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## Toxicological appraisal of the chemical fractions of ambient fine (PM<sub>2.5-0.3</sub>) and quasi-ultrafine (PM<sub>0.3</sub>) particles in human bronchial epithelial BEAS-2B cells

Ghidaa Badran, Anthony Verdin, Céline Grare, Imane Abbas, Djamel Achour, Frédéric Ledoux, Mohamad Roumie, Fabrice Cazier, Dominique Courcot, Jean-Marc Lo Guidice, et al.

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1 **TOXICOLOGICAL APPRAISAL OF THE CHEMICAL FRACTIONS OF AMBIENT**  
2 **FINE (PM<sub>2.5-0.3</sub>) AND QUASI-ULTRAFINE (PM<sub>0.3</sub>) PARTICLES IN HUMAN**  
3 **BRONCHIAL EPITHELIAL BEAS-2B CELLS**

4  
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24 **ABSTRACT**

25

26 New toxicological research is still urgently needed to improve the current knowledge about  
27 the induction of some underlying mechanisms of toxicity by the different chemical fractions of  
28 ambient particulate matter (PM). This *in vitro* study sought also to better evaluate and compare the  
29 respective toxicities of fine particles (PM<sub>2.5-0.3</sub>) and their inorganic and organic chemical fractions,  
30 and the respective toxicities of the organic chemical fractions of PM<sub>2.5-0.3</sub> and quasi-ultrafine  
31 particles (PM<sub>0.3</sub>). Human bronchial epithelial BEAS-2B cells were also exposed for 6 to 48 h to  
32 relatively low doses of PM<sub>2.5-0.3</sub> and their organic extractable (OEM<sub>2.5-0.3</sub>) and non-extractable  
33 (NEM<sub>2.5-0.3</sub>) fractions, and the organic extractable fraction (OEM<sub>0.3</sub>) of PM<sub>0.3</sub>. We reported that not  
34 only PM<sub>2.5-0.3</sub>, but also, to a lesser extent, its inorganic chemical fraction, NEM<sub>2.5-0.3</sub>, and organic  
35 chemical fraction, OEM<sub>2.5-0.3</sub>, were able to significantly induce ROS overproduction and oxidative  
36 damage notwithstanding the early activation of NRF2 signaling pathway. Moreover, for any  
37 exposure, inflammatory and apoptotic events were noticed. Similar results were observed in  
38 BEAS-2B cells exposed to OEM<sub>0.3</sub>, rich of polycyclic aromatic hydrocarbons and their nitrated  
39 and oxygenated derivatives. In BEAS-2B cells exposed for 24 and 48 h to OEM<sub>2.5-0.3</sub> and OEM<sub>0.3</sub>,  
40 to a higher extent, there was an alteration of the levels of some critical proteins even though crucial  
41 for the autophagy rather than a real reduction of autophagy. It is noteworthy that the toxicological  
42 effects were equal or mostly higher in BEAS-2B cells exposed for 6 and/or 24 h to PM<sub>2.5-0.3</sub> from  
43 those exposed to NEM<sub>2.5-0.3</sub> or OEM<sub>2.5-0.3</sub>, and in BEAS-2B cells exposed for 6 and/or mostly 24  
44 h to OEM<sub>0.3</sub> from those exposed to OEM<sub>2.5-0.3</sub>. Taken together, these results revealed the higher  
45 potentials for toxicity, closely linked to their respective physical and chemical characteristics, of  
46 PM<sub>2.5-0.3</sub> vs NEM<sub>2.5-0.3</sub> and/or OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> vs OEM<sub>2.5-0.3</sub>.

47 **CAPSULE:** Inorganic and organic chemical fractions from ambient fine and ultrafine particles  
48 differentially triggered some critical underlying mechanisms of toxicity in BEAS-2B cells.

49

50 **KEYWORDS:** Fine particles; quasi-ultrafine particles; extractable and non-extractable chemical  
51 fractions; normal human bronchial epithelial BEAS-2B cells; toxicity; underlying mechanisms

52



## 53 INTRODUCTION

54

55 Epidemiological evidence supported the association between exposure to ambient particulate  
56 matter (PM) and hospital admissions, morbidity and mortality related to chronic inflammatory  
57 lung diseases (e.g., asthma and chronic obstructive pulmonary disease) and lung cancer (Beelen et  
58 al. 2013; Doiron et al. 2019; Hamra et al. 2014; Jerrett et al. 2016; Lelieveld et al. 2019; Raaschou-  
59 Nielsen et al. 2016; Loomis et al. 2014). Fine (i.e., PM<sub>2.5</sub>) and mostly ultrafine (i.e., PM<sub>0.1</sub>) particles  
60 have generally been reported as the major contributors to the adverse health effects of air pollution  
61 (Abbas et al. 2018). Several experimental studies have notified the harmful lung effects of ambient  
62 PM, mostly through their ability to trigger some critical underlying mechanisms of toxicity, such  
63 as oxidative stress-induced airway inflammation (e.g., Abbas et al. 2013, 2016, 2019; Bocchi et  
64 al. 2016; Garçon et al. 2006; Gualtieri et al. 2010, 2011; Halonen et al. 2015; Leclercq et al. 2016,  
65 2017, 2018; Longhin et al. 2013, 2016, Sotty et al. 2019). However, although significant progress  
66 has been made during the last decade, a considerable effort will continue to be required to better  
67 identify some typical features of *in vitro* toxicological responses, which could be related to some  
68 of the specific physical and chemical characteristics of ambient PM.

69 A careful consideration has already been devoted to the types of toxicological responses  
70 induced by ambient PM, since their knowledge is greatly complicated by the fact that it is a very  
71 heterogeneous and often poorly described pollutant (Perrone et al. 2013). Despite the particular  
72 attention of the scientific community about the types of toxicological responses depending on the  
73 sources of ambient PM, this question is still far from being completely resolved. Gualtieri et al.  
74 (2010), Lonkar and Dedon (2011), and Perrone et al. (2013) have all used the inclusion of the  
75 grouping of inorganic constituents to provide useful information on the differential effects of the

76 components and/or the sources of ambient PM. Hence, it is worthy to extend this approach to  
77 organic components. Among them, polycyclic aromatic hydrocarbons (PAH) were identified as  
78 major redox-active components, which, through their metabolic activation into highly biologically  
79 reactive electrophile metabolites, largely contribute to the overall toxicity of ambient PM (Abbas  
80 et al., 2018; Boublil et al. 2013; Longhin et al. 2013, 2016; Sotty et al. 2019; Zhou et al. 2016).  
81 Other authors reported the critical role of the organic extractable matter (OEM) derived from  
82 ambient PM in its overall toxicity but failed to fully clarify them (Akhtar et al. 2010; Alessandria  
83 et al. 2014; De Brito et al. 2013, Oh et al. 2011, Xiang et al. 2018).

