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Exhaled breath condensate as a matrix for combustion-based nanoparticle exposure and health effect evaluation.

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Exhaled breath condensate for nanoparticle evaluation

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Abstract

Background

Health Assessment and medical surveillance of workers exposed to combustion nanoparticles is challenging. The aim was to evaluate the feasibility of using exhaled breath condensate (EBC) from healthy volunteers for 1) assessing the lung deposited dose of combustion nanoparticles; 2) determining the resulting oxidative stress by measuring H_2O_2 and MDA.

Methods

15 healthy non smoker volunteers were exposed to three different levels of side-stream cigarette smoke under controlled conditions. EBC was repeatedly collected before, during and 1 and 2 hours post exposure. Exposure variables were measured by direct reading instruments and by active sampling. The different EBC samples were analyzed for particle number concentration (light-scattering based method) and for selected compounds considered oxidative stress markers.

Results

Subjects were exposed to an average airborne concentration up to 4.3×10^5 particles/cm^3 (average geometric size ~60-80 nm). Up to 10×10^8 particles/ml could be measured in the collected EBC with a broad size distribution (50th percentile ~160 nm) but these biological concentrations were not related to the exposure level of cigarette smoke particles. While H_2O_2 and MDA concentrations in EBC increased during exposure, only H_2O_2 showed a transient normalization 1 hour after exposure and increased afterward. In contrast, MDA levels stayed elevated during the 2 hours post-exposure.

Conclusions

The use of diffusion light scattering for particle counting proved to be sufficiently sensible to detect objects in EBC but lacked the specificity for carbonaceous tobacco smoke particles. Our results suggest two phases of oxidation markers in EBC: first, the

initial deposition of particles and gases in the lung lining liquid, and later the start of oxidative stress with associated cell membrane damage. Future studies should extend the follow-up time and should remove gases or particles from the air to allow differentiating between the different sources of H_2O_2 and MDA.

Keywords

Introduction

Based on epidemiological studies, the fine and ultrafine fraction of ambient particles are of particular concern as they are associated to adverse health effects such as cancer, cardiovascular and pulmonary diseases [1]. In the case of engineered nanomaterial, their increasing use leads to concern regarding their toxicity. Indeed, *in vivo*[2] and *in vitro*[3] studies suggest that such materials may be hazardous to living organisms. The potential health effects of manufactured nanomaterials are thus currently being investigated in large research projects [4]. However, results from human studies are still scarce and need to be created [5]. Among the different mechanisms proposed to explain these adverse effects, is the production of reactive oxygen species (ROS) which contribute to oxidative stress [6]. This mechanism has been proposed as an unifying concept [7] applicable to ambient as well as for engineered nanoparticles. The lungs are the principal entry portal for particles and after they deposit, a rapid build-up of oxidative stress in the thin liquid layer of the alveolar region has been suggested, leading to epithelial cell damage and the release of pro-inflammatory mediators [8]. Oxidative stress induces and is induced by airway inflammation [9] and this perpetuation increases the oxidative stress further, forming a vicious cycle [10]. Both, oxidative stress and sustained inflammation can thus contribute to the onset of particle related pathophysiological effects including cardiovascular effects [11-15].

Most of the human studies regarding the determination of the dose-response due to inhalation of particles may use radiolabeled particles [16] and collect the lung fluid by invasive techniques (e.g. bronchoalveolar lavage fluids or sputum induction) [9], all of which are not totally harmless to the volunteers. In addition, the measurement of inflammatory markers in urine or plasma after particle exposure reflects a systemic rather than an organ specific inflammation. Exhaled breath condensate (EBC), consisting in condensing the exhaled air of living beings [17], is an emerging and non invasive technique allowing to study lung processes. EBC sampling is easy to be

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performed and may give valuable information about inflammatory events in the respiratory tract [18-20]. As EBC is a highly diluted aqueous media, no matrix effect are reported [21, 22] but challenging concerning analytical techniques which must be highly sensitive. From a toxicological and occupational risk assessment perspective [23], EBC could be an ideal biological matrix as it could simultaneously provide information about the deposited dose and on the subsequent biological effects such as inflammation. EBC is thus proposed as a good methodology to study pulmonary biomarkers of exposure and effects in occupational situations [24, 25].

Whereas the measurement of different metals in EBC of workers exposed to pneumotoxic metal particles has been reported several time [21, 26, 27], very few studies have tried to determine the dose of particulate matter deposited in the lungs by using EBC. Falgayrac [28] presented preliminary results indicating that the combination of EBC collection and Raman spectroscopy could be useful to identify micrometric size mica particles in lungs of exposed workers. By using nuclear microprobe techniques, Pinheiro [29] demonstrated the feasibility of detecting and characterizing the elemental composition of particles in dried EBC samples collected from workers in a smelting factory.

As oxidative stress is an important mechanism explaining the biological effect of deposited particles in lungs, corresponding relevant biomarkers of effect in EBC have to be considered. Superoxide plays a central role in the generation of oxidative stress, as it is one of the species which can be catalytically induced by particles. In biological tissues, this radical is rapidly transformed to the stable hydrogenperoxide (H_2O_2) or may oxidise cells constituents like lipids. Increased levels of H_2O_2 and malondialdehyde (MDA, considered as a marker of lipoperoxidation) have been reported in EBC of healthy volunteers after wood-smoke exposure [30] or for asthmatic children exposed to ambient particle [31, 32]. Compared to controls, patients with asthma [33] and asbestosis or silicosis [34] presented also higher levels in EBC for such

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compounds. These two molecules are thus often considered as good oxidative stress biomarkers in EBC [18].

The aim of this study was to evaluate the feasibility of using EBC for 1) assessing the deposited dose of combustion-based nanoparticles (NP) in the lungs of healthy volunteers and 2) determining the resulting oxidative stress level by measuring H_2O_2 and MDA. We used cigarette smoke as a model for NP that we expected to induce oxidative stress in the lungs [35]. We tested the hypothesis that EBC of healthy non-smokers exposed to side smoke cigarette would contain increased levels of particles and oxidative stress biomarkers compared to their own control measurements before exposure.

Materials and methods

The chosen study design was a repeated exposure of healthy volunteers to three different levels of cigarette side smoke in an inhalation chamber with repeated EBC collection.

Study participants

The study was approved by the Ethics Committee of the Faculty of Biology and Medicine from the University of Lausanne. Fifteen healthy non-smoker volunteers were included. Criteria for participating in the study were: male; non-smoker (or ex-smoker for more than 10 years); no chronic or acute respiratory illness; no current use of any medication (including vitamins); no diabetic problems; no allergies; BMI smaller than 30; no coffee, tea or alcohol consumption 24 hours before the first EBC collection. The inclusion process consisted of a first visit with a medical examination, the presentation of the exposure cabin and instructions on how to use the EBC collection system. After the medical staff confirmed that the subject could be included, written informed consent was obtained and a second visit was scheduled for the exposure experiment.

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Study design

The exposure level (defined by the number of cigarettes to be burned simultaneously) was randomly assigned to each study participant before the experiment. The exposure chamber [36] (total volume of 10 m³) had a controlled pulsing-aspiration system with an air exchange rate of 9.3 h^{-1} and a HEPA-filter for the incoming air. To exclude potential circadian effects on biomarker levels [37], the exposure started for all subjects in the morning between 8:30 and 9:30 am.

