INSIGHTS ON WOOD COMBUSTION GENERATED PROINFLAMMATORY ULTRAFINE PARTICLES (UFP)

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ABSTRACT

This study aimed to collect, characterize ultrafine particles (UFP) generated from the combustion of wood pellets and logs (softwood and hardwood) and to evaluate their pro-inflammatory effects in THP-1 and A549 cells. Both

cell lines responded to UFP producing interleukin-8 (IL-8), with wood log UFP being more active compared to pellet UFP. With the exception of higher effect observed with beech wood log UFP in THP-1, the ability of soft or hard woods to induce IL-8 release was similar. In addition, on weight mass, IL-8 release was similar or lower compared to diesel exhaust particles (DEP), arguing against higher biological activity of smaller size particles. UFP-induced IL-8 could be reduced by SB203580, indicating a role of p38MAPK activation in IL-8 production. The higher activity of beech wood log UFP in THP-1 was not due to higher uptake or endotoxin contamination. Qualitatively different protein adsorption profiles were observed, with less proteins bound to beech UFP compared to conifer UFP or DEP, which may provide higher intracellular availability of bioactive components, i.e. levoglucosan and galactosan, toward which THP-1 were more responsive compared to A549 cells. These results contribute to our understanding of particles emitted by domestic appliances and their biological effects.

Key words: wood, UFP, inflammation, cytokine, protein corona

INTRODUCTION

Domestic biomass combustion is an important emission source of air pollutants. Combustion processes can generate large quantities of fine particles, which are thought to be one of the most important health concerns worldwide (reviewed by Naeher et al., 2007; Adetona et al., 2016), and the IARC categorized household biomass fuel combustion in Category 2A, probably carcinogenic in humans, with limited human evidence but supported by animal and mechanistic evidence (IARC monograph # 95, 2010). Therefore, a strategic area of health research on ambient air and emission particles is the development and use of clean combustion and emission control technologies.

Even if the combustion of woods can generate a variety of compounds, fine and ultrafine particles (UFP, i.e. particles with aerodynamic diameter lower than 100 nm) are thought to be the best single indicator of the health impacts of most combustion sources. Wood smoke particles are usually within the size range thought to be most damaging to human health, their chemical composition can be different depending on the specific wood used and the combustion conditions, and they differ from those derived from fossil fuel combustion (Lee et al., 2005).

Inhaled particles deposited in the lung can interact with resident alveolar macrophages and epithelial cells to induce the release of a cascade of inflammatory signals, including cytokines and chemokines (Salvi and Holgate, 1999). Among the pro-inflammatory mediators induced by particles, the measurement of IL-8 is of particular relevance as this chemokine is a neutrophil attractor, and an influx of neutrophils may lead to a sustained inflammation and possible tissue damage (Harada et al., 1994; Dinarello, 2000; Chung, 2005). Increased IL-8 can thus contribute to the risk of disease development as well as exacerbate existing conditions by inducing an immunologically active state.

We have previously demonstrated - comparing the emission from conifer and beech pellets combustion - that both types of pellets can generate biologically active PM2.5 fine particles (Corsini et al., 2013). The aim of the present study was to extend these observations, collecting, characterizing and comparing ultrafine particles obtained from the combustion of conifer (soft wood) and beech (hard wood) pellets and logs, and to characterize some of the mechanisms involved in IL-8 induction. The hypothesis is that UFP may be more active compared to particles with higher size range, and that wood stoves, having in general less regular combustion in comparison with appliances fueled by wood pellets and thus emissions richer in pollutants, may generate more active UFP. Cell damage, production of inflammatory mediator, cellular uptake, and protein corona formation were evaluated as biological markers of cell activation. The effects were compared to reference diesel exhaust particles (DEP). The possible different toxicological properties of the collected wood combustion particles were evaluated by exposing two human cell lines, surrogates of alveolar macrophages and lung type II epithelial cells, namely the human promyelocytic (THP-1) and the human lung epithelial adenocarcinoma (A549) cell lines. We had focused our attention on these cells, as alveolar macrophages and epithelial cells are the first cells encountering particles in the lungs. Obtained results indicate that biomass combustion generates biologically active UFP, with UFP obtained from wood logs being more active. On a weight mass, the activity observed was comparable to the ones of DEP, arguing against a higher biological activity of UFP compared with other categories of combustion-derived particles (DEP).

METHODS

Tested particles. Wood combustion generated UFP fractions were obtained from the combustion of conifer and beech pellets and logs in domestic appliances during laboratory tests carried out at the Laboratory for Energy and Environment of Piacenza (LEAP, www.leap.polimi.it). NIES certified reference material n°8 vehicle exhaust particulates (DEP) was used as reference PM (Environment Agency NIES, Ibaraki, Japan).

