vascular function were unaltered by ozone exposure in another study. The concomitant exposure to ozone and
PM can lead to enhanced effects in terms of airway inflammation in a controlled exposure study with ozone and diesel exhaust. Further supporting evidence is provided in a controlled exposure study that showed that concomitant exposure to house dust and ozone caused a decrement in lung function as well as discomfort as compared to either isolated exposure.

It has been hypothesized that circulating endothelial progenitor cells (EPCs) are pivotal in endothelial maintenance and repair, and declining levels of EPCs have been associated with both increased carotid artery intima media thickness and increased incidents of cardiovascular events as well as related mortality. As EPCs are released into the circulation, they either express do not express the surface marker CD133 which can be used to differentiate the cells into early or late EPCs, respectively. It has been previously shown that exposure to ambient air PM is associated with increased levels of recently recruited early EPCs and decreased levels of mature late EPCs. However, knowledge on indoor air PM is very limited, although a recent study indicated that indoor levels of ultrafine particles (UFPs) was inversely associated with levels of late EPCs. Moreover, effects on EPCs of exposure to ozone are not known and co-exposure to PM might aggravate the cardiovascular effects on ozone.

The aim of the present study was to investigate the systemic effects on EPCs, inflammation and oxidative stress following 5.5 h controlled exposure to indoor house dust and ozone healthy elderly subjects who may be susceptible to effects leading to development of CVD. Systemically circulating EPCs was chosen as an intermediate mechanistic step in the development of CVD. Oxidative stress was assessed in whole blood by measuring the production of reactive oxygen species (ROS) in individual leukocyte subpopulations, both spontaneously and after stimulation with carbon black nanoparticles. In addition, oxidative stress was assessed as oxidized nucleobases and strand breaks in peripheral mononuclear blood cells (PBMCs) that are well described responses to PM exposure. Oxidatively
damaged nucleobases, including 8-oxoguanine and ring-opened purine lesions, are removed from the DNA by oxoguanine DNA glycosylase-1 (OGG1). In the whole blood gene expression of OGG1 as marker of DNA repair and oxidative stress and heme oxygenase-1 (HMOX1) a marker of oxidative stress were assessed. Finally, inflammation was assessed as changes in level of of C-C motif chemokine ligand 2 (CCL2), interleukin-8 (IL8) and tumor necrosis factor-α (TNF-α).

Methods – human exposures

Study population & blood sampling

A total of 24 healthy participants were enrolled following informed written consent. One participant did not complete the study due to personal reasons, leading to a total study population of 23 participants (14 males and 9 females). The participants were non-smoking elderly (aged 62-72 years) who all attended a medical evaluation, including medical history assessment and a clinical examination, prior to being enrolled in the study. The effect of exposure on the airways is to be reported elsewhere. The exclusion criteria participation in the study were a history of medical disease that may affect cardiopulmonary measurements and abnormal lung function and/or bronchial hyper-responsiveness (diagnosed if additive provocative doses between 0.063 - 0.241 mg methacholine induce a ≥20% decrease in forced expiratory volume in first second - FEV₁). In addition, all participating subjects were free from upper airway infections or airway symptoms for at least one week and had not taken any drugs for 48 h prior to the exposure. They study was approved by The committees on Health Research Ethics in the Central Region of Denmark (file no. 1-10-72-439-12) and registered as a controlled clinical trial at ClinicalTrials.gov (file no. NCT02017782).