After arrival on the day of exposure, information about health status, food consumption (coffee,…) and particulate matter exposure during the 24 hours before the experiment were obtained from each subject through a questionnaire. After entering the exposure chamber, the subject was asked to sit on a chair to provide the first EBC sample (named EBC 1, considered as control). The EBC was collected by breathing for 10 minutes through a pre-cooled (at -20°C) RTube$^{®}$ (Respiratory Research Inc, Austin Tx, USA). Next, depending on the planned exposure level, a varying number of cigarettes (1 to 3, with one volunteer exposed to 4 cigarettes for each burning cycle) were lit inside the cabin near the air entry using a house-made system. The cigarette brand used was Marlboro red (tar: 10 mg, nicotine: 0.8 mg, carbon monoxide: 10 mg – Philip Morris data). Cigarettes were burned passively. After completion and an additional waiting time of 10 minutes, the subject was asked to recharge the lightning system with new cigarettes (same number) to start a second cycle of cigarette smoke generation. Immediately after the second set of cigarettes finished burning, a second 10 minutes EBC collection (EBC 2) was done. After another two burning cycles, a third 10 minutes EBC was collected (EBC 3). Depending on the exposure level, each volunteer was exposed to the combustion of a total of either 4, 8, 12 or 16 cigarettes. The subject stayed two more hours inside the cabin (filtered air) and EBC 4 and EBC 5 were collected one and two hours, respectively, after the last particle exposure. The subjects

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were allowed to read or work with a computer inside the cabin, and to drink non sparkling mineral water.

Exposure characterisation

Active sampling equipment and direct reading instruments were used to characterize the exposure inside the cabin. The mass of airborne particulate matter (PM) was determined as PM_4 (PM smaller than 4 µm, referred to as the "alveolar fraction" in occupational health, sampled with using a Casella cyclone head onto 37 mm Teflon filter-SKC Inc, Eighty Four USA at a flow of 2 L/min) and as $PM_{2.5}$ (PM smaller than 2.5 µm corresponding to the "alveolar fraction" in environmental health, sampled with an impactor head-PEM® SKC Inc, Eighty Four USA onto a 37 mm Teflon filter-SKC Inc, Eighty Four USA - at a sampling flow of 10 L/min). At the beginning and at the end of the sampling, the air flow of the pumps was controlled. All filters were conditioned before and after sampling for at least 24 hours at constant humidity (60±10%) and ambient temperature before weighting.

Organic (OC), elemental (EC) and total (TC, the sum of OC plus EC) carbon content of the particles collected with the PM_4 sampler on plasma treated quartz filters (Whatmann QM-A, 37 mm, WWR International, Geneva, Switzerland) was analyzed using a coulometric method [38] accredited following the ISO/IEC 17025 norm. As a qualitative control for potential interferences of airborne compounds with the H_2O_2 and MDA measurements, air was sampled at 0.5 L/min through an impinger filled with 10 ml water. This aqueous solution was included in the MDA and H_2O_2 analyses.

Direct reading instruments were used to observe the conditions the subjects experienced inside the cabin. Temperature and humidity was monitored with an Ecolog sensor (Elpro, Buch, Switzerland). $PM_{2.5}$ was monitored with a personal Data Ram (pDR 1000 instrument; Thermo Electron Corporation, Contrec, Dietikon, Switzerland), which was zeroed before each use in a HEPA-filtered hood. A Scanning Mobility

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Particle Sizer (SMPS, Grimm Aerosol Technik, model 5403 with long column, Ainring, Germany) was used to monitor the particle size distribution and to calculate the total particle surface of the particles in the cabin. It was configured to monitor the particle size distributions between 11 and 1083 nm. Finally, a photoelectric aerosol sensor (PAS 2000; EcoChem Meβtechnik, Dietikon, Switzerland) was used to assess the time-course of particle-bound PAH concentration.

Spirometry and EBC collection

Forced expiratory maneuvers were done before exposure and at the end of the experiment (2 hours post-exposure) under the supervision of the same technician using a MicroLab spirometer (Micro Medical Ltd, Rochester, UK). Spirometric parameters retained were forced expiratory volume in the first second (FEV_1), the forced vital capacity (FVC), the forced expiratory ratio (FEV_1/FVC), and the forced expiratory flow (FEF_{25-75}) expressed in percent of the predicted values following the European Respiratory Society recommendations [39].

Before use, the RTube® was thoroughly rinsed with ultrapure water and air dried in a clean environment. Volunteers were asked to breathe normally for 10 minutes through a mouthpiece while wearing a nose clip. Up to 2 ml of condensate was collected. About 500 µl was used immediately to determine the particle size-number distribution and the total reducing capacity. The rest was aliquoted for H_2O_2 and MDA analysis. H_2O_2 was analysed the same day and the aliquot for MDA was stored at -78°C and analyzed within 1.5 months.

Particle size-number distribution in EBC

The particle size-number distribution (sphere equivalent hydrodynamic diameter) in EBC was determined within 30 minutes after EBC collection by nanoparticle tracking analysis (NTA, Nanosight LM20, Nanosight Ltd, Salisbury, UK). About 300 µl of EBC

was directly injected in the flow cell of the viewing unit without any further treatment. For each sample, 3 to 5 videos of 30 seconds duration were recorded and analyzed (NTA software version 2.0).

The expected dose of cigarette smoke particles deposited in the lungs was estimated by entereing the airborne size distribution obtained with the SMPS equipment into the Multiple Path Particle Dosimetry model (MPPD software version 2.0, RIVM/CIIT). The model used the Yeh/Schum symmetric, and assumed a fractional residual capacity volume of 3300 ml, upper respiratory tract volume of 50 ml, breathing frequency of 12/min, tidal volume of 65 ml, and nasal breathing. The particle size distribution was split into logarithmically-spaced particle size bins; each bin was considered monodisperse and the MPPD was run separately for each size bin. The obtained head, trachea-bronchial and alveolar deposition percentage were then applied to the corresponding particle number concentration for each bin and summed up to obtain the total deposition.

Measurement of oxidative stress biomarkers in EBC

H_2O_2 was measured in EBC based on a chemiluminescent-based procedure described by Zappacosta [40] and Navas-Diaz [41]. Briefly, 10 µl of a freshly prepared mixture containing luminol 3.3 mM (Sigma-Aldrich, Buchs, Switzerland) with horse radish peroxidase 3.3 U/ml (acidic isoenzyme type X, Sigma-Aldrich, Buchs, Switzerland) was injected sequentially in a 96 well containing 300 µl EBC or H_2O_2 standard solution and thermostatised at 37°C. The produced luminescence was recorded every 20 second during 5 minutes using a Tecan infinite M200 (Tecan, Männedorf, Switzerland).

Malondialdehyde (MDA), considered as a marker for lipoperoxidation, was analysed following the procedure described by Larstäd [22]. After MDA derivatisation with thiobarbituric acid (Sigma-Aldrich, Buchs, Switzerland) at pH 3.9 and 95°C during 60 minutes, the reacting mixture was analyzed by injecting 10 µl of the sample in a HPLC

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system (Varian 9012) equipped with a Nucleosil 100 C18 column (MachereyNagel, MSP, Zollikofen, Switzerland) in isocratic mode (0.8 ml/min phosphate buffer 20 mM, pH 6.9:acetonitrile 80:20) and a fluorescent detector (excitation 532 nm; emission: 553 nm, Shimazu). Calibration was done with MDA tatrabutylammonium salt (5-100 nM in water, Sigma-Aldrich, Buchs, Switzerland).

The total reducing capacity was measured using an electrochemical based technique (EDELSCAN, Edeltherapeutics SA, Lausanne, Switzerland), as described in Sauvain [42]. The EBC sample was diluted 2 times in phosphate buffer pH 7.4 0.1M.