Tested appliances and combustion cycles. Given the strict relationship between combustion technology, burning cycle (real-world vs. standard) and emission levels (Ozgen et al., 2014), the study focused on UFP sampling from both automatic (pellet stove) and manually fed (wood stove) appliances. UFP samples were obtained from combustion experiments on two types of commercial stoves fed with two wood species (softwood-conifer and hardwood-beech). The 11 kW top-feed wood pellet stove, with fan assisted flue discharge and internal fuel hopper was operated with 3 to 4 hour combustion cycles designed to represent real-life usage of the combustion appliance. To this aim, the heat output was modulated between high (75% of the nominal) and low (~33 % of the nominal) loads. Startup and shutdown periods were excluded from the measurement period. The wood log stove had a nominal heat output of 8 kW and was equipped with a state-of-the-art triple air supply system. The primary and secondary airflows were adjusted manually during the tests. Test cycles applied represented the real life operating conditions (total run duration about 5 to 7 hours) including the cold start, nominal and transitory periods. The burning cycle started with the ignition of the wood logs from the top including the wooden kindling material and the kerosene based fire starter. This ignition period was integrated when needed with a preheating period loading few small batches. Stove was tested under hot furnace conditions with 1 or 2 consecutive batches of nominal load (2 kg/h). The burning cycle was concluded with a higher load batch (1.2 to 1.5 times the nominal load), sometimes with slightly bigger logs. The start-up and loading followed the prescriptions of the manufacturer. Both stoves were placed on a weight scale for continuous fuel consumption determination.

UFP sample collection for chemical characterization and toxicological analyses. Samples for chemical characterization and toxicological analyses were collected at LEAP. The experimental setup is shown in the supplemental material figures 1 and 2. UFP were sampled from diluted flue gas streams using three multistage impactors operating parallel. The stack gas was diluted from 90 to 150 times in case of the pellets stove, while for the wood stove the dilution ratios range from about 400 to 1000. For all multistage impactors, only UFP collected on the two lower impaction stages and the back-up filter were considered to select particles with d_{ae}<100nm for each sampling. The multistage impactors operated on different substrates, depending on the subsequent analysis to be performed. The first impactor (Small Deposit Impactor SDI, Dekati, details in Bernardoni et al., 2011) collected UFP on polycarbonate impaction stages and on PTFE back-up filter for elemental analysis (all these substrates were also weighed for gravimetric determination). The second impactor (micro-orifice, uniform deposit impactor MOUDI, MSP corporation) operated with quartz fiber filters: half of each filter was devoted to the determination of ions, total carbon, and levoglucosan and its isomers, whereas the other half was devoted to polycyclic aromatic hydrocarbons (PAH) analysis. In the last impactor (MOUDI) two aluminum foils were used as impaction stages and a PTFE was the back-up filter to collect UFP for gravimetric determination and toxicological tests.

UFP characterization. The UFP mass was determined from the aluminum foils and PTFE filters gravimetrically using a microbalance (MX5 Mettler-Toledo, sensitivity 1 μg) located in a controlled weighing chamber (T = 20±2°C and RH = 50±5%) where the filters were conditioned for 48 h before weighing. Metals (Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, P, Pb, Sr, Ti, V, Zn) were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (Varian 720-ES). Solubilization procedure followed the EN14902:2005 methodology by using concentrate sub-boiled distilled HNO_3 and 30% ultrapure H_2O_2 in a microwave oven, at 220°C for 25 min (P= 55 bar) (Perrone et al., 2013). TC analysis was performed by TOT (Thermal Optical Transmittance, Sunset Laboratory) on a quartz fiber filter punch (1.5 cm^2) taken from each filter, using analysis protocol NIOSH870a.

For the analysis of ions and levoglucosan in UFP, 4.5 cm^2 of the three quartz fiber filters were put together in a test tube and extracted with 5 mL of MQ water in an ultrasound bath for 1 hour. Ion Chromatography (IC) has been employed for the quantification of the main ions. Measurements of cationic (Na^+, K^+, Ca^{2+}, Mg^{2+} and NH_4^+) and anionic (NO^{3-}, NO^{2-}, SO_4^{2-}, Cl^-, acetate, propionate, formiate, methansulphonate and oxalate) species were carried out using an ICS-1000 HPLC system equipped with a conductivity system detector. Anions analysis was carried out with a Ion Pac AS11 column using KOH from 1 to 17 mM, flow rate=1 mL/min, for the detection a conductivity system detector working with an anion self-regenerating suppressor AERS 500 was used. Cations determination was performed using a CS17 (Dionex) column and methanesulfonic acid (MSA) from 0.5 to 10 mM as eluent at a flow rate=0.25 mL/min and for the detection a conductivity system equipped with a cation self-regenerating suppressor CERS 500. Levoglucosan and its isomers analysis were carried out by HPAEC–PAD using an ion chromatograph Dionex ICS1000. Different anhydrosugars (i.e. levoglucosan, mannosan and galactosan) were separated using a Carbopac PA-20 guard column and a Carbopac PA-20 anion exchange analytical column. As eluent, NaOH 18 mM was used with a flow rate of 0.5 mL/min. An amperometric detector (Dionex ED50) equipped with an electrochemical cell was used.

Among the 17 priority PAHs defined by the United States Agency for Toxic Substances and Disease Registry (Ravindra et al., 2008), the following PAH were evaluated by mass spectrometry: benzo[α]anthracene, benzo[α]fluoranthene, fluoranthene, phenanthene, chrysene, benzo[α]pyrene, pyrene, anthracene. Results are presented as sum of all the measured PHAs (ΣPHA, ng/μg of UFP).

UFP sample extraction for biological studies. UFP for biological studies were obtained by extraction from the aluminum foils and PTFE filter in 50% ethanol in water using ultrasonic bath for 60 minutes. After extraction, samples were evaporated under a nitrogen flow for at maximum 8 hours. Extracted UFP were then resuspended in sterile PBS at 1 mg/ml. In order to evaluate the contribution of the blank, unexposed filters were extracted under the same conditions.