Exposures and design
The study was designed as a randomized, double-blind, controlled cross-over exposure experiment. The exposures were all conducted in a highly regulated welded stainless steel climate chamber of 79 m³, optimized for experiments with gasses and particulate air pollution including exposure generators for airborne house dust and ozone. The participants were exposed in groups of 3 to 5 with each participant attending every different exposure setting, with at least two weeks between every exposure, thereby acting as their own control. The participants were instructed to take a shower, carry clean clothes and not use perfume or cosmetics on exposure days. In the exposure chamber, the participants wore clean-suits (Clean Guard T65XP) and were seated at predefined seats. The exposure periods started with an acclimatization period of 30 min with clean filtered air, followed by 30 min build up to the actual exposure-setting. After reaching this setting the participants were exposed at rest for further 5 to 5.5 h. As a side note, refreshments and light meals were served following 2 h of exposure. The exposure settings included: clean filtered air, house dust (target PM₂.₅ concentration of 250 µg/m³), ozone (target concentration of 100 ppb) and a combination of house dust and ozone (target of PM₂.₅ concentrations of 250 µg/m³ and ozone concentrations of 100 ppb). The re-suspension rate was regulated during the exposure sessions to maintain a constant concentration of PM₂.₅ of 250 µg/m³ monitored by a Dusttrak Aerosol Monitor 8520 equipped with a PM₂.₅ inlet (TSI, St. Paul, MN, USA)). The chamber air was further monitored for particle number concentrations in the aerodynamic diameter range 20-1000 nm by a P-Trak condensation particle counter (TSI, St. Paul, MN, USA) and for number distribution of particles with aerodynamic diameter up to 300 nm, between 300 and 500 nm, between 500 and 700 nm and between 700 and 1000 nm by a Lighthouse Solair (Lighthouse Worldwide Solutions, Freemont, CA). PM₂.₅ from the exposure chamber was further collected on glass fiber filters (47 mm) by means of a PM₂.₅ sampler (BGI Inc., Waltham, MA, USA) for mass analysis. The ambient exposure conditions, in terms of temperature (device, producent, land), relative humidity (device, producent,
land), ventilation rates (device, producent, land) and CO₂ concentrations (device, producent, land), were monitored during the different exposure scenarios.

Following exposure, 8 ml of peripheral venous blood was sampled in K₃-tubes (BD Bioscience, Denmark) containing EDTA as anticoagulant for EPCs and ROS measurements. Next, blood samples for measurements of genotoxicity were collected in PBMC isolation tubes (Vacutainer, BD, Denmark). Following PBMC isolation by gradient centrifugation, the PBMC samples were stored at -80°C in a premade freezing medium containing 50% fetal bovine serum (FBS) (GibcoRBL), 40% non-supplemented RPMI1640 cell culture medium (GibcoRBL) and 10% dimethyl sulphoxide. Finally, for the gene expression measurements, 7 ml of peripheral whole-blood was sampled in Tempus™ RNA tubes (P/N 4342792, Applied Biosystems, Denmark).

Collection and characterization of house dust

The house dust used for the exposure sessions was collected just prior to the study and represented a realistic composition of house dust in the homes of the general population. The house dust was collected from three vacuum cleaner bags by participants in the study living in homes without any known indoor climate problems, such as mold, moisture damage, pets and smoking. These participants were instructed to avoid any unusual objects when vacuuming (such as plant soil). Following collection of the vacuum cleaner bags, the contents were processed in order to separate the house dust into fine and coarse fractions as previously described 16. The vacuum cleaner bag contents were passed through a filter with 2.5 mm pores (producent, land). The filtered fraction was stored in vacuum-alubags (producent, land) and composed the bulk fraction of house dust used in the experimental settings. This final fraction used for the controlled exposures was characterized for level of endotoxin and glucan
contamination as well as certain allergens (pollen, mold, can and Der.P.) (device, producent, land). Furthermore, the hydrodynamic size distributions of samples in ELGA® water (ultrapure water of resistivity of 18.2 Ω cm⁻¹, by ELGA maxima water systems) and RPMI1640 (Gibco, Denmark) cell culture medium was analyzed by Nanoparticle Tracking Analysis using a Nanosight LM20 (NanoSight, Amesbury, United Kingdom). In addition, we applied transmission electron microscopy (TEM maskine eller lign.) for size and shape characterization of the house dust samples.