Statistical analysis

Pre-post exposure differences in the respiratory parameters were compared using a paired T-test. The oxidative stress biomarkers in EBC were modeled as a linear function of the cumulative exposure and two indicators of the post-exposure EBCs in the framework of a linear mixed model with random subject-specific intercept. The exposure coefficient can thus be interpreted as a mean within-subject increase of the biomarker per unit exposure. The parameters of the two indicators can be interpreted as the "recovery", that is decrease in the biomarker concentration, one hour (respectively two hours) after the cessation of exposure.

Results

Demographic and functional characterization of the participants.

All 15 subjects successfully terminated their participation in the experiments. Their demographic and functional characteristics are summarized in Table 1. We did not detect any significant changes in the spirometric values from before to post exposure (T-test). The mean collected EBC volume was 1.34±0.22 ml from a minimum of 0.93 ml to a maximum of 2.05 ml.

Exposure characterization

The exposure levels followed the combustion pattern of the cigarettes (Figure 1).

The particle mass concentration in the chamber was linearly correlated with the number of cigarettes burned, each generating on average about 120 µg/m^3 of PM$_{2.5}$. Table 2 presents the average values of the different parameters measured during the exposure of the volunteers. The average background number concentration measured in the cabin during these experiments was 2'600±1'100 particles/cm^3. Smoke generation at days with high relative humidity tended to result in lower than expected number concentrations and particle surface. The particle size distribution was mainly monomodal with an average geometric size of 65 ± 12 nm and a mean geometric standard deviation of 1.88 ± 0.06 nm. Considerable variations in the particle size were observed when single cigarettes were burned (range from 30 to 82 nm).

The collected cigarette smoke particles were composed mainly of organic carbon. The average OC/TC ratio for all the exposure experiments was 0.89±0.01. The average PAH signal obtained from the PAS-device was not linearly correlated to the particle mass concentration, but rather to the particle surface (see the supplemental material for a detailed analysis of the exposure conditions). All the cumulative exposure variables (total number and surface of particles; mass and PAH concentration) were strongly correlated to each other ($r^2 > 0.83$).

Estimation of particle number concentration in EBC

Figure 2A presents the measured particle concentration in the collected EBC, which ranged from 0.3E^8 to 9.9E^8 particles/ml. 50% of the particles observed in EBC 1 (considered as the control) have a diameter smaller than 161±47 nm, with the 90th percentile of the particles being smaller than 320±146 nm. These percentile values are not statistically different for the other EBC samples (see supplemental material Figure S3).

The MPPD model resulted in an average total deposited particle number at the end of the exposure of $(4.6\pm1.7)E^{12}$; $(6.3\pm0.9)E^{12}$ and $(7.0\pm1.9)E^{12}$ particles for respectively low (total of 4 cigarette burned per volunteers), medium (total of 8 cigarette burned per volunteers) and high exposure level (total of $\geq$ 12 cigarette burned per volunteers). If assuming a lung lining fluid volume of 12 ml [43] and no agglomeration of the particles, the expected particle concentration in the lung lining fluid would be between 3.8 - $5.8E^{11}$ particle/ml. Neither airborne particle counts nor the modeled deposition numbers were correlated with the EBC number concentrations.

Oxidative stress biomarkers in EBC

Table 3 presents the average concentrations of the different biomarkers before exposure (EBC 1). 8% of all the samples were below the limit of detection (LOD) of H_2O_2 (0.1 µM, corresponding to three time the blank variability) and 11% were below the LOD for MDA (3.4 nM). The inter-individual variability (ratio of the standard deviation divided by the mean value expressed as percentage) of EBC 1 for H_2O_2, MDA and total reducing capacity was 55%, 87% and 13%, respectively.

Figure 2 shows the concentrations of the different markers in EBC during the course of the experiment. For the statistical analysis, the experiment was divided into two phases: Phase I corresponded to the exposure status lasting about 1 hour (EBC 2 and 3); and Phase II corresponded to the post-exposure situation (EBC 4 and 5). Both, MDA and H_2O_2 increased significantly (Table 4) with the total cumulative number of particles (consider to be a proxy of all other exposure variables) during the exposure. The general pattern consisted of an increase of these two biomarkers in EBC during the exposure time (phase I), followed by a decrease one hour after cessation of the exposure (phase II). This decrease was not statistically significant for MDA but was clearly significant for H_2O_2 (p=0.001, Table 4). In addition, an increasing H_2O_2 concentration was observed again after 2 hours post-exposure. Two hours post

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exposure, the MDA and H_2O_2 levels in EBC were statistically different from the control (EBC 1, Table 4). The total particle number concentration and the total reducing capacity did not change by the exposure. However, a tendency to increase was observed for the total reducing capacity levels in the two post-exposure EBC (Table 4).

Discussion

This study investigated the deposition of ultrafine particles in the lungs and the resulting oxidative stress at the example of tobacco smoke. While nanoscaled particles were detected in EBC, it was not possible to relate their concentration to the exposure levels. Both, H_2O_2 and MDA concentrations in EBC were significantly increased during exposure to cigarette smoke. H_2O_2 levels showed a bi-phasic pattern with an increase during exposure, a significant decrease in the first hour after exposure, and a second increase 2 hours post-exposure. In contrast, MDA did not decrease during the post-exposure time with all subjects having very similar MDA-levels at the past EBC value, as indicated by the small interquartile range (Figure 2C). Neither the total reducing capacity in the EBC nor any of the spirometric parameters were significantly associated to any of the exposure parameters.

The particles were composed mostly of organic compounds with a size range in the sub-micrometer and nanoscale. Cigarette smoke is a complex mixture of different pollutants as it contains large amounts of gas and particle phase radicals [44] and other oxidants capable of inducing oxidative stress [35]. The $PM_{2.5}$ levels used in this study corresponded to levels than can be observed in restaurants and bars in Switzerland [45]. The aerosol characteristics in our study were not identical when a different number of cigarette were burned (Table 1 and supplemental material). The differences are not extreme and systematic; for example a geometric mean size of 58 nm for one cigarette compared to 64-77 nm for multiple cigarettes or the ratio of PAH/mass decreasing from

0.73 for one cigarette to 0.51 for ≥ 3 cigarettes. Thus, it is unlikely that these differences prevented the detection of effects in the statistical analysis.

Evaluation of deposited particles

To our knowledge, this is the first study to investigate the particle concentration in EBC in connection with NTA. Large amounts of particles were detected in EBC with this technique. However, the total number of particles in EBC was not significantly changed by the tobacco smoke exposure. The MPPD deposition model suggests that the resulting particle dose after exposure correspond to about $3.8 - 5.8E^{11}$ particle/ml in the lung lining liquid. The measured particle concentrations in EBC were by a factor of thousand smaller. This corresponds to the lower dilution factor reported for lung lining fluid in EBC [46]. Thus one would have expected to see an increase of particles following exposure. The pre-exposure analysis shows that the condensed liquid contains already a considerable number of small particles, possibly some originating from the body such as cell debris, large proteins, and also ambient air pollution particles. Tobacco particles are relatively small and composed of a combination of solid and soluble organic compounds. These latter can quickly dissolve in the lung lining fluid, thus diminishing the number of particles that are sufficiently large to be detected with the NTA instrument. The smallest carbonaceous particle size which can reliably be detected with this technique is around 60 nm [47], which may be the key limiting factor. The size-number distribution determined with the NTA technique uses light diffusion, which is a generic property of particles. Without measuring a specific physical property of the cigarette smoke particle, the unambiguous determination of the deposited dose cannot be done with the described protocol. This uncertainty could be solved in laboratory settings by using non-toxic fluorescent nanoparticles and tracking their Brownian motion with a specific excitation and emission wavelength, or by using methods that include the chemical analysis of particles. In the case of metallic particles

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for example, the use of single-particle inductively coupled plasma mass spectrometry (sp-ICP-MS) could be an interesting technique [48]. An additional phenomena that could have prevented the detection of smoke particles is the particle growth in the airways [49] followed by their internalization into vesicles or micelles. It was suggested that exhaled lining fluid aerosols have the form of vesicles [50] and NTA does detect such structures [51]. Thus, the agglomerated smoke particles could be internalized in these vesicles and could not be detected by NTA as a consequence of insufficient high numbers but because they were no longer individual particles.