84 New toxicological research is therefore requested to improve the current knowledge about the  
85 specific involvement of the inorganic and organic chemical fractions of ambient PM in its overall  
86 toxicity. Recently, OEM derived from ambient  $PM_{2.5-0.3}$  was recognized as triggering oxidative,  
87 inflammatory, and genotoxic events: a combination thereof may be the same or different in  
88 comparison with those triggered by the whole  $PM_{2.5-0.3}$ . However, further works are still needed to  
89 better compare the respective roles of each chemical fraction: it might also be more appropriate to  
90 study the toxicity of  $OEM_{2.5-0.3}$  together with this of the whole  $PM_{2.5-0.3}$ , from which it was  
91 extracted, and the toxicity of non-extractable matter ( $NEM_{2.5-0.3}$ ; i.e., residual  $PM_{2.5-0.3}$  after  
92  $OEM_{2.5-0.3}$  extraction). The comparison between the specific toxicities of  $OEM_{2.5-0.3}$  and  $OEM_{0.3}$ ,  
93 respectively prepared from fine ( $PM_{2.5-0.3}$ ) or quasi-ultrafine ( $PM_{0.3}$ ) particles, could also be very  
94 interesting to better assess the chemical compounds mainly involved. Hence, in this work, we  
95 sought to better investigate: (i) the toxicological effects of  $OEM_{2.5-0.3}$  and  $NEM_{2.5-0.3}$  with a  
96 particular attention being paid to oxidative, inflammatory, autophagic, and apoptotic events, and  
97 thereafter compare them to those of  $PM_{2.5-0.3}$  in its entirety, and (ii) the toxicity of  $OEM_{0.3}$  which  
98 would lastly be related to that of  $OEM_{2.5-0.3}$ .

## 100 MATERIALS AND METHODS

101

### 102 Chemicals

103 Sigma-Aldrich (Saint-Quentin Fallavier, France) provided BEAS-2B cells (ATCC® CRL-  
104 9609™) and chemicals. ThermoFisher scientific (Villebon-sur-Yvette, France) provided hydro-  
105 ethidium (HE), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA),  
106 Pierce™ BCA protein assay kits, Click-iT™ Plus EdU Alexa Fluor™ 647 flow cytometry assay  
107 kits, Single-channel dead cell apoptosis kits with Annexin V alexa fluor™ 488, and SYTOX™  
108 green dyes, and other biological reagents. Qiagen (Courtaboeuf, France) provided RNeasy mini  
109 Kits. Promega (Charbonnière-les-Bains, France) provided CellTiter-Glo® luminescent cell  
110 viability, GSH/GSSG-Glo™ assay, and Caspase Glo® 3/7, Caspase Glo® 8, and Caspase Glo® 9  
111 assays. Gentaur (Paris, France) provided Highly sensitive 8-OHdG check kits. Abcam (Cambridge,  
112 UK) provided Protein carbonyl content assay kits and 8-isoprostane ELISA kits. Active Motif (La  
113 Hulpe, Belgium) provided Nuclear extract and TransAM® NRF2 kits. Merck-Millipore (St  
114 Quentin-en-Yvelines, France) provided MILLIPLEX® MAP human cytokine/chemokine  
115 magnetic bead panel-immunology multiplex assays. Bio-techne (Rennes, France) provided  
116 TACS® TdT *in situ*-fluorescein system and mouse monoclonal anti-human actin antibody  
117 (MAB8929), rabbit polyclonal anti-human ATG5 (NB110-53818), rabbit polyclonal anti-human  
118 LC3-B antibody (NB100-2220), rabbit polyclonal anti-human beclin antibody (NB500-249),  
119 mouse monoclonal anti-human p62/SQSTM1 antibody (H00008878-M01), and goat polyclonal  
120 anti-rabbit IgG, HRP-linked antibody (NB7160). Cell Signaling Technology (Montigny-le-  
121 Bretonneux, France) provided rabbit monoclonal anti-mouse IgG, HRP-linked antibody (S7076).

122

123        **Sampling site description, PM sampling methods, PM physicochemical characterization,**  
124 **and PM fraction preparation**

125        Sampling site description, sampling procedure, physicochemical characterization of PM<sub>2.5-0.3</sub>  
126 and PM<sub>0.3</sub>, and preparation of NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> have been published elsewhere  
127 (Badran et al. 2020; see also supplemental data: Tables S1 and S2, and Figure S1).

128

129        **Cell culture and exposure**

130        Normal human bronchial epithelial BEAS-2B cells (ATCC® CRL-9609™) were cultured as  
131 published elsewhere (Leclercq et al. 2017). Just before reaching the confluence (< 80%), BEAS-  
132 2B cells were incubated either with LHC-9 with dimethylsulfoxide (DMSO) at 0.5% (v/v), as a  
133 negative control, or with PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub>, prepared in LHC-9 with  
134 DMSO ≤ 0.5% (v/v), at concentrations ranging from 3 to 96 µg Eq. PM/cm<sup>2</sup>, as detailed elsewhere  
135 (Badran et al. 2020). Aliquots of cell-free culture supernatants were collected after 6, 24 and/or 48  
136 h, and quickly frozen at -80°C. Adherent cells were washed twice with 1 mL-aliquots of cold  
137 sterile PBS, and either immediately fixed or quickly frozen at -80°C, as published elsewhere (Sotty  
138 et al. 2019).

139

140        **Cytotoxicity**

141        Intracellular ATP concentrations of BEAS-2B cells were determined using the CellTiter-  
142 Glo® luminescent cell viability (Promega). This assay system used the properties of a proprietary  
143 thermostable luciferase to enable reaction conditions that generate a stable “glow-type”  
144 luminescent signal. The mono-oxygenation of luciferin was catalyzed by luciferase in the presence

145 of Mg<sup>2+</sup>, ATP, and molecular oxygen, and resulted in the emission of light, directly linked to the  
146 ATP concentration and also the number of living cells.

147

## 148 **Oxidative stress**

149 Firstly, the fluorescence of either HE (1 μM) or carboxy-H<sub>2</sub>DCFDA (1 μM) was kinetically  
150 monitored at 37°C within BEAS-2B cells. Secondly, the nuclear factor erythroid 2-related factor  
151 2 (NRF2) DNA binding activity was studied using TransAM® NRF2 from Active Motif.  
152 Associated gene expressions of some members of the NRF2 signaling pathway (i.e., *NRF2*; *Kelch-*  
153 *like ECH-associated protein 1*, *KEAP-1*; *heme oxygenase 1*, *HMOX*; *NAD(P)H quinone*  
154 *dehydrogenase 1*, *NQO1*; *superoxide dismutase*, *SOD*) were evaluated by RT-qPCR using specific  
155 Taqman™ gene expression assays (*NRF2*: hs00975961\_g1, *KEAP-1*: hs00202227\_m1, *HMOX*:  
156 Hs01110250\_m1, *NQO1*: Hs01045993\_g1, *SOD*: hs00162090\_m1, *ARN18S*: Hs99999901\_s1), a  
157 StepOnePlus™ Real-Time PCR System, and the Expression Suite Software (ThermoFisher  
158 scientific). Thirdly, the status of glutathione (GSSG/GSH), and the concentrations of carbonylated  
159 protein (CO-protein), 8-isoprostane (8-Isop), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were  
160 carried out using GSH/GSSG-Glo™ assay (Promega), Protein carbonyl content assay kits  
161 (Abcam), 8-isoprostane ELISA kits (Abcam), and Highly sensitive 8-OHdG check enzyme  
162 immunoassays (Gentaur), respectively, as published elsewhere (Leclercq et al. 2016). Menadione  
163 (2.5 mM, 4 h)-treated BEAS-2B cells served as a positive control.

164

## 165 **Inflammation**

166 The secretion of tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and  
167 monocyte chemoattractant protein 1 (MCP-1) in the cell-free culture supernatants of BEAS-2B

168 cells was detected by MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel-  
169 Immunology Multiplex Assay (Merck-Millipore). Lipopolysaccharide (LPS from E. coli, 50  
170 µg/mL, 24 h)-treated BEAS-2B cells served as a positive control.