**Endothelial progenitor cells**

The collected EPCs were analyzed using polychromatic flow cytometry, defining the cells as events within the leukocyte gate with a CD34⁺KDR⁺ antigenic profile, as previously described 19 with a few modifications. We further used the presence or absence of the differential progenitor marker CD133 to separate the EPCs into early or late sub-populations, respectively 20. Blood samples from the study participants were immunostained with anti-human monoclonal antibodies (15 min, 25°C, dark) for CD34 (PE-CY™ conjugated, BD Biosciences, Denmark), CD133 (APC conjugated, Miltenyi Biotec, Sweden) and CD309/KDR (PE conjugated, BD Biosciences, Denmark), followed by haemolysis (20 min, 25°C, dark) using Pharmlyse™ buffer (BD Biosciences, Denmark). Finally, samples were washed and re-suspended in a Stain buffer (DPBS, pH 7.4, 2% FBS, 0.09% sodium azide, BD Pharmingen™, Denmark). The samples were analyzed utilizing a BD Accuri C6 flowcytometer (BD Biosciences, Denmark) using a flow rate of 66 µl/min and a threshold of 1×10⁵ events above 5×10⁵ FSC-H to avoid contribution from lysed erythrocytes and thrombocytes with relevant isotypes used as controls and positive events defined by Boolean logics.

**DNA damage**
The alkaline comet assay was used to detect levels of DNA strand breaks (SB) and oxidised nucleobases; the latter measured as an additional induction of DNA strand breaks following enzymatic treatment with formamidopyrimidine DNA glycosylase (FPG) as previously described\(^\text{29}\). In brief, PBMC samples were re-suspended in 37°C agarose and transferred to a Gelbond® Film (Lonza, Switzerland). The cells were lysed overnight (1% Triton X-100, 2.5 M NaCl, 100 mM Na\(_2\)-EDTA, 10 mM Tris, pH 10, 4°C, dark) and subsequently incubated with FPG (enzyme was a gift from Professor Andrew Collins, University of Oslo, Norway) for 45 min (dark, 37°C). Following enzymatic treatment, the samples were submerged in an alkaline (pH 13) electrophoresis buffer for 40 min at 4°C before they were electrophoresed (25 V, 200 mA, 20 min, 4°C). Next, they were washed in a neutral buffer (0.4 M Trizma base, pH 7.5, 15 min, 25°C) and finally fixed with 96% ethanol (2 h, 25°C). The nucleoids were stained with YOYO-1 (Life Technologies, Denmark) and damage scored using in a fluorescence microscope (Olympus CX40) by visual classification according to a 5-class scoring system and transformed to lesions/10\(^6\) base pairs by an investigator specific calibration curve as described previously\(^\text{30}\). One arbitrary unit in a 0-100 arbitrary scale corresponds to 0.0195 lesions/10\(^6\) base pairs. The samples were scored in duplicates, and the damage expressed as mean lesions/10\(^6\) base pairs. The final data is expressed as SB and FPG sensitive sites (FPGss). The latter represents the net level of lesions that have been detected by the FPG enzyme (difference in DNA damage between samples that have been treated with FPG enzyme or buffer). The FPGss represents oxidized DNA nucleobases, including 8-oxoGua and ring-opened formamidopyrimidine lesions. In all experiments, a positive reference control was included - PBMCs treated with photosensitizer Ro19-8022 and exposed to white light, which induces FPGss. The photosensitizer was a gift from F.Hoffmann-La Roche (Basel, Switzerland).
Production of reactive oxygen species