Oxidative stress biomarkers in EBC

We had the hypothesis that inhaled particles will induce an oxidative stress in the lung lining fluid upon inhalation. H_2O_2 and MDA increased in EBC during the phase I. This is concordant with already published data showing an early increase of H_2O_2 levels 30 minutes after cigarette smoke exposure [37, 52]. This could indicate that our hypothesis is correct. However, alternative explanations need to be assessed as well. When passing the air during the exposure phase through an impinger filled with water, we detected compounds present in the gaseous phase that could be interpreted as H_2O_2 or MDA. Cigarette smoke contains large amount of H_2O_2 in it gaseous phase [53] and associations between H_2O_2 levels in ambient air and H_2O_2 levels in EBC were reported [54]. However, it remains unclear whether the H_2O_2 increase in EBC 2 and 3 (collected during exposure) is related solely to gaseous H_2O_2 or if some was transported by or generated on the particles' surface [55]. Cigarette smoke is chemotactic to neutrophils and macrophages and can activate them [35]. However, the activation of immune cells usually takes several hours. Thus, the initial burst in H_2O_2 is most likely not related to immune cells, whereas such a mechanism could well explain the delayed increase of H_2O_2 during phase II (EBC 5). This increase could also result from the direct production of reactive oxygen species by the deposited cigarette smoke particles or could be the

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sign of a transient inflammatory process in the lungs produced by airway epithelia [56] or alveolar macrophages and type-II pneumocytes [54]. Normalisation of H_2O_2 levels after the cessation of cigarette smoke exposure has been reported to last 10-12 hours for children exposed to passive smoke [57] or for smokers having stopped smoking for at least 10h [52]. In addition to the changes related to the exposure, H_2O_2 in EBC seem to show a circadian pattern [37]. However, it would not interfere with the dose-effect evaluation because we controlled for such patterns by standardizing the time of exposure and health effect assessment.

Acrolein is an important carbonyl emitted by cigarettes [58] and is known to react with thiobarbituric acid, the derivating agent used for the MDA measurement [59]. To confirm that acrolein can interfere in the MDA detection, we proceed a pure water-based acrolein standard with the analytical method for MDA and indeed observed a dose dependent signal (see suppelementary material, Figure S4). This suggests that acrolein interfered with the MDA measurement. The acrolein concentration in tobacco smoke would be sufficiently large to explain most of the elevated MDA values in EBC 3 (supplemental material, Table S1). MDA was proposed to be a product from lipid peroxidation that occurs during oxidative damage to cell membranes and it is unclear if lipoperoxidation would be sufficiently rapid to increase MDA in the time frame of phase I. Additional studies are needed to clarify if some of the signal still could be related to lipoperoxidation.

We observed a statistically significant MDA increase between EBC 1 and EBC 5 (collected 2 hours post-exposure Fig 4C), but, contrary to H_2O_2, no post-exposure decrease could be observed until the last EBC-sample. MDA is a small molecule and it should start to disappear if it was only an exposure marker for acrolein. However, if the lipoperoxide degradation process was slow, it is possible that the EBC 2 and 3 mostly reflected exposure to acrolein, while after 2 hours, the MDA production resulting from the lipoperoxidation was the predominant source [30]. The mixed effect model only

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confirms that the MDA signal is correlated to exposure. However, the above suggested timing would be in agreement with another study that investigated ambient air particles (up to 250'000 PM_1 particles/cm^3 during moderate exercise) and found an increase in MDA levels in EBC 30 minutes post-exposure compared to pre-exposure [60].

We did not observe any change in the total reducing capacity in the collected EBC and also the mixed model did not find any association with exposure. This suggests that the healthy volunteers maintained the redox balance at the organ level even though local oxidative damage did occur, as suggested by the MDA findings. However, we cannot exclude that the exposure may have triggered the production of antioxidants because for that to occur, activation of antioxidant-related genes is required [61]. In human monocyte cell lines and primary blood mononuclear cells stimulated with cigarette smoke extract, a 1.5 fold upregulation of genes related to antioxidants was observed 8 hours post-exposure [62].

In summary, we found that EBC contains large numbers of sub-micron particles but these could not be related to the level of cigarette smoke the volunteers were exposed to. On the contrary, H_2O_2 and MDA concentration in EBC showed significant changes in the hours following exposure to cigarette smoke. This seems to represent initially the deposition of particles and gases in the lung lining liquid, and later the start of oxidative stress with associated cell membrane damage. Future studies should extend the follow-up time and should remove gases or particles from the air to allow differentiating between the different sources of H_2O_2 and MDA. This would also help to determine in which conditions both compounds could be considered as biomarkers of exposure or effect. In addition, it would be worth measuring other biomarkers of exposure/effect in EBC, including for example 8-isoprostane [63, 64] and small molecular weight metabolites by NMR spectroscopy [65, 66]. The use of breath analysis by electronic noses could also help the monitoring of lung damage [67,68], as it has been reported to be a reproducible technique [69].

Acknowledgments

Author Disclosure Statement

No conflicts of interest exist for any of the authors.

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Tables

Table 1: Characteristics of volunteers and their lung parameters.

	All Pre-exposure	All Post-exposure	p-value
Age	26.3 ± 6.5 (15)	-	-
BMI	23.1 ± 2.6 (13)	-	-
FEV1 (L)	4.4 ± 0.4 (12)	4.4 ± 0.5 (13)	0.97
FEV1 (% pred)	100.3 ± 8.4 (12)	100.1 ± 9.7 (13)	0.97
FVC (L)	5.0 ± 0.5 (12)	5.0 ± 0.8 (13)	0.97
FVC (% pred)	97.4 ± 8.6 (12)	96.2 ± 11.0 (13)	0.76
FEV1/FVC	87.0 ± 5.0 (12)	87.7 ± 5.7 (13)	0.75
FEF25-75 (L)	4.9 ± 1.0 (12)	4.9 ± 1.0 (13)	0.96
FEF25-75 (% pred)	96.9 ± 21.1 (12)	98.1 ± 19.7 (13)	0.88

Table 2: Average exposure conditions (± standard deviation) during the 15 experiments, grouped in function of the number of cigarettes burned.

	Low (n=5)	Medium (n=5)	High (n=5)
# cigarettes	4 x 1	4 x 2	4 x ≥ 3
T [°C] [a]	22.2 ± 0.12	23.6 ± 0.12	22.7 ± 0.43
Humidity [% rH] [a]	29.9 ± 1.4	35.5 ± 1.0	27.9 ± 3.3
$PM_{2.5}$ (PDR) [µg/m^3]	117 ± 4	240 ± 25	370 ± 18
OC [µg/m^3][a]	71 ± 2	119 ± 4	170 ± 8
EC [µg/m^3][a]	10 ± 1	15 ± 1	18 ± 2
PAH (PAS) [ng/m^3]	85 ± 2	162 ± 2	188 ± 9
Total # [/cm^3]	225'000 ± 15'200	307'500 ± 13'400	428'600 ± 29'400
Geom size [nm]	58 ± 3	64 ± 1	77 ± 2
Total surface [mm^2/m^3]	4'920 ± 203	8'270 ± 340	15'500 ± 640

[a] : corresponds to the average over all the time the volunteer stayed in the cabin (exposure + post-exposure).