Cells. The human lung epithelial cell A549 and the human monocytic THP-1 cell lines were obtained from Istituto Zooprofilattico (Brescia, Italy). Cell culture media and all supplements were from Sigma (St Louis, MO, USA). For IL-8 production, THP-1 cells were diluted to 10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50 μM 2-mercaptoethanol, supplemented with 10 % heated-inactivated fetal calf serum (media) and cultured at 37°C in 5% CO_2 incubator. While A549 cells were diluted to 2.5 x 10^5 cells/mL in 24-well plates, and after 48 h, treated in 0.3 ml of RPMI 1640 containing 2 mM L-glutamine,

155 0.1 mg/ml streptomycin, 100 IU/ml penicillin, supplemented with 10% heated-inactivated fetal calf serum (media)
156 and cultured at 37C in 5% CO_2.

157 *Treatments and cell viability.* Cells were treated with increasing concentrations of UFP (0-100 μg/ml) or DEP
158 (100 μg/ml) for 24 h. This time point was chosen based on previuos experiments as optimal time to assess IL-8
159 release in both cell lines (Corsini et al., 2013). We have data of IL-8 release at 3 h for some of the UFPs, but the
160 release was minimal. Therefore, due to the small amount of sample available, we decided to compare the release
161 at 24 h. Cell viability was assessed by lactate dehydrogenase (LDH) leakage from damaged cells. LDH is a well-
162 known indicator of cell membrane integrity and cell viability. LDH activity was determined in cell-free
163 supernatants using a commercially available kit (Takara Bio Inc., Japan). Results are expressed as percentage of
164 control.

165 In order to evaluate the signal transduction pathways involved in UFP-induced IL-8 induction, the role of p38
166 mitogen-activated protein kinase (p38MAPK) was investigated. The levels of IL-8 were measured in the
167 supernatants of cells treated with UFP (100 μg/ml) or DEP (100 μg/ml) in the presence and absence of SB202190
168 (SB, 0.1 μM) as a highly selective, potent and cell permeable inhibitor of the p38 MAPK. SB was added to cell
169 culture 1 h prior to UFP or DEP, and the IL-8 release was assessed 24 h later. The concentration of 100 μg/ml
170 was chosen based on its ability to induce IL-8 production in both cellular models.

171 To investigate a possible presence of endotoxin, UFP generated form beech wood logs were pre-incubated with
172 polymixin B sulfate (15 μg/ml final concentration) for 1 h at room temperature and then added to THP-1 cells for
173 24 h. Lipopolysaccharide (LPS) from Escherichia coli serotype 0127:B8 (Sigma) was included as a positive
174 control. LPS (10 ng/ml) was incubated with polymixin B as described for particles.

175 To investigate the uptake of UFP in THP-1 cells, the NPs uptake method described by Suzuki et al. (2007), based
176 on the measurement of the laser light intensity scattered by particulates inside of the cell (SSC: Side SCatter), was
177 used. Briefly, THP-1 cells were treated for 24 h with 100 μg/ml UFP generated from the combustion of conifer
178 and beech wood logs and DEP. The amounts of particles taken up by the cells were analyzed using a flow
179 cytometer (FACS) (FACScan, BD, Italy). The laser beam (488 nm) illuminates cells in the sample stream, which
180 go through the sensing area. The laser light scattered at narrow angles to the axis of laser beam is called forward-
181 scattered light (FSC). The laser light scattered at about a 90° angle to the axis of the laser beam is called side-
182 scattered light (SSC). The intensities of FSC and SSC are proportional to the size of cells and the intracellular
183 density, respectively. Results are expressed percentage of gated cells.

184 Finally, in order to investigate the role of serum in UFP-induced cellular uptake and IL-8 release, THP-1 cells
185 were treated with beech wood log UFP (100 μg/ml) resuspended in culture media with or without 10 % FCS for
186 24 h.

187 *Chemokine production.* IL-8 release was assessed in cell-free supernatants obtained by centrifugation at 2500 rpm
188 for 3 min and stored at –20 °C until measurement by a commercially available sandwich ELISA (ImmunoTools,
189 Friesoythe, Germany), with 15.6 pg/ml as the limit of detection. Spike experiments were conducted to exclude
190 possible interference of UFP with the ELISA: the addition of 100 μg/ml of UFP to IL-8 standard (250 pg/ml)

didn't change its detection as evaluated by back calculation (242 pg/ml in the presence of UFP). Results are expressed as pg/ml.

Protein corona. 50 µg of UFP obtained from conifer and beech wood logs, or DEP were resuspended in 0.5 mL of culture media containing 10% FCS in microtubes and incubated for 60 min at room temperature with gentle rocking. Tubes were centrifuges at 5000 rpm for 5 min, pellets washed three times by resuspension in 0.5 mL of PBS with vortexing, followed by centrifugation and aspiration of the supernatant. Protein were desorbed from UFP by sonication in 100 uL of Laemli loading buffer for 20 min followed by incubation in boiling water for 5 min. Samples were then centrifuged at 12000 rpm for 5 min to sediment UFP. Supernatants (20 µL) were analysed by 12 % SDS-polyacrylamide gel under reducing conditions (SDS-PAGE). Separated proteins were visualized by Coomassie blue staining.