ROS production was measured by flow cytometry in three leukocyte sub-types (lymphocytes, monocytes and granulocytes) categorized by their size and granularity as previously described \(^\text{28}\). In brief, two different measurements of ROS production were obtained, termed basal and capacity for ROS production. Intracellular ROS production was measured following exposure to Printex 90 carbon black (CB) from Evonik Industries, Frankfurt, Germany (primary particle size 14 nm; surface area 300 m\(^2\)/g). This type of CB has been shown to increase ROS production in primary endothelial cells and monocytes during \textit{in vitro} \(^\text{31}\). ROS measurements were performed on the same batches of peripheral blood that were used for testing of EPC levels. CB was suspended in ELGA® water at a concentration of 1 mg/ml using a Branson Sonicator at 450W and 10% amplitude (24 cycles of 10 sec sonication and 10 sec break to avoid overheating). Following suspension, CB was diluted in a Stain buffer containing DPBS (pH 7.4), 2% FBS and 0.09% sodium azide (BD Pharmingen™, Denmark). The cells were exposed (dark, 25°C) for 1 h to CB at concentrations of 0, 2, 5 and 15 µg/ml. Following CB exposure, samples were incubated (20 min, 25°C, dark) with a haemolysis buffer (BD PharmLyse™, BD Bioscience, Denmark). Subsequently, samples were centrifugated (5 min, 300 g, 25°C) and washed in Stain buffer twice before incubation in Stain buffer containing 2 µM of 2,7-dichlorodihydrofluorescein (DCFH) diacetate (Sigma-Aldrich, Denmark) (15 min, 25°C, dark). The samples were once again centrifugated (5 min, 300 g, 25°C), washed and re-suspended in Stain buffer before being run in a BD Accuri™ C6 flow cytometer installed with BD Accuri CFlow®Plus software for data analysis (BD Bioscience, Denmark). The median DCFH fluorescence was used as a measure of ROS production for each leukocyte population (Figure 1). ROS production at 0 µg/ml was defined as the basal/resting level,
whereas the capacity for ROS production was assessed by area-under-curve of CB concentrations of 0, 5 and 15 µg/ml, baseline adjusted to represent the increase subtracted the basal levels.

*Gene expression*

Next, the expression of the genes related to DNA repair (*OGGI*, GenBank sequence accession ID: 4968) and oxidative stress (*HMOX1*, Gene ID: 3262), as well as the genes related to inflammation *IL8* (Gene ID: 3576), *TNFA* (Gene ID: 7124) and *CCL2* (Gene ID: 6347) were analysed. Total RNA was isolated using Tempus Spin RNA Isolation Kit (4380204), with approximately 0.1 µg DNase-treated RNA used for construction of cDNA in a volume of 20 µl using High-Capacity cDNA Reverse Transcription Kits (P/N 4368813) TaqMan Gene-Amp RT-PCR kit as recommended by the manufacturer (Applied Biosystems). The quantitative PCR reactions were carried out in ABI PRISM® 7900HT (Applied Biosystems), using probes and primers from Applied Biosystems. The assay IDs for the genes were as follows: CCL2, Hs00234140_m1; IL6, Hs00985641_m1; IL8, s00174103_m1; TNF, Hs00174128_m1; HMOX1, Hs00157965_m1; OGG1, Hs01114116_gl. The 18S rRNA was used as a reference gene (Eukaryotic 18S rRNA Endogenous Control, 4352930E, Applied Biosystems). The PCR reactions were performed as previously described. The level of gene expression is reported as the ratio between the level of the target gene and the 18S rRNA reference gene using the comparative $2^{-\Delta\Delta Ct}$ method.

*Biological in vitro screening of house dust*

An array of *in vitro* biological end-points were investigated to establish the toxicological and inflammamogenic potential of the house dust on macrophages (THP-1a cells) and primary endothelial cells (HUVECs). These assays included the WST-1 cytotoxicity assay, the intracellular DCFH-DA
assay and 8 cytometric bead array flex sets. The cytotoxicity, ROS production and cytokine release was measured following 24 h exposure of cells and described in detail in the supplementary information.

Statistics

All statistics were performed using STATA/IC v.13.2. For the human exposures, we used mixed-model linear regression. The in vitro characterization of house dust samples was tested for significant results using one-way analysis of variance. The data was accepted as statistically significant at 5% level. The results from the human exposures were all expressed as % change ± CI relative to the negative control (clean air exposure) and the in vitro data expressed as mean ± SD.

Results

Exposures and house dust characterization

During the four different exposure scenarios the temperature, relative humidity, air exchange rate, CO₂, ozone and house dust concentrations were measured and summarized in table 1. Overall, the target concentrations of ozone and house dust in terms of PM₂.₅ as mass were achieved in the exposure scenarios. During ozone exposure the particle number concentrations were high, in all probability due to reactions with organic compounds as indicated by peak levels during meals (data not shown). During dust exposure more than 90% of the mass appeared to have aerodynamic diameter above 700 nm. Measurements of hydrodynamic size distributions (supplemental figure S1) showed a median size range of around 146 to 247 nm, depending on dust concentration and vehicle used for suspension. In the high dust suspension in RPMI1640 medium agglomerates of dust particles were clearly visible. TEM measurements (supplemental figure S2) showed house dust fractions of varying size with many
particles in the low nm size range and high electron density, whereas other slightly larger particles also had lower electron density.