Table 3: Average concentration of biomarkers in the control EBC (EBC 1).

Variable	Mean	Std deviation	Min	Max
MDA [nM]	5.6	4.9	< LOD (3.4 nM)	21.6
H_2O_2 [µM]	0.21	0.14	< LOD (0.1 µM)	0.43
Total reducing capacity [a.u.]	52	7	43	67

Table 4: Mixed models – Phase I and II based on the cumulative total number of particles in the air as the exposure variable.

	Exposure (cumulative total number)	1 hour post exposure	2 hours post exposure	Constant
Model for particle number in EBC				
Coefficient	$(0.19\pm2.3)E^{-8}$	-0.01±0.66	0.31±0.72	3.17±0.44
p	0.935	0.985	0.662	0.001
Model for Log MDA				
Coefficient	$(2.0\pm0.6)E^{-8}$	-0.22±0.17	-0.13±0.17	1.67±0.14
p	0.001	0.185	0.473	0.001
Model for Log H_2O_2				
Coefficient	$(5.2\pm1.3)E^{-9}$	-0.17±0.03	-0.03±0.04	0.26±0.03
p	0.001	0.001	0.394	0.001
Model for Log Total reducing capacity*				
Coefficient	$(0.98\pm1.32)E^{-9}$	0.02±0.03	0.06±0.04	3.94±0.04
p	0.457	0.474	0.129	0.001

*: without one outlier.

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Figure legends

Figure 1: Typical exposure levels (number concentration: left axis; PAH concentration and PM$_{2.5}$ concentration: right axis) encountered for an experiment where 1 cigarette was burned 4 times. The collection of the five EBC samples (10 minute duration) is indicated with the grey bars. Abbreviations: SMPS: Scanning Mobility Particle Sizer; PAS: Photoelectric Aerosol Sensor; pDR: Personal DataRam.

Figure 2: Variation of the different markers measured in EBC in function of the sampling (EBC N° 1 corresponding to the before exposure situation – control; EBC N° 2-3 corresponding to EBC collected during exposure; EBC N° 4-5 corresponding to post-exposure samples). Panel A: Particle number concentration; Panel B: H$_2$O$_2$ concentration; Panel C: MDA concentration; Panel D: Total reducing capacity.

*: difference with EBC 1 statistically significant ($p < 0.05$, multiplicity corrected p-values).

¶: difference with EBC 2 statistically significant ($p < 0.05$, multiplicity corrected p-values).

†: difference with EBC 3 statistically significant ($p < 0.05$, multiplicity corrected p-values).

‡: difference with EBC 4 statistically significant ($p < 0.05$, multiplicity corrected p-values).
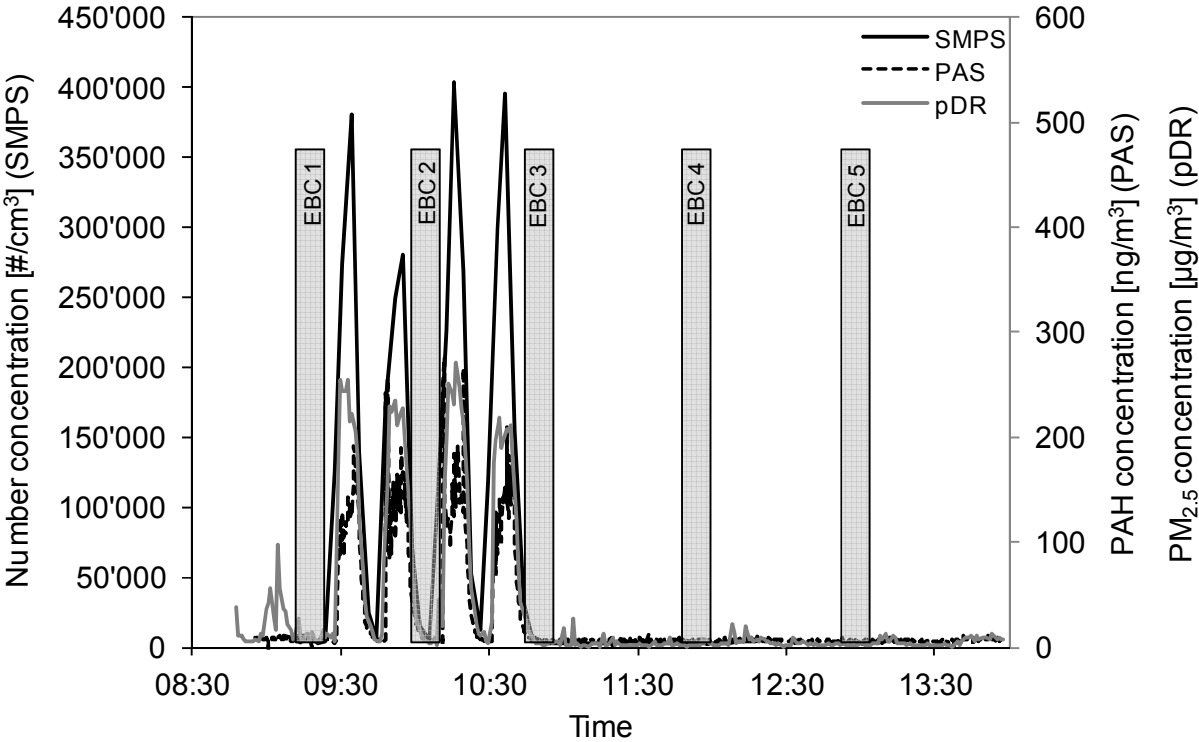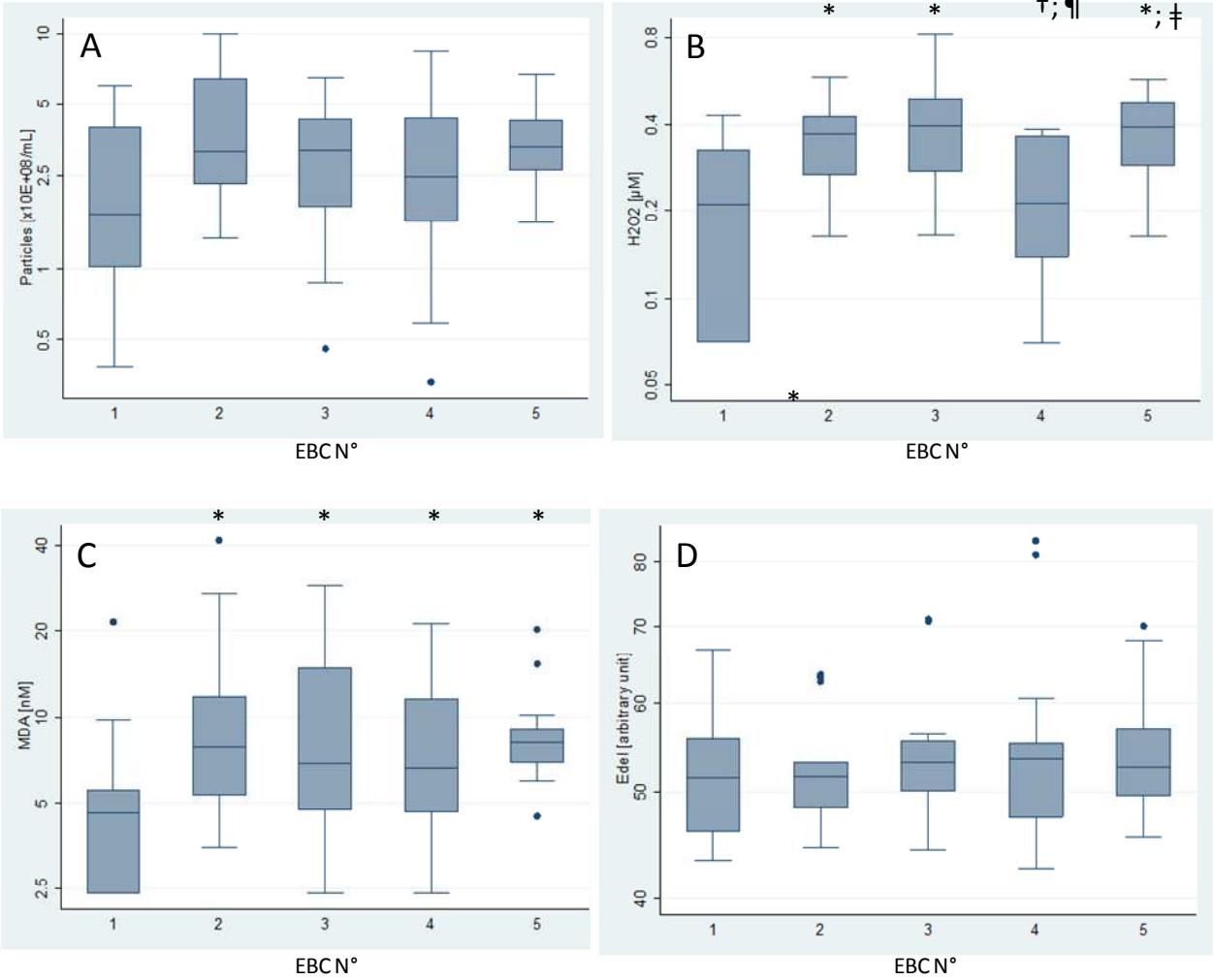
Figures