To confirm that the major protein visible with an apparent molecular weight of 63 kDa was albumin, Western blot analysis using an anti-bovine albumin (BSA) antibody was performed. Proteins desorbed from beech wood logs and DEP were electrophoresed into a 12 % SDS-PAGE. The proteins were then transferred to PVDF membrane (Amersham, Little Chalfont, UK), and BSA visualized using primary antibody for BSA (Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000), and developed using enhanced chemiluminescence (Pierce, Thermo Scientific, Rockford, IL, USA). The image of the blot was acquired and analyzed with the Molecular Imager Gel Doc XR (BioRad).

Statistical analysis. A total of fourteen UFP samples obtained from independent combustion tests were assayed: 4 for conifer pellets, 3 for beech pellets, 3 for conifer logs and 4 for beech logs. In some experiments, UFP were pooled as specified in the captions. Statistical analysis was performed using InStat software version 3.0a (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined using ANOVA followed by a multiple comparison test as indicated in the captions. Effects were designated significant if $p < 0.05$.

RESULTS

Chemical composition of UFP generated from pellets and logs combustion

UFP were obtained from fourteen independent combustion experiments carried out as described in the Methods section. The full analysis of the chemical composition and physical characterization of the UFP used in this study has been published elsewhere (Ozgen et al., 2017). Following the most salient findings will be discussed. In the Supplemental Figure 3, mean, median, 10th and 90th percentile particle number size distributions are reported. Average UFP number concentrations for the pellet stove were $1.1 \cdot 10^8$ particles/cm^3 (geometric mean aerodynamic diameter: 38 nm) for beech pellets and $1.1 \cdot 10^8$ particles/cm^3 (geometric mean aerodynamic diameter: 75 nm) for conifer pellets. The average particle number concentration was comparable for beech and conifer pellets; however, the number size distribution (Supplemental figure 3, upper panel) is shifted towards larger particles, still in the UFP size range, in the case of conifer pellets. The average contribution of NP to UFP ranged from 36% in conifer pellet UFP to 82% in beech pellet UFP. The average UFP number concentrations were $7.0 \cdot 10^8$ particles/cm^3

(geometric mean aerodynamic diameter: 50 nm) for beech logs and $1.9 \cdot 10^8$ particles/cm^3 (geometric mean aerodynamic diameter: 99 nm) for conifer logs. The particle number size distribution is shown in Supplemental Figure 3 lower panel. A higher variability was observed with respect to pellet experiments, with an average contribution of NP to UFP range from 52% in conifer wood UFP to 67% in beech wood UFP.

The chemical composition (mean±SD) of the UFP fractions for each configuration (conifer/beech pellets, conifer/beech wood logs) is reported in Table 1. The mass of UFP recovered was not significantly different among conifer/beech pellets or conifer/beech wood logs. Chemical composition showed some interesting differences:

- TC was the main relative component in wood log UFP (>40% of UFP mass), whereas its contribution was far lower in pellet samples (<15%);

- ions dominated UFP from pellet combustion (about 40%) whereas their contribution was generally below 10% of UFP mass from wood log combustion;

- PAH concentration was significantly higher in UFP from wood logs, with conifer wood logs generating approximately twenty times more PAH compared to beech wood logs ($p<0.01$).

Comparing the average relative composition of UFP particles generated from experiments carried out with different wooden fuels it results that those generated from pellets had significantly (beyond 1 standard deviation) higher K^+, SO_4^{2-}, Cl^- e Ca^{2+}, Al, Cd, Cu, Mn, Pb concentrations while TC, all PAH and Mn were much higher in UFP from wood log combustion. Focusing on levoglucosan, which represents the marker for biomass burning in particulate matter emissions (Piazzalunga et al., 2010; Belis et al., 2011) it was negligible (0.005% of UFP mass) in the samples from pellet combustion while it accounted for about 5% of the UFP mass in samples from wood log combustion, with beech wood logs generating approximately three-four times more levoglucosan or galactosan compared to conifer wood logs ($p<0.01$). Overall, these results are in accordance with a more efficient combustion process in the pellet stove reducing carbonaceous particulate components.

Effects of UFP generated from pellets and logs combustion

To investigate and compare the effects of UFP generated from the combustion of conifer and beech pellets and wood logs on the induction of IL-8 or CXCL8, cells were exposed to UFP (25-100 µg/ml) or DEP (100 µg/ml) for 24 h. LDH leakeage and IL-8 production were assessed as indicators of cytotoxicity and pro-inflammatory effects. In Figure 1 the effects on THP-1 are reported. No significant cytotoxicity was observed following exposure to UFP generated by conifer and beech pellets (Figure 1 A) or conifer and beech log wood (Figure 1 B), while a modest but statistically significant increase (< 10 %) was observed following treatment with DEP (Figure 1 A and B last column). While UFP did not affect cell viability, they induced IL-8 release, with different effects observed as shown in Figure 1 and 2. Each dot represents the effect obtained from UFP collected from different combustions, the responses were very similar within each groups with the exception of one sample obtained for conifer pellet UFP (Figure 1C). Based on the mean values, UFP generated from conifer, both pellets (Figure 1 C) and logs (Figure 1 D), induced a similar release of IL-8, which was also comparable with the release observed with DEP (last group on Figure 1C and 1D). On the contrary, UFP generated from the combustion of beech pellets

failed to induce statistically significant release of IL-8 (Figure 1C), while UFP generated from beech logs induced higher amounts of IL-8 compared to control, conifer wood and DEP treated cells (Figure 1 D, third group). The release of IL-8 induced by both conifer pellets UFP (Figure 1E) and beech log wood UFP (Figure 1 F) was dose-related.