171

## 172 **Autophagy**

173 ATG5, BECN1/Beclin 1, SQSTM1/p62 protein, and microtubule-associated protein 1 light  
174 chain 3 b (MAP1LC3B/LC3B) were studied within BEAS-2B cells. Protein extracts (20 µg) of  
175 BEAS-2B cells were prepared using protease and phosphatase inhibitor cocktail (Sigma-Aldrich)-  
176 supplemented RIPA lysis/extraction buffer (ThermoFisher scientific). Western-blot method  
177 consisted of: separation by electrophoresis on SDS-PAGE gels, transfer on nitrocellulose  
178 membranes, blockage in 5 % (m/v) non-fat dry milk for 45 min, primary antibody incubation  
179 overnight at +4°C (i.e., rabbit polyclonal anti-human ATG5, rabbit polyclonal anti-human LC3-B  
180 antibody, rabbit polyclonal anti-human Beclin antibody, mouse monoclonal anti-human  
181 p62/SQSTM1 antibody, mouse monoclonal anti-human actin antibody (Bio-technie), secondary  
182 antibody incubation for 1 h at room temperature (i.e., rabbit monoclonal anti-mouse IgG, HRP-  
183 linked antibody, Cell Signaling Technology; goat polyclonal anti-rabbit IgG, HRP-linked  
184 antibody, Bio-technie), and revelation by ECL prime Western Blotting Detection reagent (GE  
185 Healthcare). Chemiluminescence was recorded by Fusion FX Spectra (Vilbert-Lourmat, Marne-  
186 la-Vallée, France). Rapamycin (10 µM, 24 h)-treated BEAS-2B cells served as a positive control.

187

## 188 **Apoptosis**

189 Caspase Glo® 3/7, Caspase Glo® 8, and Caspase Glo® 9 Assays (Promega) were used as  
190 recommended by the manufacturer. TACS® TdT *in situ*-Fluorescein System (Bio-technie) was

191 used according to the manufacturer's recommendations before BEAS-2B cell observations using  
192 an EVOS® FL Cell Imaging System (ThermoFisher scientific). Single-channel dead cell apoptosis  
193 kit with Annexin V Alexa Fluor™ 488 and SYTOX™ green dyes, and Click-iT™ Plus EdU Alexa  
194 Fluor™ 647 flow cytometry assay kits (ThermoFisher scientific) were used as recommended by  
195 the manufacturer, before BEAS-2B cell analysis using an Attune™ NxT Acoustic Focusing  
196 Cytometer (ThermoFisher scientific) to study apoptosis and cell cycle, respectively. Staurosporine  
197 (2.5 µM, 24 h)-treated BEAS-2B cells served as a positive control.

198

### 199 **Statistical analysis**

200 Results were expressed as mean values and standard deviations. Comparisons were performed  
201 between data from exposed BEAS-2B cells (i.e., PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub> OEM<sub>2.5-0.3</sub>, or OEM<sub>0.3</sub>) and  
202 those from negative controls. Significant differences between results from BEAS-2B cells exposed  
203 either to NEM<sub>2.5-0.3</sub> or OEM<sub>2.5-0.3</sub> vs PM<sub>2.5-0.3</sub>, and to OEM<sub>0.3</sub> vs OEM<sub>2.5-0.3</sub> were reported. The non-  
204 parametric Mann-Whitney U-test with correction for multiple comparisons ( $p < 0.05$ ) was used to  
205 perform statistical analyses (Software: SPSS for windows).

206

## 207 **RESULTS AND DISCUSSION**

208

### 209 **PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub>-induced cytotoxicity**

210 As shown in Figure S2 (see also supplemental data), significant decreases of intracellular  
211 ATP concentrations were reported in BEAS-2B cells 6, 24 and 72 h after their exposure to PM<sub>2.5-</sub>  
212 <sub>0.3</sub>, from the dose of 6 µg/cm<sup>2</sup>. Almost a similar profile of intracellular ATP concentrations was  
213 seen in BEAS-2B cells after their exposure to NEM<sub>2.5-0.3</sub>. In contrast, both OEM<sub>2.5-0.3</sub> and  
214 particularly OEM<sub>0.3</sub> significantly altered intracellular ATP concentrations of BEAS-2B cells only  
215 for the highest doses, almost since 48 µg Eq. PM/cm<sup>2</sup>. The dose- and/or time-dependent  
216 cytotoxicity of PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> observed in BEAS-2B cells allowed  
217 to choose the concentrations (i.e., 3 and 12 µg Eq. PM/cm<sup>2</sup>) and kinetic (from 6 to 48 h) to apply  
218 to the further study of the other toxicological endpoints. It is noteworthy that both these  
219 concentrations were among the lowest shown as toxicologically active without, however,  
220 triggering massive regulated cell death (RCD) (Abbas et al. 2019; Badran et al., 2020; Gualtieri et  
221 al. 2010, 2011; Leclercq et al. 2018; Longhin et al., 2013, 2016; Platel et al. 2019; Sotty et al.  
222 2019).

223

### 224 **PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub>-induced oxidative stress**

225 Oxidative stress is one of the primary critical underlying mechanisms of toxicity activated by  
226 the inhalation of ambient PM. A ROS overproduction by the BEAS-2B cells 6 and 24 h after their  
227 exposures to PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, or OEM<sub>0.3</sub> was shown in Figure 1. Indeed, HE  
228 fluorescence intensity increased in a dose-dependent manner in BEAS-2B cells 6 and 24 h after  
229 their exposures to NEM<sub>2.5-0.3</sub> and OEM<sub>2.5-0.3</sub>, and more markedly PM<sub>2.5-0.3</sub> and OEM<sub>0.3</sub> (Figure 1-



230 A, B). It is noteworthy that, after 6 and 24 h, HE fluorescence was significantly higher in BEAS-  
231 2B cells exposed to  $PM_{2.5-0.3}$  from those exposed to  $NEM_{2.5-0.3}$  or  $OEM_{2.5-0.3}$ , and in BEAS-2B cells  
232 exposed to  $OEM_{0.3}$  from those exposed to  $OEM_{2.5-0.3}$ . Carboxy- $H_2DCFDA$  fluorescence revealed  
233 almost similar changes (Figure 1-C, D). The ahead-reported intracellular overproduction of ROS  
234 in BEAS-2B cells could be related either to the inorganic chemicals (e.g., Al, Cr, Cu, Fe, Mn, Ni,  
235 Pb, Ti, and Zn) detected within  $PM_{2.5-0.3}$  and  $NEM_{2.5-0.3}$ , or to the organic chemicals (i.e., PAH, O-  
236 PAH, and N-PAH) found within  $PM_{2.5-0.3}$ ,  $OEM_{2.5-0.3}$ , and rather  $OEM_{0.3}$ , also usually associated  
237 with redox reactions (Abbas et al. 2019, Badran et al. 2020; Gualtieri et al. 2011, Leclercq et al.  
238 2018; Longhin et al. 2013, Oh et al. 2011, Saint-Georges et al. 2008, 2009). Accordingly, due to  
239 the presence of both inorganic and organic chemical fractions within the whole  $PM_{2.5-0.3}$ , there was  
240 also a higher ROS overproduction in BEAS-2B cells exposed to  $PM_{2.5-0.3}$  from those exposed only  
241 to  $NEM_{2.5-0.3}$  or  $OEM_{2.5-0.3}$ . Interestingly, the higher ROS overproduction reported in BEAS-2B  
242 cells exposed to  $OEM_{0.3}$  from those exposed to  $OEM_{2.5-0.3}$  could be explained by its higher contents  
243 of total carbon, PAH, O-PAH, and N-PAH (Badran et al. 2020, see also supplemental data: Figure  
244 1, Tables S1 and S2).