Figure 1:

Supplemental material

Exhaled breath condensate as a matrix for combustion-based nanoparticle exposure and health effect evaluation.

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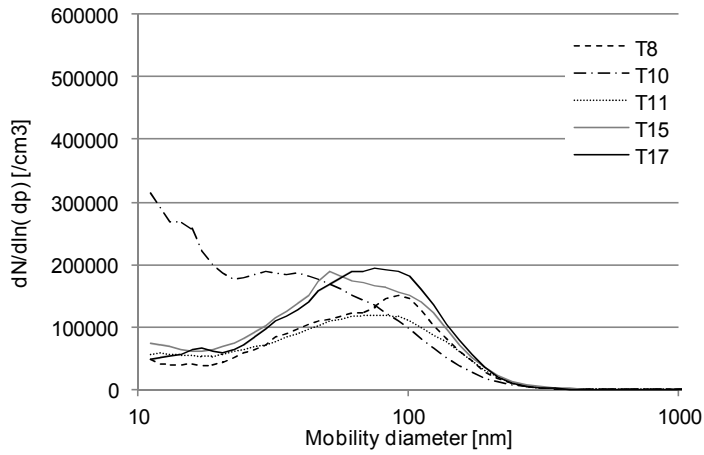[2] Institute for Research and Safety, Rue du Morvan, CS60027, 54519 Vandoeuvre (France).

Size distribution of the different exposure experiments

Figure S1 presents the averaged size distribution of the cigarette smoke particles measured during the exposure of the different volunteers. Each size distribution corresponds to the average of the four ignition of a different number of cigarette (low=1 cigarette/ignition; medium=2 cigarettes/ignition; high=3 cigarettes/ignition, with one volunteer exposed to 4 cigarettes/ignition – Test N° T2). Measurements have been done with a SMPS (Grimm Aerosol Technik, model 5403 with long column, Ainring, Germany).
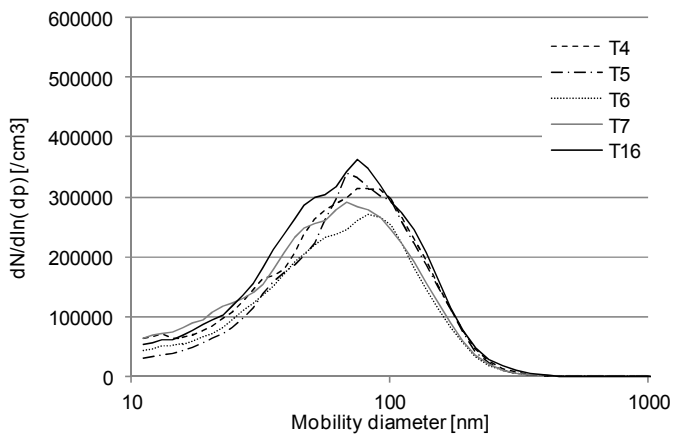
The exposure conditions for the test N° T10 were different from the other (Figure S1, A: Low exposure) and corresponded to a rather high generation of small particles. Whereas temperature and relative humidity during this generation were around the average value, this difference could be due to the fact that all the cigarettes were not conditioned in a controlled environment, possibly generating variability in the burning conditions.

1

Figure S1: Average size distribution of the cigarette smoke during the different generations. Each test ID are given on the upper right part of each graph