In A549 cells, similar results were observed. In Figure 2A, the effects obtained for UFP generated from the combustion of pellets are reported. Cells were treated for 24 h with UFP (100 μg/ml) or DEP (100 μg/ml). Conifer UFP induced a similar IL-8 release compared to DEP, while beech UFP failed to induce a statistically significant release of IL-8 as observed in THP-1 cells. On the contrary, UFP generated for the combustion of wood logs from both soft and hard woods induced a statistically significant release of IL-8 compared to control (Figure 2B). The release was also comparable to the one observed following treatment with DEP, indicating that, on a mass basis, UFP are not more dangerous compared to other categories of combustion-derived particles. In A549, differently from THP-1 cells, beech wood UFP induced a similar release of IL-8, suggesting a different sensitivity to UFP constituents in the two cell lines.

Role of MAPK p38 in UFP-induced IL-8 production

We next investigated the molecular mechanism of action involved in UFP-induced IL-8 production. We previously demonstrated a role of p38MAPK in IL-8 release induced by PM2.5 obtained from pellet combustion (Corsini et al., 2013). Therefore, we investigated if p38MAPK was also involved in UFP-induced IL-8. Cells were incubated in absence or presence of the selective inhibitor of p38 MAPK SB202190 (0.1 μM) for 1 h, then conifer pellet UFP (100 μg/ml) or DEP (100 μg/ml) were added for 24 h. The concentration of the inhibitor used was not cytotoxic, as assessed by LDH leakage (data not shown). As shown in Figure 3, IL-8 release could be significantly reduced in both THP-1 (Figure 3A) and A549 (Figure 3B) cells, confirming the involvement of p38MAPK activation in UFP-induced IL-8 production.

UFP uptake, protein corona formation and role of serum

The most striking difference we observed was the higher production of IL-8 in THP-1 cells exposed to UFP generated from the combustion of beech wood logs. To exclude a possible role of endotoxin contamination, which could explain the response in THP-1 and not in A549 (epithelial cells in general poorly respond to endotoxin), we used polymixin B to sequester LPS. Endotoxin in ambient air particles has been associated with inflammatory responses in vivo (Schins et al., 2004; Alexis et al., 2006), and with release of pro-inflammatory cytokines in vitro (Becker et al., 2005; Imrich et al., 2000). UFP generated from beech woods were pre-incubated with polymixin B sulfate (15 μg/ml final concentration) for 1 h at room temperature and then added to THP-1 cells for 24 h. LPS (10 ng/ml) was incubated with polymixin B as described for particles and used as a positive control. The release of IL-8 induced by UFP was 568±57 and 589±27 pg/ml in the presence of polymixin B, excluding a role of endotoxin contamination. On the contrary, as expected in the same experimental condition, the release of IL-8

induced by LPS (3265±87 pg/ml) was significantly reduced by polymixin B (217±19 pg/ml). Each value represents the mean±SD, n=3.

Considering the lower geometric mean aerodynamic diameter (50 nm) of beech log UFP compared to conifer UFP (99 nm), we then tested the hypothesis that a different UFP uptake in THP-1 may explain the different response observed. We assess UFP uptake by FACS analysis as described by Suzuki et al. (2007) for nanomaterials by measuring changes in size and intracellular density of cells. It is important to emphasize that the method does not distinguish particles that are internalized from particles attached to the external side of the cell membrane. THP-1 cells were treated for 24 h with conifer and beech log wood UFP (100 μg/ml) or DEP (100 μg/ml). Despite the limitation of the method used, as shown in Figure 4A, the % of gated cells did not differ between conifer and beech groups: 56.3±0.8 % and 53.4±1.4%, respectively. The % of gated cells was lower in DEP treated cells compared to UFP treated cells, indicating that UFP are better internalized compared to DEP, which also includes larger size particles.

In parallel, we speculated that a different protein corona formation might explain the different response observed in THP-1 cells. The presence of FCS is important, as the response to beech wood UFP was significantly reduced in the absence of FCS as clearly shown in Figure 4B, where both uptake and IL-8 release were reduced in the absence of serum. Interestingly, the qualitative FCS protein absorption profiles (Figure 4C) shows less proteins absorbed to beech wood UFP compared to conifer wood UFP or DEP, which may result in greater and more rapid intracellular availability of 'active' ingredients, towards which THP-1 are more susceptible compared to A549 cells.

Looking at the chemical composition (Table 1), UFP generated from the combustion of beech wood logs contains higher amount of levoglucosan and galactosan compared to conifer wood logs. While levoglucosan, mannosan and galactosan are used as wood burning tracers (Simoneit et al., 1999), there are few reports on their biological activity (Miyakawa et al., 1999; Saffari et al., 2013; Sarigiannis et al., 2015; Hamad et al., 2016). We decided to investigate the ability of these compounds to induce IL-8 production. Both THP-1 and A549 cells were exposed to increasing concentrations of levoglucosan and galactosan for 24 h. At concentrations found in beech wood log UFP, only THP-1 cells responded inducing a concentration-related release of IL-8. In A549, only levoglucosan at the highest concentration tested (four time higher of the average concentration found in beech wood log UFP) induced IL-8 release, while galactosan was ineffective. The effects of levoglucosan and galactosan were not due to endotoxin contamination (data not shown). These results provide a clear explanation to the higher biological activity observed with beech wood log UFP in THP-1 cells.