245 In addition, significant increases of the gene expression and binding activity of NRF2 were  
246 reported in BEAS-2B cells exposed to  $NEM_{2.5-0.3}$  or  $OEM_{2.5-0.3}$ , and more markedly to  $PM_{2.5-0.3}$  or  
247  $OEM_{0.3}$  (Figure 1-E, F, G, H). All these changes were generally higher at 6 than 24 h. NRF2,  
248 because of its crucial function as a primary regulator of cellular redox homeostasis, is devoted to  
249 counteract the cytosolic overproduction of ROS and, therefore, to maintain redox balance within  
250 the cell (Wardyn et al. 2015). However, 24 h after any exposure, there was only a relatively low  
251 activation of NRF2 transcription factor, which could be explained by its possible relocalization,  
252 binding inactivation or degradation (Leclercq et al. 2018). It is important to note that, according to

253 ROS overproduction, the activation of the NRF2 signaling pathway was significantly higher in  
254 BEAS-2B cells exposed for 6 and 24 h to PM<sub>2.5-0.3</sub> from those exposed to NEM<sub>2.5-0.3</sub> or OEM<sub>2.5-0.3</sub>,  
255 and in BEAS-2B cells exposed for 6 h to OEM<sub>0.3</sub> from those exposed to OEM<sub>2.5-0.3</sub> (Figure 2).  
256 Once again, all these results underlined the higher toxicity potentials of PM<sub>2.5-0.3</sub> vs NEM<sub>2.5-0.3</sub>  
257 and/or OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> vs OEM<sub>2.5-0.3</sub>, also closely linked to their respective physical and  
258 chemical characteristics.

259 Remarkably, for any exposure, despite the early activation of NRF2 signaling pathway,  
260 oxidative alterations of glutathione, DNA and proteins significantly occurred in a dose- and time-  
261 dependent manner (Figure 3). Overall, the highest oxidative damage to target macromolecules was  
262 reported in BEAS-2B cells 24 h after their exposures to PM<sub>2.5-0.3</sub>, and interestingly OEM<sub>0.3</sub> rather  
263 than NEM<sub>2.5-0.3</sub> and OEM<sub>2.5-0.3</sub>, thereby underlining the higher toxicity potentials of PM<sub>2.5-0.3</sub> vs  
264 NEM<sub>2.5-0.3</sub> and/or OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> vs OEM<sub>2.5-0.3</sub>. Overall, these results supported the  
265 occurrence of a remarkable cytosolic oxidative stress in BEAS-2B cells after their exposures to  
266 PM<sub>2.5-0.3</sub> and OEM<sub>0.3</sub>, and NEM<sub>2.5-0.3</sub> and OEM<sub>2.5-0.3</sub>, to a lesser extent, and the inability of the  
267 NRF2 signaling pathway to totally counteract ROS overproduction. Oxidative damage to DNA,  
268 proteins and/or lipids can also disrupt the cell homeostasis, thereby contributing to gene expression  
269 deregulation, DNA mutation, protein alteration with loss of function, and lipid peroxidation with  
270 loss of membrane integrity and/or fluidity (Abbas et al. 2019; Dergham et al., 2012, 2015; Garçon  
271 et al., 2006; Gualtieri et al., 2010, 2011; Leclercq et al. 2016, 2017, 2018; Longhin et al. 2013,  
272 2016).

273

274 **PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub>-induced cytokine secretion**

275 The four cytokines under study, TNF- $\alpha$ , IL-6, IL-8 and MCP-1, were significantly secreted  
276 by BEAS-2B cells 6 and 24 h after their exposures to PM<sub>2.5-0.3</sub>, and NEM<sub>2.5-0.3</sub>, to a lesser extent,  
277 but only 6 h after their exposure to OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub> (Figure 4). Once again, except for TNF-  
278  $\alpha$ , the highest concentrations of the three other cytokines have been secreted by BEAS-2B cells  
279 exposed for 24 h to PM<sub>2.5-0.3</sub> from those exposed to NEM<sub>2.5-0.3</sub> and/or OEM<sub>2.5-0.3</sub> (Figure 4-D, F,  
280 H). In keeping with the current literature, a significant secretion of proinflammatory cytokines by  
281 BEAS-2B cells or other lung cell models, acutely or repeatedly exposed to relatively low doses of  
282 ambient PM, has already been reported (Abbas et al. 2019; Dergham et al., 2012, 2015; Boulbil et  
283 al. 2013; Garçon et al., 2006; Ghio et al. 2013; Gualtieri et al. 2010, 2011; Leclercq et al. 2016,  
284 2018; Longhin et al. 2013, 2016; Loxham et al. 2015; Sotty et al. 2019; Zhou et al. 2016). The  
285 relationship between the contents of redox-active compounds in ambient PM and the secretion of  
286 proinflammatory cytokines by lung cells might closely rely on the regulation of NF- $\kappa$ B (Lodovici  
287 and Bigagli, 2011).

288 However, in this work, despite the significant secretion of these cytokines by BEAS-2B cells  
289 exposed for 6 h to OEM<sub>2.5-0.3</sub> and OEM<sub>0.3</sub>, no significant secretion of them was still observed after  
290 24 h. Interestingly, other authors, contributing to better decipher the underlying mechanisms of  
291 toxicity of PAH-rich PM in different mouse or human cell models reported a negative association  
292 between the PAH contents of ambient PM and the secretion of inflammatory cytokines (Jalava et  
293 al. 2009; Kaur et al., 2019; Manzano-León et al. 2016).

294 Moreover, although the endotoxin contents have not been determined in PM<sub>2.5-0.3</sub>, PM<sub>0.3</sub>, nor  
295 their NEM nor OEM, their possible influences on the secretion of the proinflammatory mediators  
296 could not be excluded.

297 Another explanation supporting the difference between the results arising from cells exposed  
298 to metal-rich PM ( $PM_{2.5-0.3}$  and  $NEM_{2.5-0.3}$ ) vs PAH-rich PM ( $OEM_{2.5-0.3}$  and  $OEM_{2.5-0.3}$ ) in terms  
299 of cytokine secretion could be related to the bioavailability and the bioaccessibility of the organic  
300 compounds, whether those adsorbed-on core PM, or found in OEM (Garçon et al. 2004a, 2004b).  
301 The cell absorption of native particles by endocytosis, and the vector role of core PM could also  
302 facilitate the absorption of organic compounds, while PAH within OEM could be directly diluted  
303 in the culture medium. At once, PAH-coated onto PM could be only progressively released in cell  
304 cytoplasm after particle internalization, and progressively metabolized, thereby contributing to  
305 prolong their adverse effects within target cells. In contrast, the specific chemical properties of  
306 OEM, which regrouped lipophilic compounds that can easily and even rapidly go through the  
307 cytoplasmic membrane and could be freely, quickly, metabolized, within the cell, could contribute  
308 to shorten their adverse effects within the target cells.