A: Low exposure
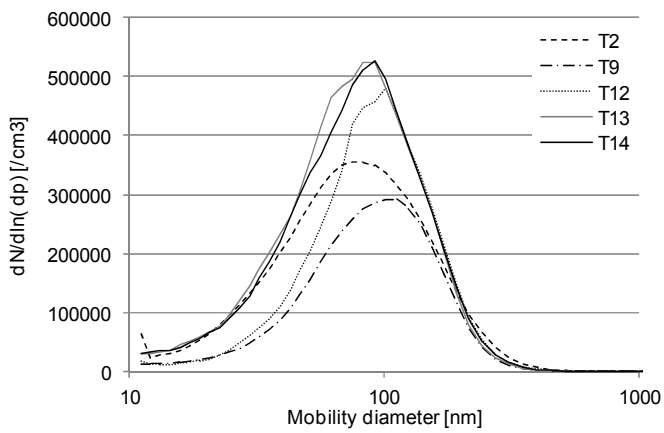

B: Medium exposure

C: High exposure

<u>Figure S2</u>: Association between the different variables measured by direct reading instruments during each cigarette burning. Numbering corresponds to the different tests done.
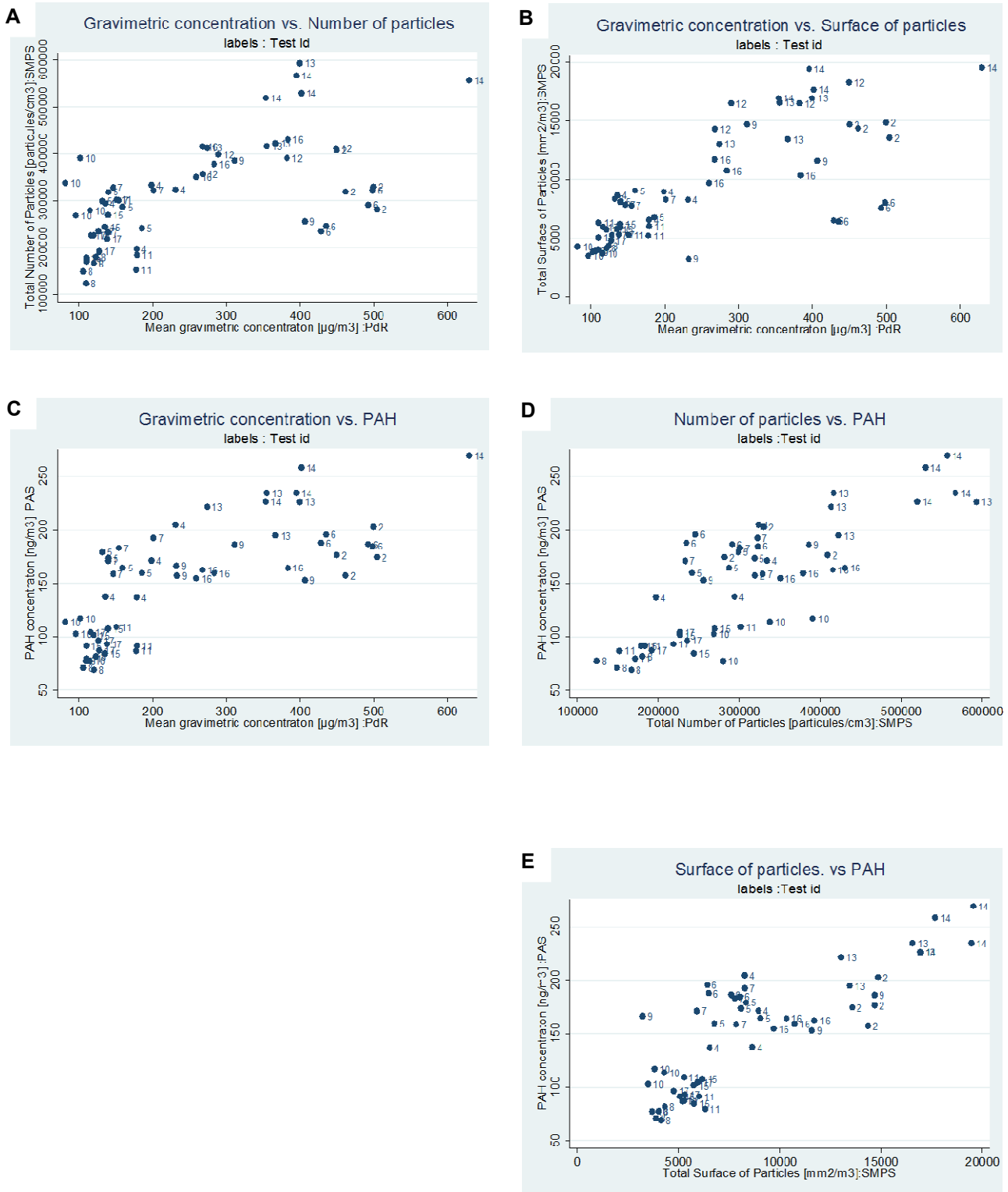
Figure S2 presents the association between the different exposure variables recorded by direct reading instruments. Each point corresponds to the averaged concentration measured during each cigarette burning (4 point for each test). The label of each point corresponds to the test ID. A statistically significant non-linear relationship is observed for the particle surface and PAH content in function of the mass (p<0.011 for a square term in the regression model, see panel B and C respectively), suggestive of an agglomeration process in the air at high particle concentrations. On the contrary, when the SMPS-based surface is considered, a linear relationship (p<0.001) is observed with the particulate PAH concentration (Panel E). This linear relationship is expected based on the measurement principle of the PAS instrument [1]. As the surface variable is calculated based on the particle number measured with the SMPS, it is not surprising that a similar linear relationship is observed between the PAS signal and the particle number in the air (Panel D).
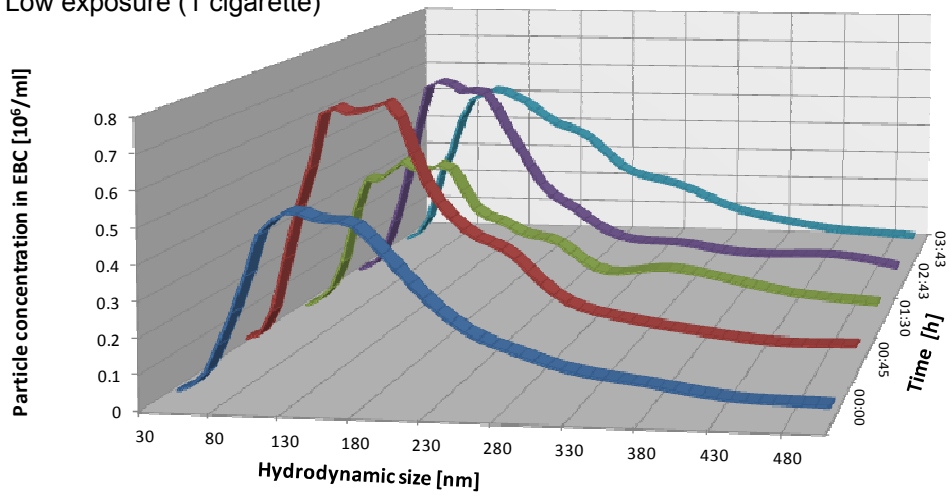
Size distribution of the particles in EBC

Figure S3 illustrates the different averaged size distribution in EBC. In Figure S3, the size distribution at 00:00 min corresponds to EBC 1 (control); the second and third curves correspond to EBC 2 and 3 (just after exposure episodes) whereas the fourth and fifth curves correspond to EBC 4 and 5 (about 1 and 2 hours post exposure).
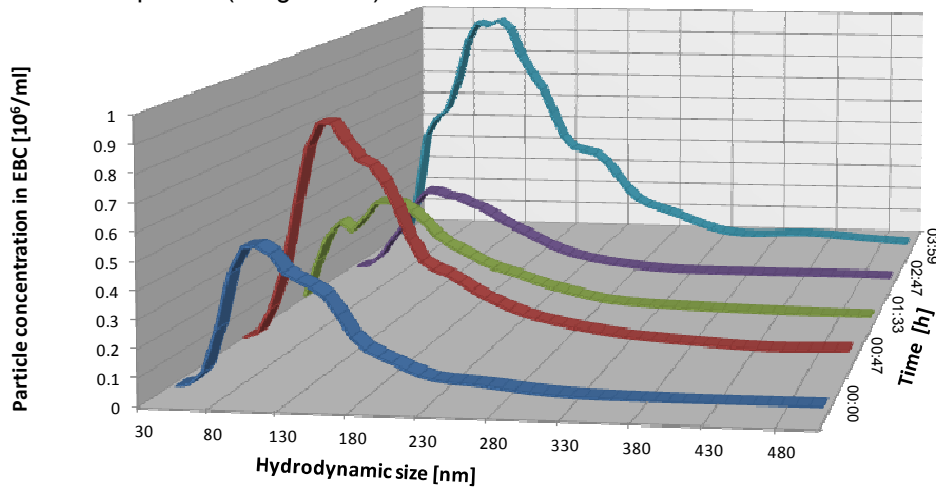
The averaged coefficient of variation (CV) on the particle concentration (corresponding to the ratio of the standard deviation for each size range divided by the averaged particle concentration, expressed in percent) for all these size distribution was 117%. This rather high CV could be due to the variability observed between the different smoke generations and with an additional contribution from the inter-individual variability.