DISCUSSION

The hypotheses driving this study were that: (1) UFP may be more biologically active compared to particles with higher size range (DEP); (2) wood stoves may generate more biologically active UFP compared to pellet stoves; (3) different wood types may generate UFP of different composition and characteristics. We investigated the chemical composition and biological activity of UFP generated from the combustion of wood pellets and logs, and compared conifer-soft and beech-hard woods. The biological activity was evaluated in two human cell lines

surrogate of alveolar macrophages and lung epithelial type II cells. Both cell lines responded to wood generated UFP producing IL-8, with UFP obtained from wood logs combustion being more active compared to UFP generated from pellets. With the exception of the higher effect of beech wood log UFP only in THP-1 cells due to the presence of the pyrolysis products levoglucosan and galactosan, toward which THP-1 were more responsive compared to A549 cells, the ability of soft or hard woods to induce the release of IL-8 was similar. In addition, on a mass basis the release of IL-8 induced by UFP was also similar or even lower compared to DEP, arguing against a higher biological activity of UFP compared to other particles such as DEP. We did not measure the surface area, however, considering that UFP are expected to have a much greater surface area, on surface base one could expect their biological effect to be less than that induced by particulate matters of larger size. Being the inflammatory potential of UFP generated from the combustion of logs, comparable to that of traffic-derived particles, our data support epidemiological and human inhalation studies, which suggest that wood smoke may cause health effects similar to particles from other sources (Naeher et al., 2007; Boman et al., 2003; Barregard et al., 2006). It is important to mention that there are, however, studies showing no effect of wood smoke on human lung functions (Sehlstedt et al., 2010; Forchhammer et al., 2012), and further studies are required to address this point.

In our experiments, we used relatively high particle concentrations to stress the influence of different particles on the inflammatory response. The concentrations used (25-100 μg/ml) should, however, be considered as relevant for human exposure, as the use of deposition models suggested that in vitro concentrations of 100 μg/ml may be representative for the amount of particles deposited in the human lungs after 24 h inhalation of ambient air concentrations of 100-150 $\mu g/m^3$ (Veronesi et al., 2002; Fujii et al., 2002).

The in vitro proinflammatory effects of fine wood smoke particles have been previously described in several studies using different cellular models, including A549 (Danielsen et al., 2009 and 2011; Corsini et al., 2013), THP-1 cells (Kocbach et al., 2008; Danielsen et al., 2009 and 2011; Corsini et al., 2013), RAW264.7 (Jalava et al., 2012), and endothelial cells (Forchhammer et al., 2012b). Our results are overall consistent with such findings, demonstrating that also nanoparticulate materials have effects overall comparable to larger particles. In addition, we demontrated that UFP generated from wood logs are more active compared to UFP-derived from pellets, most likely due to a more complete combustion in pellet stove than in wood stove, resulting in the generation of particles with less inflammatory activity. Particularly important is also the lower quantity of PAHs found in UFP generated by the pellet stove.

In a previous study (Corsini et al., 2013), we demonstrated the PM2.5-induced IL-8 production could be blocked by a specific inhibitor of p38MAPK. Several studies have identified a function for MAPK signaling in regulating IL-8 mRNA stability in different cell types (Dean et al. 2004; Muselet-Charlier et al. 2007). Similarly, we found in both A549 and THP-1 cells that the selective inhibitor SB203580 could block UFP-induced IL-8 release, confirming the involvement of p38MAPK activation also in the molecular mechanism underlying UFP-induced IL-8 production.

The most striking difference we observed was the effect of beech wood log UFP on IL-8 release in THP-1 cells, which was ten times higher compared to the release induced by conifer wood logs UFP or by DEP. The easiest explanation could have been an endotoxin contamination: THP-1 being monocyte-like cell line is very sensitive to LPS compared to epithelial cells, which barely respond to LPS due to a very low expression of TLR4. This was