309

### 310 **$PM_{2.5-0.3}$ , $NEM_{2.5-0.3}$ , $OEM_{2.5-0.3}$ , and $OEM_{0.3}$ -induced autophagy**

311 Mounting evidence further suggests that the autophagy machinery is closely involved in  
312 extensive crosstalk with several chronic inflammatory lung diseases, including asthma and COPD,  
313 and several, if not all forms of RCD, either by hyperactivation or by inhibition of the autophagy  
314 flow (Racanelli et al. 2016; Napoletano et al. 2019; Zhou et al. 2016). The evaluation of some  
315 hallmarks (i.e., BECN1/Beclin 1, ATG5, SQSTM1/p62 protein, and MAP1LC3B/LC3B) was also  
316 undertaken to better decipher the possible alteration of the autophagy process within BEAS-2B  
317 cells 24 and 48 h after their exposures to  $PM_{2.5-0.3}$ ,  $NEM_{2.5-0.3}$ ,  $OEM_{2.5-0.3}$ , or  $OEM_{0.3}$  (Figure 5).  
318 Significant decreases of ATG5 and Beclin1 were noted in BEAS-2B cells 24 h after their exposures  
319 only to  $OEM_{2.5-0.3}$  and  $OEM_{0.3}$ , thereby supporting the reduction of two critical proteins, even

320 though crucial for the autophagy process. Remarkably, the highest decreases of these autophagy  
321 hallmarks have been observed in BEAS-2B cells exposed for 24 h to OEM<sub>0.3</sub> from those exposed  
322 to OEM<sub>2.5-0.3</sub> (Figure 5-B, D). Two ubiquitin-like conjugation systems, LC3B and ATG5-12, up-  
323 regulated by another critical autophagy protein, Beclin, are generally highly required prior to  
324 autophagosome formation (An et al. 2019). One of the well-described hallmarks of autophagosome  
325 formation consists of the conversion of LC3-I unconjugated cytosolic form to LC3-II  
326 autophagosomal membrane-associated phosphatidylethanolamine-conjugated form.  
327 Interestingly, LC3B-II/LC3B-I protein ratio appeared to be specifically reduced in BEAS-2B cells  
328 only 48 h after their exposures to OEM<sub>2.5-0.3</sub> and mostly OEM<sub>0.3</sub>. Overall, the alterations of these  
329 critical proteins closely involved in the autophagy process revealed differential responses of whole  
330 PM or NEM towards PAH-rich OEM. However, according to Yoshii and Mizushima (2017), while  
331 LC3 is currently the most widely used autophagosome marker, one must keep in mind that the  
332 LC3-II amount at a given time point does not necessarily estimate the autophagic activity, because  
333 not only autophagy activation but also inhibition of autophagosome degradation could greatly  
334 increase the amount of LC3-II. Also, LC3-II can ectopically localize on non-autophagosome  
335 structures that are not turned over in the lysosome. Taken together, the present results supported  
336 rather an alteration of the levels of some critical proteins even though crucial for the autophagy  
337 rather than a real reduction of autophagy itself. Nevertheless, the essential role of autophagy in  
338 regulating inflammation induced by PM containing environmentally persistent free radicals was  
339 reported by Chen et al. (2016), and Xu et al. (2017). Their statements supported the alteration of  
340 some current hallmark proteins of autophagy and at once the relatively low secretion of  
341 proinflammatory cytokines, both noticed in this work in BEAS-2B cells exposed to OEM<sub>2.5-0.3</sub> and

342 OEM<sub>0.3</sub>, at a higher extent, despite the absence of additional observations proving the alteration of  
343 the autophagy itself.

344

#### 345 **PM<sub>2.5-0.3</sub>, NEM<sub>2.5-0.3</sub>, OEM<sub>2.5-0.3</sub>, and OEM<sub>0.3</sub>-induced apoptosis**

346 Recent data suggested divergent cell responses towards ambient PM resulting in RCD (i.e.,  
347 apoptosis, autophagy, and necrosis) (Peixoto et al. 2017). To go further, apoptosis was also  
348 investigated. As depicted by Figure 6-A, B, C, the caspases 8, 9, and 7/ activities showed almost  
349 similar changes in BEAS-2B cells 24 and 48 h after any exposure, thereby supporting the  
350 progressive occurrence of molecular apoptotic events.

351 However, as shown by Figure 5-D, E, F, TUNEL, Annexin-V immunostaining, and cell cycle  
352 phases of BEAS-2B cells, indicated rather no clear morphological characteristics of RCD, even  
353 after 48 h. An et al. (2019), studying the critical interplays between oxidative stress, autophagy  
354 and apoptosis induced by oxidized black carbon, noticed that it was able to activate each of them  
355 in a dose-dependent manner. Other authors reported the proapoptotic effects of ambient PM<sub>2.5</sub>  
356 within different human epithelial bronchial cell models, including BEAS-2B cells (Boublil et al.  
357 2013; Gualtieri et al. 2011; Leclercq et al. 2018; Longhin et al. 2013; Lee et al. 2019).

358 Peixoto et al. (2017) recently published relevant data about the relationships between the  
359 exposure to ambient PM and the underlying mechanisms related to RCD, with a particular attention  
360 devoted to the pivotal role played by oxidative stress in the determination of cell fate. Indeed, the  
361 cellular fate was progressively directed toward RCD, and notably autophagy, when the levels of  
362 ROS were overwhelming for the cells, and lead to an inhibition of the apoptotic pathway. **In this**  
363 **work, for any exposure, while no clear autophagy process has been identified, molecular but not**  
364 **morphological evidence of apoptosis has been noted.**

365

366 **CONCLUSION**

367

368 Overall, the present results clearly demonstrated that whole  $PM_{2.5-0.3}$  and both its inorganic  
369 and organic chemical fractions were able to induce some underlying mechanisms of toxicity  
370 relying on ROS overproduction, inflammation, and, with regards to RCD, apoptosis rather than  
371 autophagy. Despite the capacity of  $NEM_{2.5-0.3}$ , and  $OEM_{2.5-0.3}$  to activate them, their combination  
372 thereof may generally be lower comparing with those triggered by the whole  $PM_{2.5-0.3}$ . Similar  
373 data, even with equal or higher extent from those of  $OEM_{2.5-0.3}$ , were reported in BEAS-2B cells  
374 exposed to  $OEM_{0.3}$ . Taken together, these results highlighted the higher toxicity potentials, closely  
375 linked to their physical and chemical characteristics, of the  $PM_{2.5-0.3}$  vs  $NEM_{2.5-0.3}$  and/or  $OEM_{2.5-0.3}$ ,  
376 and  $OEM_{0.3}$  vs  $OEM_{2.5-0.3}$ . Future works are still needed to go further in the better knowledge  
377 of the toxicity potentials of the different chemical fractions of ambient PM.

378

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555

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563



564 **Figure Legend**

565

566 **Figure 1:** Fluorescence intensity of hydroethidium (HE) (Figure 1-A, B) and 6-carboxy-2',7'-  
567 dichlorodihydrofluorescein diacetate (carboxy-H<sub>2</sub>DCFDA) (Figure 1-C, D), and gene expression  
568 and/or protein binding activity of nuclear factor erythroid 2-related factor 2 (NRF-2) (Figure 1-E,  
569 F and Figure 1-G, H, respectively), 6 (Figure 1-A, C, E, G) and 24 h (Figure 1-B, D, F, H) after  
570 BEAS-2B cell exposure to fine particles (PM<sub>2.5-0.3</sub>) and their extractable (OEM<sub>2.5-0.3</sub>) and non-  
571 extractable (NEM<sub>2.5-0.3</sub>) fractions, and to the extractable fraction (OEM<sub>0.3</sub>) of the ultrafine particles  
572 (PM<sub>0.3</sub>). Menadione (100 μM, 4 h) was used as positive control. Results are normalized to controls  
573 and presented as means and standard deviations of 6 or 3 replicates from three independent  
574 experiments. (Mann-Whitney U-test; vs. controls: \* =  $p < 0.05$ , and \*\*\* =  $p < 0.001$ ; vs. PM<sub>2.5-0.3</sub>  
575 or vs OEM<sub>0.3</sub>: c =  $p < 0.05$ ).