**Endothelial Progenitor cells**

Concomitant exposure to house dust and ozone caused a 48% (95% confidence interval, CI: [-24% to -65%]; p=0.001) decrease in the late EPC (CD34+/KDR+) levels (Figure 2A). Neither of the single-factor exposures induced statistically significant changes in EPC levels, although subjects exposed to ozone alone displayed a non-significant decrease in late EPC levels of 32% (95% CI: [-54% to 0.2%]; p=0.051). The levels of early EPCs (CD34+/CD133+/KDR+) showed no significant change after any of the exposures (Figure 2B).

**Genotoxicity**

The individual exposure to house dust or ozone did not change the levels of genotoxicity in PBMCs. However, concomitant exposure to house dust and ozone decreased the levels of both oxidized purines (FPGss) and total genotoxicity defined as the sum of FPGss and SB (FPG) by 56% (95% CI: [-86% to -0.4%]; p=0.049) and 39% (95% CI: [-63% to -7%]; p=0.021), respectively (Figure 3A-C). The assay controls for SB and FPGss (i.e. Ro19-8022 treated cells) were 0.05 ± 0.04 and 0.44 ± 0.08 lesions/10^6 base pairs, respectively.

**Production of reactive oxygen species**

The subjects exposed to a combination of house dust and ozone showed significantly increased capacity for ROS production in monocytes (p=0.01) and granulocytes (p=0.005) by 30% (95% CI: [7% to 53%]) and 25% (95% CI: [8% to 43%]), respectively. In contrast, subjects exposed to ozone alone
showed decreased basal levels of ROS production in monocytes (p=0.006) and granulocytes (p=0.03), by -16% (95% CI: [-5% to -27%]) and -14% (95% CI: [-1% to -26%]), respectively. Furthermore, monocytes from subjects exposed to ozone alone displayed a -23% (95% CI: [-1% to -46%]) change capacity for ROS production (p=0.039). Exposure to house dust alone caused no significant change in ROS production (Figure 4 and 5).

**Gene expression**

Exposure to house dust and ozone in combination caused significantly increased mRNA levels of *IL-8* and *OGG1* by 59% (95% CI: [15% to 120%]; p=0.005) and 31% (95% CI: [5% to 62%]; p=0.015), respectively. Subjects exposed to ozone had significantly down-regulated levels of *TNF-α* mRNA by -9% (95% CI: [-16% to -0.8%]; p=0.033) and *CCL2* mRNA by -7% (95% CI: [-12% to -2%]; p=0.008). Exposure to house dust alone caused no significant change in gene expression (Figure 6A-E).

**Biological characterization of house dust**

The *in vitro* cytotoxicity measurements showed that 24 h exposure to house dust changed the viability of THP-1a cells and HUVECs exposed to 1000 µg/ml, by -73% (95% CI: [-133; -14]; p=0.009) and -46% (95% CI: [-84; -9]; p=0.01), respectively. The positive controls (ZnO nanomaterials) changed the viability of THP-1a cells and HUVECs, by -63% (95% CI: [-123; -4]; p=0.032) and -90% (95% CI: [-128; -53]; p>0.0001), respectively. Neither THP-1a cells nor HUVECs changed their ROS production in response to house dust exposure (supplemental figure S3). In terms of cytokine secretion, THP-1a cells appeared to increase secretion of pro-inflammatory and vascular growth and adhesion cytokines, in a concentration dependent manner (supplemental figure S4). In the HUVECs an increase in the
secretion of adhesion molecule ICAM-1 and pro-inflammatory cytokine IL-6 was noted (supplemental figure S5).
Discussion

The concomitant exposure of elderly subjects to house dust and ozone for 5.5 h caused a 48% reduction in levels of late EPCs, whereas ozone alone had a smaller effect of borderline statistical significance. The concomitant exposure was furthermore accompanied by increased capacity for ROS production in monocytes and granulocytes, as well as upregulated expression of genes related to inflammation and oxidative stress in PBMCs. In contrast, downregulated expression of inflammatory genes and decreased basal ROS production in monocytes and granulocytes were observed following exposure to ozone alone. Only the concomitant exposure had significant effect on genotoxicity, which was observed as decreased DNA lesions related to oxidative stress. The exposure to house dust alone did not result in any significant effects on any end-point measured.