not, however, the case: while polymixin B almost completely blocked the effect of LPS no modulation was observed with beech wood logs UFP, thus excluding an endotoxin contamination. We then speculated that a higher cellular uptake of beech wood log UFP might explain the difference. However, by assessing cellular uptake, no differences were observed between beech and conifer wood logs UFP that could explain the different IL-8 response. Once particles are exposed to biological environments, they are modified by adsorption of biomolecules, mainly proteins (Dobrovolskaia et al., 2009), and it is known that protein adsorption on nanoparticles may facilitate their uptake. This is consistent with what we observed: UFP are better internalized in the presence of serum. But one difference we found between beech and conifer wood UFP is the protein corona. At present, we only have a qualitative FCS protein adsorption profile, which clearly show that beech log wood UFP have less adsorbed proteins compared to conifer wood UFP or DEP, which may result in lower shielding of 'active' ingredients and increased intracellular bioavailability. We speculate that lower content of proteins may result once inside the cell in a higher or more rapid availability of reactive compounds, resulting from the dissolution of the biomolecule coating within the lysosome following cellular internalization (Wang et al., 2014). Looking at the chemical composition, the UFP obtained from beech wood logs have approximately three-four times higher levels of levoglucosan and galactosan compared to conifer wood logs UFP. Levoglucosan, mannosan and galactosan are typically used as wood burning tracers (Simoneit et al., 1999). There are, however, few reports showing a biological activity or at least showing a possible correlation with these pyrolytic products and adverse effects (Miyakawa et al., 1999; Saffari et al., 2013; Sarigiannis et al., 2015; Hamad et al., 2016). Miyakawa et al. (1999), in a two-stage mouse skin carcinogenesis model using 12-O-tetradecanoylphorbol-13-acetate as the promoter, showed that levoglucosan as well as other pyrolysates of carbohydrates potentially possess tumor initiating activity, based on the incidences and development of the skin papillomas and/or carcinomas. Saffari et al. (2013) reported a strong association between reactive oxygen species (ROS) production and the concentrations of levoglucosan, galactosan, and potassium, underscoring the potential impact of wood smoke on PM-induced toxicity during the winter months. Hamad et al. (2016) reported similar results. Sarigiannis et al. (2015) observed that polycyclic aromatic hydrocarbons and levoglucosan levels were highly correlated, we could not confirm this association. In our experimental models, both levoglucosan and galactosan were able to induce in a dose-related manner IL-8 production in THP-1 cells, whereas A549 slightly responded only at the highest concentration of levoglucosan tested and galactosan was inactive. The differences in protein corona, the different chemical composition and cellular reactivity towards these components are the most likely explanation of the different reactivity observed. At the levels measured in UFP, levoglucosan and galactosan were able to induce IL-8 release only in THP-1. In pellet-derived UFP these products are barely detectable, indicative of a more complete combustion. Interestingly, the qualitative FCS protein adsorption profile observed is overall similar to the ones reported for other nanomaterials, with BSA being the major protein adsorbed (Dutta et al., 2007). Further investigations are required to determine this interaction in order to understand its contribution to the higher effects of beech wood UFP.

Combustion technology has been shown to greatly affect the emission and the concomitant toxicological responses. Open fireplaces and domestic log wood stoves have in general less regular combustion in comparison with appliances fueled by wooden pellet, and thus emissions richer in pollutants (Nussbaumer, 2010). Commercial pellet stoves are expected to substitute older stoves and fireplaces in countries where laws and regulations force towards lower emission levels. Jalava et al. (2012) demonstrated in vitro, using the murine macrophage cell line

RAW264.7, that modern automated stoves were the least potent inducers of most of the toxicological endpoints investigated (i.e. cytokine production, genotoxicity) if compared to the emissions from the old technology log wood stoves.

In conclusion, in our study we confirmed that pellet stoves generate less active UFP compared to the ones generate from wood stoves. Even if pellet stoves have reduced in vitro activity when compared to wood stoves, they still can occasionally generate biologically active particulate. Therefore, additional efforts should be devoted to identify optimal combustion conditions in order to reduce the health impact of wood smoke emissions. The strategy we applied in the current manuscript can allow the characterization of the chemical composition and biological activity of particulates obtained from wood combustion. The present data revealed clear differences in the emissions and their toxicological effects.

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531 **Table 1** - Chemical compositions of UFP obtained from the combustion of conifer and beech pellets and logs.

Compounds	Unit	Beech pellets	Conifer pellets	Beech log wood	Conifer log wood
UFP	mg	1.282±0.071	1.643±0.867	1.114±0.482	0.575±0.192
Levoglucosan	%	0.005±0.001	0.006±0.003	**6.548±0.263****	1.470±0.632
Mannosan	%	0.003±0.001	0.005±0.001	0.451±0.048	0.487±0.232
Galactosan	%	0.001±0.001	n.a.	**0.233±0.017****	0.076±0.049
Al	ppm	2118±963	2554±2435	329±35	385±17
As	ppm	14±4	10±5	21±2	27±13
Ba	ppm	59±11	111±42	32±20	30±16
Cd	ppm	5±1	9±2	26±11	33±14
Co	ppm	3±1	3±5	2±9	5±5
Cr	ppm	<LOD	<LOD	407±316	438±320
Cu	ppm	217±42	428±225	104±39	78±40
Fe	ppm	2160±2230	2560±3529	833±1111	883±690
Mn	ppm	221±41	633±370	28±15	29±12
Mo	ppm	**373±46****	9±8	11±6	7±10
Ni	ppm	90±94	423±197	123±134	65±54
P	ppm	239±42	169±72	349±54	340±140
Pb	ppm	**317±20***	195±68	57±35	104±47
Sr	ppm	21±9	7±12	35±11	42±21
Ti	ppm	26±3	43±59	29±26	21±8
V	ppm	3±1	4±3	10±7	21±2
Zn	ppm	**2396±663****	4931±406	1860±183	2787±1087
TC	%	**3.643±0.422****	12.741±0.810	**48.796±4.615****	87.113±2.613
Na^+	%	0.170±0.029	0.231±0.067	0.180±0.050	0.158±0.100
NH_4^+	%	0.016±0.002	0.024±0.014	0.300±0.031	0.362±0.047
K^+	%	25.443±4.350	23.180±1.748	**2.255±0.549****	0.319±0.047
Mg^{++}	%	0.034±0.003	0.044±0.035	0.002±0.001	0.001±0.001
NO_3^-	%	0.626±0.097	0.891±0.125	**0.540±0.110***	0.317±0.062
SO_4^{--}	%	9.188±1.832	11.920±0.689	3.585±0.901	4.033±0.671
ΣPAH	‰	0.148±0.018	0.133±0.090	**2.063±1.834****	35.908±6.591