576

577 **Figure 2:** Gene expression of Kelch-like ECH-associated protein 1 (KEAP-1) (Figure 2-A, B),  
578 heme oxygenase 1 (HMOX) (Figure 2-C, D), NAD(P)H quinone dehydrogenase 1 (NQO1) (Figure  
579 2-E, F), and superoxide dismutase (SOD) (Figure 2-G, H) in BEAS-2B cells, 6 (Figure 2-A, C, E,  
580 G) and 24 h (Figure 2-B, D, F, H) after BEAS-2B cell exposure to fine particles (PM<sub>2.5-0.3</sub>) and  
581 their extractable (OEM<sub>2.5-0.3</sub>) and non-extractable (NEM<sub>2.5-0.3</sub>) fractions, and to the extractable  
582 fraction (OEM<sub>0.3</sub>) of the ultrafine particles (PM<sub>0.3</sub>). Results are normalized to controls and  
583 presented as means and standard deviations of 3 replicates from three independent experiments.  
584 (Mann-Whitney U-test; vs. controls: \* =  $p < 0.05$ ; vs. PM<sub>2.5-0.3</sub> or vs OEM<sub>0.3</sub>: c =  $p < 0.05$ ).

585

586 **Figure 3:** Glutathione status (Figure 3-A, B), 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Figure 3-  
587 C, D), carbonylated protein (CO-protein) (Figure 3-E, F), and 8-isoprostane (8-Isop) (Figure 3-G,

588 H) in BEAS-2B cells, 6 (Figure 3-A, C, E, G) and 24 h (Figure 3-B, D, F, H) after BEAS-2B cell  
589 exposure to fine particles ( $PM_{2.5-0.3}$ ) and their extractable ( $OEM_{2.5-0.3}$ ) and non-extractable  
590 ( $NEM_{2.5-0.3}$ ) fractions, and to the extractable fraction ( $OEM_{0.3}$ ) of the ultrafine particles ( $PM_{0.3}$ ).  
591 Results are normalized to controls and presented as means and standard deviations of 3 replicates  
592 from three independent experiments. (Mann-Whitney U-test; vs. controls:  $* = p < 0.05$ ; vs.  $PM_{2.5-0.3}$   
593  $0.3$  or vs  $OEM_{0.3}$ :  $c = p < 0.05$ ).