The house dust had limited inherent capacity of inducing oxidative stress as indicated by the ROS measurements in our in vitro data, though we observed a non-significant concentration-dependent increase following exposure to high concentrations of dust in THP-1a cells. Previous studies have shown pro-inflammatory effects of house dust in terms of IL-8 production in cultivated pneumocytes and monocytes \(^{33-35}\). Moreover, the dust exposure mainly comprised airborne particles with aerodynamic diameter between 0.7 and 2.5 \(\mu\)m with usual alveolar deposition fraction around 10% (ICRP deposition model). TEM analysis demonstrated that the dust consisted of particles with diameter as small as 10 nm and variable electron density, whereas in suspension the largest fraction of particles measured at hydrodynamic diameters of around 146 to 247 nm. Due to small size and large surface area these particles are more than capable of alveolar with potential for cellular interactions.
The inhalation of ozone may induce local oxidative stress within the respiratory lining fluid, although it is not readily available as an oxidant within the systemic circulation following exposure; therefore systemic effects including CVD might be caused by a spill-over of pulmonary oxidant reaction products and/or mediators from activation of inflammation upon local cell injury. This theory is supported by a study of short-term exposure to ozone in healthy adults that showed increased expression of vascular endothelium adhesion molecules P-selectin and ICAM-1. Another study showed that serum from ozone exposed mice contained bio-reactive molecules that promoted endothelial dysfunction in the aorta rings from non-exposed mice. The observation that ozone exposure lowered inflammatory gene expression in our study could be a possible explanation for the reduction in ROS production in monocytes and granulocytes. This reduction was more profound in monocytes as they not only displayed a status of decreased basal ROS levels, but also a decreased ability to up-regulate their ROS production. Indeed this could be attributed to the observed reduction of TNF-α and CCL2 expression with the forming being crucial to governing overall acute phase inflammation, which includes the recruitment of granulocytes and monocytes. Additionally, CCL2 is a pro-inflammatory chemokine involved in recruitment and activation of monocytes (Gu et al, 1999).

The decline of late EPC levels after the combined exposure to house dust and ozone at realistic environmental concentrations for 5.5 h indicates at an adverse effect on the endothelium, which could be due to acute endothelial damage and the requirement for the the recruitment of circulating late EPCs essential in the repair process. This finding is in keeping with animal and observational studies reporting decreased levels of late EPCs associated with ambient PM2.5 exposure. Importantly, the level of circulating late EPCs has been shown to be an independent predictor of cardiovascular events. It has been shown that exposure to ambient PM2.5 decreased the level of late EPCs in mice
attributed to VEGF desensitization, which resulted in turn increased levels of EPC generation within
the bone marrow. In a cross-sectional study, a comparison between two Chinese cities with similar
mass-based levels of ambient air PM$_{2.5}$, but very different levels of metal content, showed that both
ey early and late EPC levels were lower in women from the city with high metal content. We have
previously found that late EPC levels are inversely related to indoor levels of UFP, but not outdoor
levels of UFP, among healthy subjects highlighting the importance of indoor air exposure on late EPC
levels. It is possible that the responses reported in the present study were due to the particular doses
and exposure periods selected and the possibility that that a higher concentration of ozone might have
elicited a significant change of late EPCs. In particular, the insignificant reduction of late EPCs
following ozone exposure ($p=0.051$) suggest that the relatively short period of the exposure (5.5 h)
might be too brief to allow for significant effects in this respect. Nevertheless, it has been shown that
acute exposure to ozone did not result in microvascular dysfunction and altered heart-rate variability,
though both dose and time of exposure were considerably lower (300 ppb ozone for 75 min) than what
has been used in the current study. Furthermore, we did not observe any effect on the levels of early
EPCs (recruited from the bone marrow in response to vascular injury before maturation into the late
EPC subtype, as indicated in a time-lapse study of secondhand smoking, where the late EPCs appeared
to increase after and at the expense of the early EPC subset). Elsewhere, increased levels of early
EPCs have been reported in studies investigating exposure to outdoor air pollution.