Figure S3: Averaged (n=5) size distribution of particles in EBC for volunteers exposed to low (panel A), medium (panel B) or high (panel C) levels of cigarette smoke.
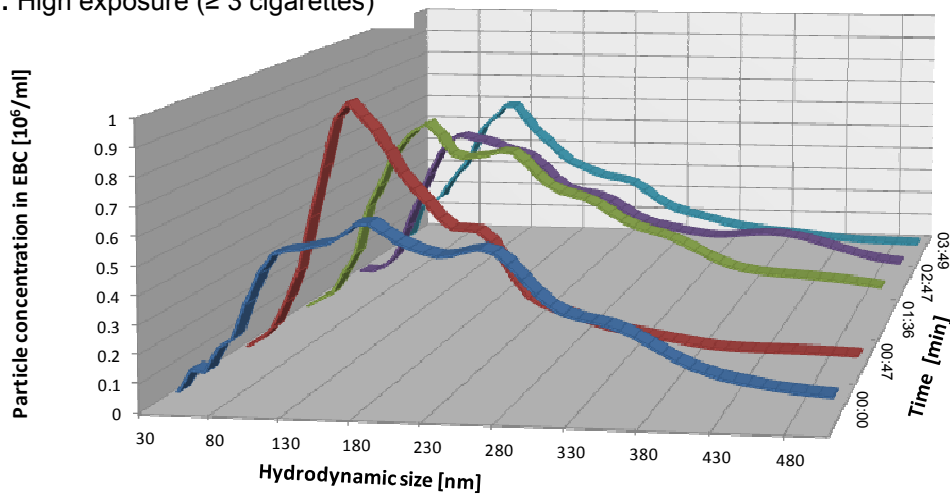
A : Low exposure (1 cigarette)

B : Medium exposure (2 cigarettes)

C : High exposure (≥ 3 cigarettes)

Measurement of acrolein with the MDA methodology

We prepared different standard solution of acrolein (>99%, for electron microscopy, Fluka) by dilution of a mother solution of 8.9 mM with water. The studied concentration was comprised between 8.9 and 225 µM. These solutions were derivatised with TBAR in the same manner as for MDA. The resulting peak at the same retention time as for the MDA was quantified with a calibration curve obtained with tetrabutyl-ammonium MDA. Figure S4 presents the resulting relationship between the acrolein concentration in the standard solutions and the equivalent MDA concentration produced when applying the analytical protocol.

Figure S4: Relationship between the acrolein concentration in the standard and the measured MDA equivalent when applying the analytical procedure described under Materials and Methods for MDA. Error bars correspond to the standard deviation of 3 independent measurements.

The plotted trend line shows the equation $y = 0.4133x^{1.3098}$ with $R^2 = 0.9893$. The y-axis is labeled "MDA conc [nM]" ranging from 0.00 to 600.00, and the x-axis is labeled "Acrolein conc [µM]" ranging from 0.00 to 250.00.
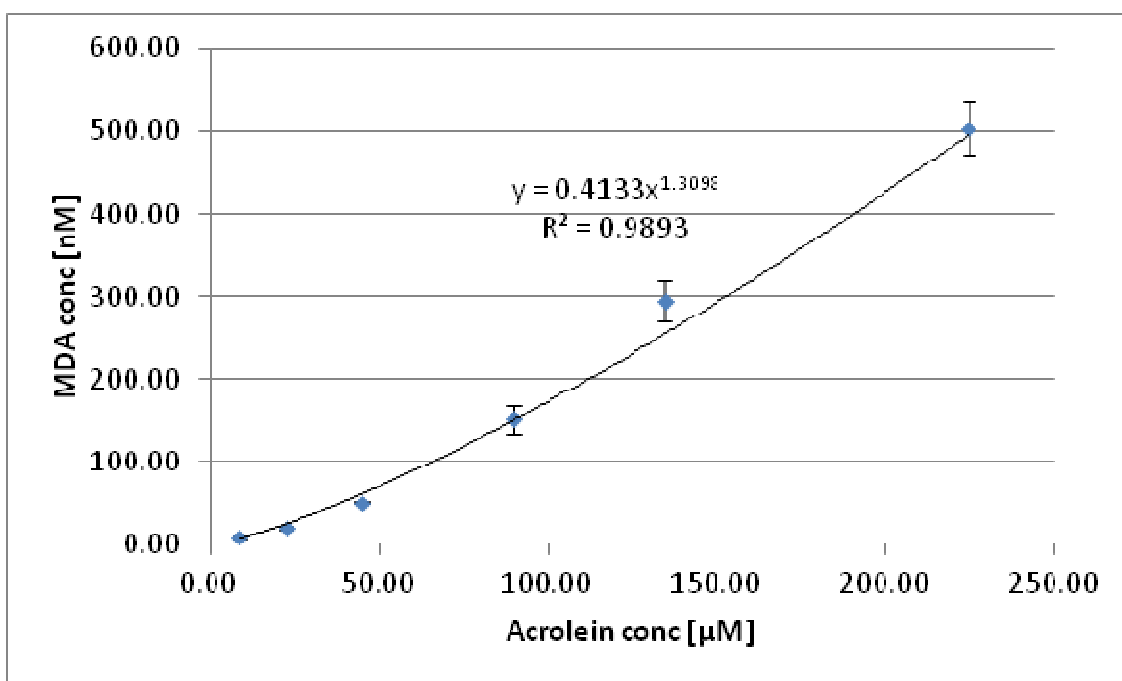
Table S1 presents the calculation done to estimate the contribution of the acrolein to the MDA concentration in EBC. This calculation indicates that the MDA increase between EBC 1 (control) and EBC 3 (just after the exposure) could be largely attributable to the inhalation of acrolein generated by the combustion of the cigarettes.

Table S1: Estimated contribution of acrolein from the cigarette combustion to the MDA level measured in EBC.

Acrolein level per cigarette [µg/cig]				Reference
Best case [µg/cigarette]	63			[2]
Worst case [µg/cigarette]	167			[2]
Concentration of acrolein during exposure				
Number of cig burned in the 10 m³ cabin	4	8	12	
Acrolein concentration in air; best case [µg/m³]	25	50	76	
Acrolein concentration in air; worst case [µg/m³]	67	133	200	
Mass of inhaled acrolein during cigarette exposure				
Volume inhalted/min [L/min]	6			
Exposure duration [min]	60			
Total deposition in the lower respiratory tract [%]	98			[3]
Mass acrol inhaled; best case [µg]	8'891	17'781	26'672	
Mass acrol inhaled; worst case [µg]	23'496	46'993	70'489	
Concentration of acrolein in EBC				
Volume of lung lining fluid [ml]	12			[4]
Dilution factor	10'000			[5]
Concentration of acrolein in EBC; best case [µg/ml]	0.07	0.15	0.22	
Concentration of acrolein in EBC; worst case [µg/ml]	0.20	0.39	0.59	
Concentration of acrolein in EBC; best case [nmol/ml]	1.32	2.65	3.97	
Concentration of acrolein in EBC; worst case [nmol/ml]	3.50	6.99	10.49	
Acrolein contribution to the MDA measurement				
Calibration curve: MDA = $0.4133 \cdot Acrol^{1.31}$				This work
Concentration MDA attributable to acrolein; best case [nM]	**0.6**	**1.5**	**2.5**	
Concentration MDA attributable to acrolein; worst case [nM]	**2.1**	**5.3**	**9.0**	
Average MDA concentration measured; control [nM]	5.6			
Average MDA concentration measured in EBC 3 [nM]	6.6	8.1	18.8	
Average measured MDA increase [nM]	**1.0**	**2.5**	**13.2**	
Acrolein contribution; best case [%]	61	60	19	
Acrolein contribution; worst case [%]	218	214	68	

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