594

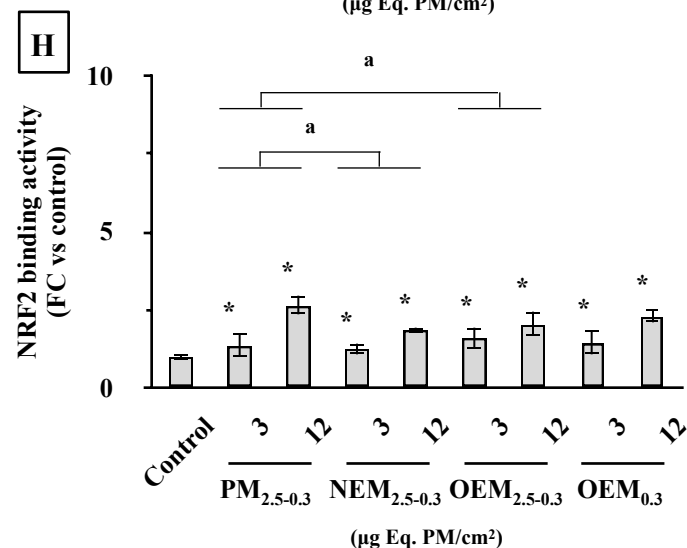
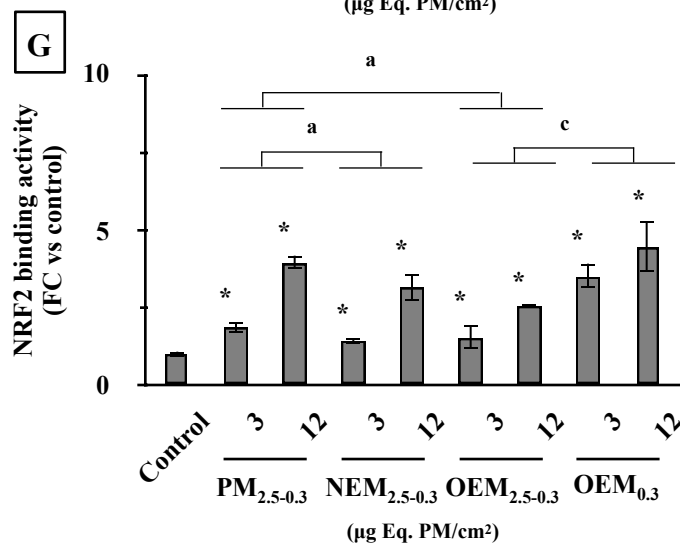
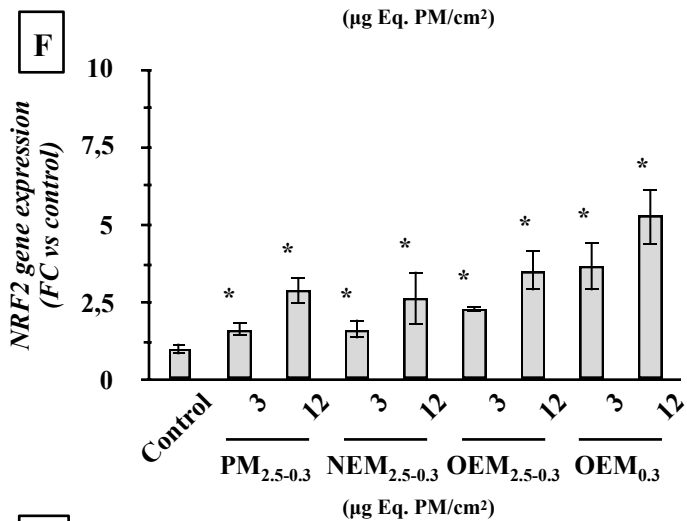
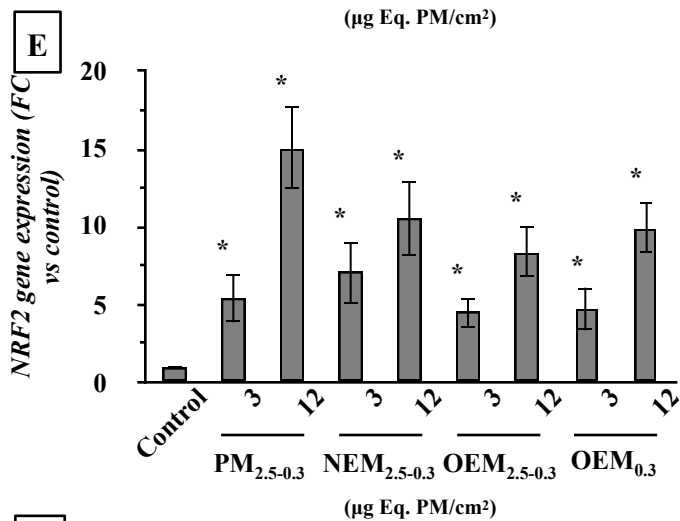
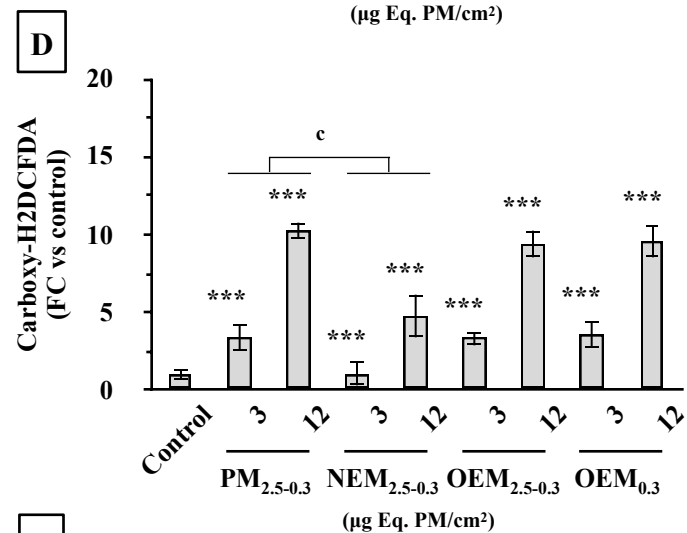
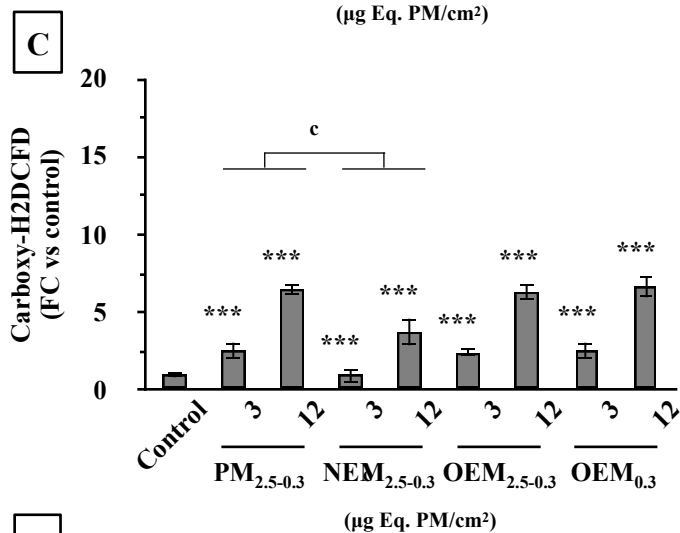
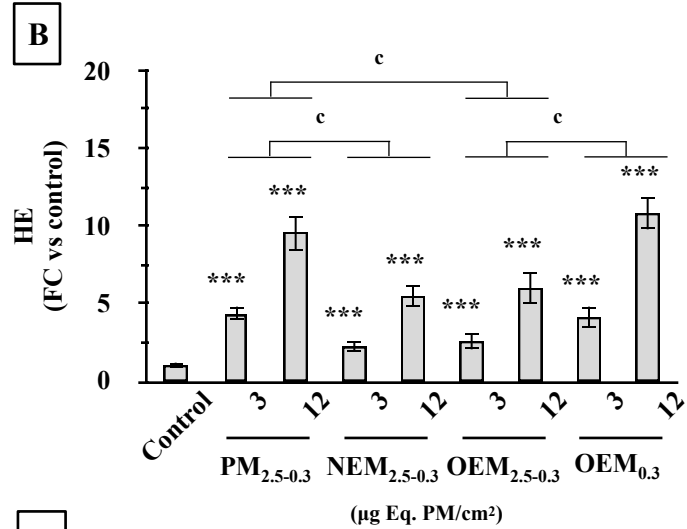
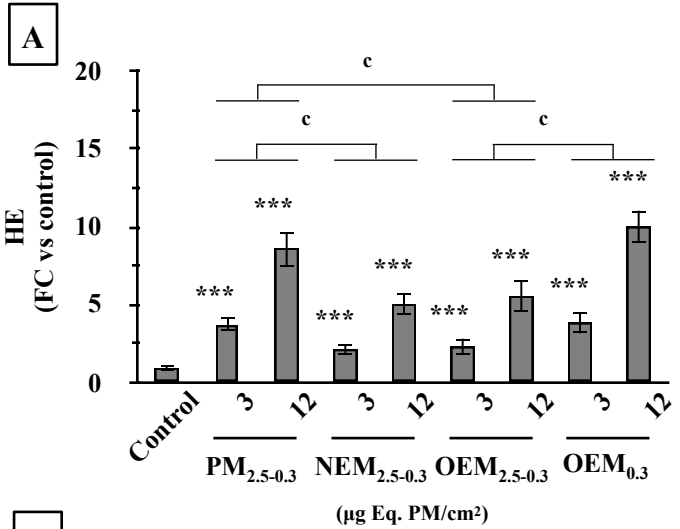
595 **Figure 4:** Concentrations of tumor necrosis factor (TNF- $\alpha$ ) (Figure 4-A, B), interleukin-6 (IL-6)  
596 (Figure 4-C, D), interleukin-8 (IL-8) (Figure 4-E, F), and monocyte chemoattractant protein-1  
597 (MCP-1) (Figure 4-G, H), in BEAS-2B cells, 6 (Figure 4-A, C, E, G) and 24 h (Figure 4-B, D, F, H)  
598 H) after BEAS-2B cell exposure to fine particles ( $PM_{2.5-0.3}$ ) and their extractable ( $OEM_{2.5-0.3}$ ) and  
599 non-extractable ( $NEM_{2.5-0.3}$ ) fractions, and to the extractable fraction ( $OEM_{0.3}$ ) of the ultrafine  
600 particles ( $PM_{0.3}$ ). Results are normalized to controls and presented as means and standard  
601 deviations of 3 replicates from three independent experiments. Baseline cytokine secretion of  
602 BEAS-2B cells for 6 and 24 h were respectively:  $36 \pm 14$  pg/mL and  $48 \pm 16$  pg/mL for TNF- $\alpha$ ,  
603  $66 \pm 20$  pg/mL and  $78 \pm 21$  pg/mL for IL-6,  $410 \pm 49$  pg/mL and  $648 \pm 74$  pg/mL for IL-8, and  
604  $973 \pm 94$  pg/mL and  $1390 \pm 173$  pg/mL for MCP-1. (Mann-Whitney U-test; vs. controls:  $* = p <$   
605  $0.05$ ; vs.  $PM_{2.5-0.3}$  or vs  $OEM_{0.3}$ :  $c = p < 0.05$ ).