Outdoor PM induces both oxidative stress and inflammation and the effects of a combination of ozone
and house dust could be similar. In these experiments, combined acute ozone-induced local oxidative
stress injury might allow for elevated cellular uptake of house dust and subsequent increased release
and systemic translocation of mediators and/or reaction products. Indeed, only the concomitant
exposure induced a systemic oxidative stress response in terms of increased **OGG1** expression and ROS production capacity in monocytes and granulocytes, combined with a proinflammatory response in terms of **IL8** expression, whereas ozone alone reduced ROS production and inflammatory gene expression. The contrasting effect of the concomitant exposure to house dust and ozone may similarly be related to changes in gene expression; highlighted by increased **IL-8** expression which can lead to the translation into IL8 protein; a highly potent chemoattractant known to induce granulocytic (mainly neutrophilic) activation via increased chemotaxis and phagocytosis.\(^4^0\) In addition, ozone may oxidize surface components of house dust, leading to an increased ability for inducing oxidative stress by house dust that has been in contact with ozone.\(^5\).

In keeping with increased ROS production in monocytes and granulocytes following the concomitant exposure scenario, levels of **OGG1** mRNA were also increased. It was noted that levels of oxidative stress related DNA lesions in terms of FPG and FPGss, decreased following the concomitant exposure. Both the **OGG1** expression level and FPGss are established biomarkers of oxidative stress\(^4^1\) and may interact because of **OGG1** being the enzyme responsible of excising 8-oxoguanine and ring-opened purine bases from the DNA strand. The decreased levels of FPGss and increased **OGG1** expression has previously been reported following 5 h exposure to wood smoke\(^4^2\). Here, we did not see any significant changes in leukocyte differential counts following exposure to ozone and house dust in combination, which indicates that the observed changes in gene expression in PBMCs are not driven by changes in the composition of leukocyte sub-populations.

In conclusion, the data shows that acute concomitant exposure to ozone and house dust may increase the risk of developing CVD in elderly healthy subjects. The mechanistic endpoints of CVDs can be attributed directly to the exposure because the subjects were their own controls; thus the study has
controlled for other risk factors of CVD. Risk factors for developing CVD are usually lifestyle
dependent, which are supported by several studies reporting that levels of EPCs can be restored to the
norm within system circulation upon the removal of the risk factor \(^{21,27,43}\). Thus, the decrease in late
EPC levels observed after the concomitant exposure may recover upon the cessation of the exposure,
although this was not investigated. However as the onset of many CVD related pathologies are
clinically silent, the use of EPCs as a biomarker may be a useful as a tool for the ongoing evaluation of
cardiovascular effects of potentially toxic compounds.
References


Figure legends

Figure 1 – The stimulated production of reactive oxygen species in leukocyte sub-populations. Carbon black was used to stimulate reactive oxygen species (ROS) production in terms of DCFH fluorescence in leukocyte subpopulations (lymphocytes, monocytes and granulocytes). The bars are median values (± standard deviation) of all conducted experiments within the subjects exposed to clean air (n=24).

Figure 2 – Endothelial progenitor cells. (A) Levels of late (CD34+KDR+ cells/events) and (B) early (CD34+KDR+CD133+ cells/events) endothelial progenitor cells (EPCs) in subjects exposed to clean air, house dust (dust), ozone (O3) and combination of house dust and ozone. The mean values are represented by horizontal bars and significant or border-line significance compared to the clean air exposure group are provided with a p-value (n=24).