535 Each value is expressed as mean±SD. The SD refers to the data obtained from UFP generated from independent
536 combustion tests (4 conifer pellets, 3 beech pellets, 3 conifer logs and 4 beech logs). Statistical analysis was
537 performed with Student's t test, with *$p<0.05$ and ** $p< 0.01$ Beech vs Conifer pellet UFP or Beech vs Conifer

538 log wood UFP. In bold the statistically different values. Concentrations lower than the limit of detection are
539 reported as <LOD, and n.a. for not available data. PAH= polycyclic aromatic hydrocarbons.
540

FIGURE LEGENDS

Figure 1. Pro-inflammatory effects of UFP obtained from independent combustions of conifer and beech pellets and logs in THP-1 cells. A) LDH leakage induced by UFP generated from the combustion of conifer and beech pellets; B) LDH leakage induced by UFP generated from the combustion of conifer and beech wood logs (log UFP); C) IL-8 secretion induced by UFP generated from the combustion of conifer and beech pellets; D) IL-8 secretion induced by UFP generated from the combustion of conifer and beech log woods; E) Dose-related release of IL-8 induced by pooled conifer pellet UFP; F) Dose-related release of IL-8 induced by pooled beech wood UFP. Cells were treated for 24 h with UFP (25-100 μg/ml) or DEP (100 μg/ml). Results are expressed as mean ± SD, n= 3. In the dot blots, each dot represents an independent UFP sample; the bar is the mean value. Statistical analysis was performed with Tukey's multiple comparison test, with *$p<0.05$ and **$p< 0.01$ vs control cells (Cont), and §$p<0.05$ and §§$p<0.01$ vs UFP or DEP treated cells.

Figure 2. Pro-inflammatory effects of UFP obtained from independent combustions of conifer and beech pellets and logs in A549 cells. A) IL-8 secretion induced by UFP generated from the combustion of conifer and beech pellets; B) IL-8 secretion induced by UFP generated from the combustion of conifer and beech wood logs (log UFP). Each dot represents an independent UFP sample; the bar is the mean value. Statistical analysis was performed with Tukey's multiple comparison test, with *$p<0.05$ and ** $p<0.01$ vs control cells (Control), and §§$p<0.01$ vs UFP or DEP treated cells.

Figure 3. Role of p38 MAPK activation in UFP or DEP-induced IL-8 secretion in THP-1 and A459 cells. A) Modulation by SB202190 on conifer pellet UFP or DEP-induced IL-8 secretion in THP-1 cells; B) Modulation by SB202190 on conifer pellet UFP or DEP-induced IL-8 secretion in A459 cells. Cells were incubated in absence or presence of the selective inhibitor of p38 MAPK SB202190 (0.1 μM) for 1 h, then UFP (100 μg/ml) or DEP (100 μg/ml) were added for 24 h. Results are expressed as mean ± SD, n=3. Statistical analysis was performed with Tukey's multiple comparison test, with **$p<0.01$ vs relative controls, and §$p< 0.05$ and §§$p<0.01$ vs cells treated with UFP or DEP alone.

Figure 4. UFP uptake, protein corona formation and role of serum in UFP-induced IL-8 production in THP-1 cells. A) Analysis of cellular uptake of conifer and beech UFP and DEP by flow cytometric light scatter. THP-1 cells were treated with UFP or DEP (100 μg/mL) for 24h. Representative FSC/SSC dot blots are shown. The % of cells in the gated area is reported in each dot blot. Results are expressed as mean ± SD, n=3. B) Role of serum in beech log UFP-induced uptake and IL-8 production in THP-1 cells. THP-1 cells were treated with beech log wood UFP or DEP (100 μg/mL) resuspended in culture media with or without 10 % FCS for 24 h. Cellular uptake was evaluated by FACS analysis, while IL-8 release by ELISA Results are expressed as mean ± SD, n=3. C) Qualitative FCS protein absorption profile associated with pooled conifer and beech logs UFP and DEP, and Western blot analysis of BSA immunoreactivity. Particles were resuspended in media containing 10 % FCS for 60 min as described in the Materials and Methods. Desorbed proteins were analysed by SDS-PAGE. Similar results were observed in two other independent experiments. Statistical analysis was performed with Tukey's multiple comparison test, with **$p<0.01$ vs relative controls, and §$p<0.05$ and §§$p<0.01$ vs cells treated with UFP or DEP alone in the absence of FCS.

Figure 5. Pro-inflammatory effects of levoglucosan and galactosan in THP-1 cells. A) IL-8 secretion following levoglucosan and galactosan treatment in THP-1 cells; B) IL-8 secretion following levoglucosan and galactosan treatment in A549 cells. Cells were treated with levoglucosan and galactosan (6.25-25 μg/mL) for 24h. Results are expressed as mean ± SD, n=3. Statistical analysis was performed with Dunnett's multiple comparison test, with *$p<0.05$ and **$p<0.01$ vs control cells (0).