Figure 3 – Genotoxicity. (A) DNA strand breaks (SB), (B) formamidopyrimidine DNA glycosylase sensitive sites (FPGss) and (C) the sum of SB and FPGss (FPG) were measured by the alkaline comet assay in peripheral blood mononuclear cells and expressed as lesions per million DNA base pairs. The subjects were exposed to clean air, house dust (dust), ozone (O3) and house dust and ozone in combination. The mean values are represented by horizontal bars and significant changes compared to the clean air exposure group are provided with a p-value (n=24).

Figure 4 – Basal levels of production of reactive oxygen species in leukocyte sub-populations. (A) Basal levels of reactive oxygen species (ROS) production in lymphocytes, (B) monocytes and (C) granulocytes, measured using flow cytometry, in terms of median DCFH fluorescence (FL) within each sub-population. The subjects were exposed to clean air, house dust (dust), ozone (O3) and house dust
and ozone in combination. Mean values are represented by horizontal bars and significant changes compared to the clean air exposure group are provided with a p-value (n=24).

Figure 5 - The capacity for production of reactive oxygen species in leukocyte sub-populations. The capacity for increasing production of reactive oxygen species in response to carbon black in (A) lymphocytes, (B) monocytes and (C) granulocytes, measured using flow cytometry, in terms of the integrated response between median DCFH fluorescence (FL) and carbon black concentrations, within the subpopulation. Investigated subjects were exposed to clean air, house dust (dust), ozone (O₃) or house dust and ozone in combination. The mean values are represented by horizontal bars and significant changes compared to the clean air exposure group are provided with a p-value (n=24).

Figure 6 – Gene expression. Expression of genes related to oxidative stress (A-B), in terms of (A) HMOX1 and (B) OGG1, and genes related to proinflammatory signaling (C-E), in terms of (C) TNF-α, (D) CCL2 and (E) IL8, expressed as ratios between mRNA of the relevant gene of investigation and an 18S rRNA control using quantitative PCR. Gene expressions were measured in total leukocytes with the investigated subjects were exposed to clean air, house dust (dust), ozone (O₃) and house dust and ozone in combination. Mean values are represented by horizontal bars and significant changes compared to the clean air exposure group are provided with a p-value.
Table 1. Overview of measured exposure conditions in the climate chamber during 5.5 h exposure of 23 healthy elderly subjects.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Clean air</th>
<th>Dust</th>
<th>Ozone</th>
<th>Dust + Ozone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>22.9±0.2</td>
<td>22.8±0.2</td>
<td>22.9±0.2</td>
<td>22.9±0.2</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>43.4±0.6</td>
<td>43.3±0.8</td>
<td>43.8±0.8</td>
<td>43.4±0.6</td>
</tr>
<tr>
<td>Rate of air exchange (h⁻¹)</td>
<td>3.6±0.2</td>
<td>3.6±0.2</td>
<td>3.7±0.2</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>CO₂ levels (ppm)</td>
<td>697±52</td>
<td>700±48</td>
<td>690±47</td>
<td>691±47</td>
</tr>
<tr>
<td>Ozone (ppb)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>100±7</td>
<td>100±6</td>
</tr>
<tr>
<td>PM₂.₅ (µg/m³)</td>
<td>11.6±6.8</td>
<td>263±26</td>
<td>9.0±7.9</td>
<td>273±23</td>
</tr>
<tr>
<td>Particle number concentration (20-1000 nm) #/cm³</td>
<td>238</td>
<td>375</td>
<td>24000</td>
<td>17026</td>
</tr>
<tr>
<td>Particles &lt;300 nm (% of mass)*</td>
<td>21</td>
<td>3</td>
<td>52</td>
<td>2</td>
</tr>
<tr>
<td>Particles 300-500 nm (% of mass)*</td>
<td>15</td>
<td>3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Particles 500-700 nm (% of mass)*</td>
<td>10</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Particles 700-1000 nm (% of mass)*</td>
<td>55</td>
<td>90</td>
<td>31</td>
<td>91</td>
</tr>
</tbody>
</table>

* Estimated from the number size distribution between 0 and 1000 nm and assuming spherical particles of identical density. Data are mean with SD where available.