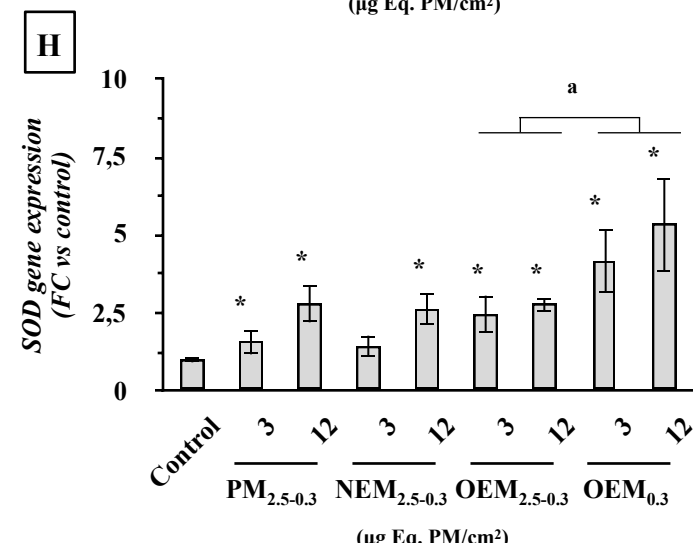
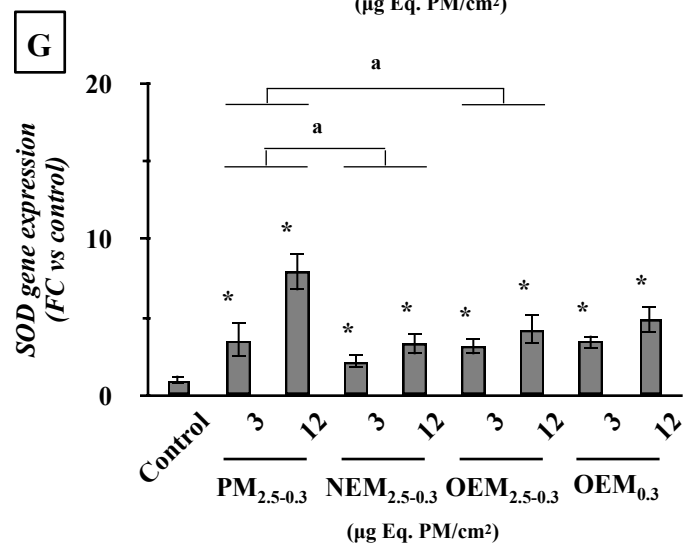
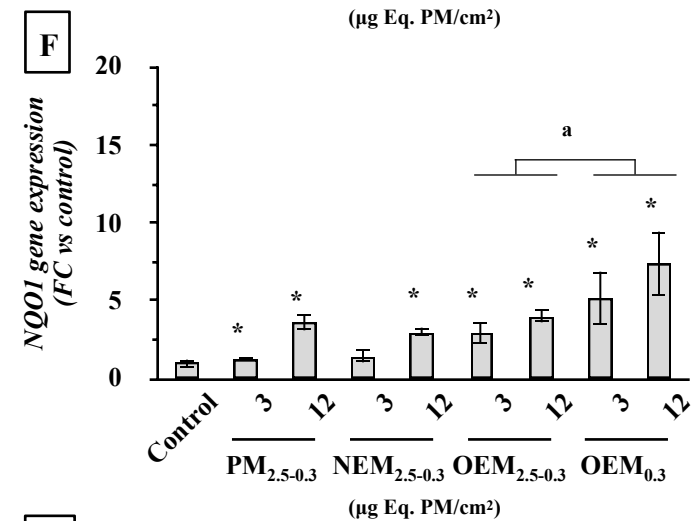
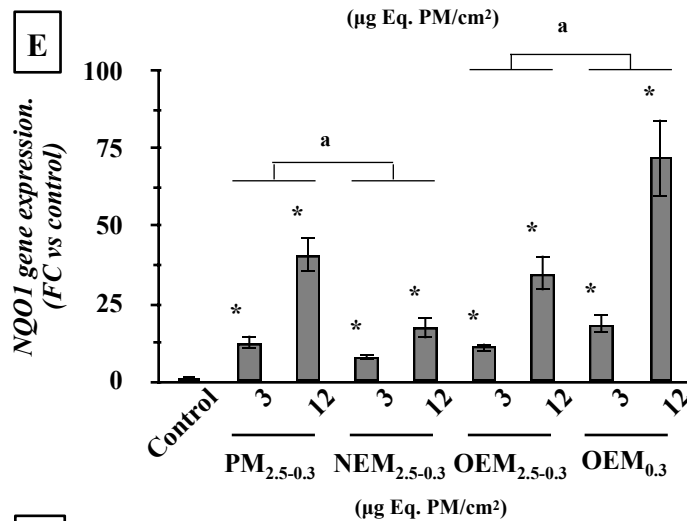
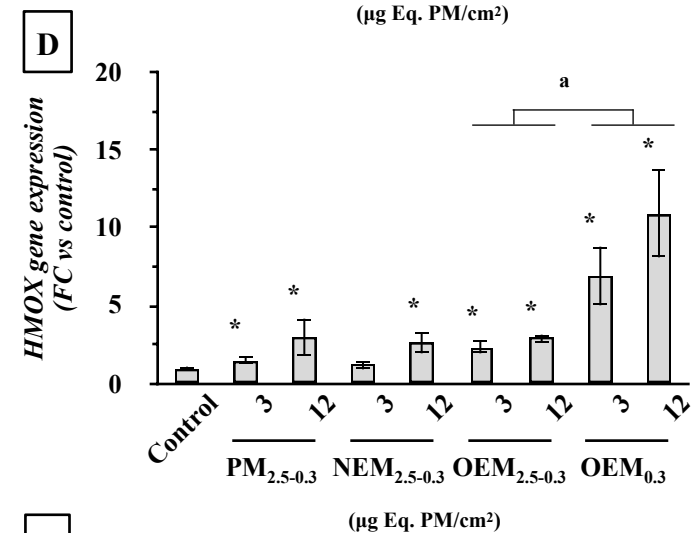
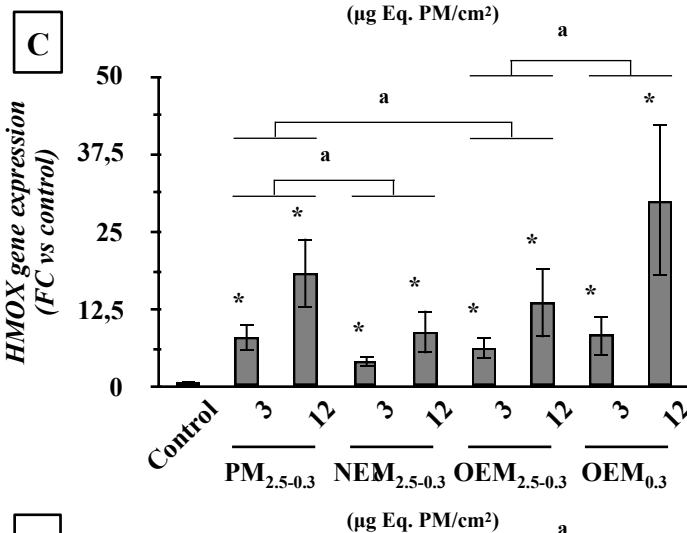
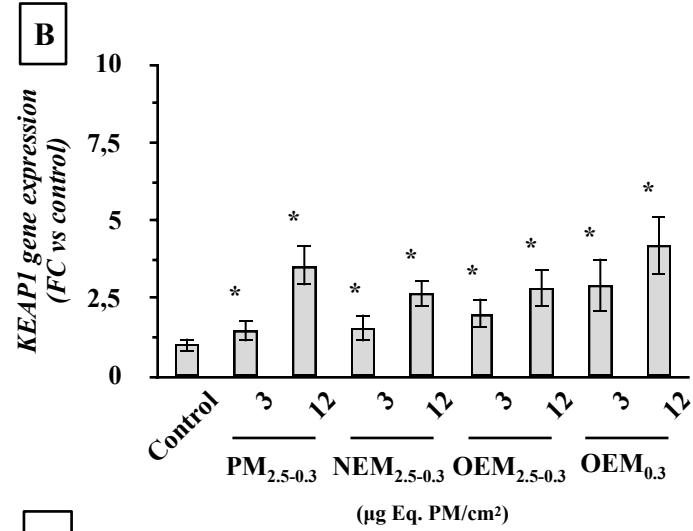
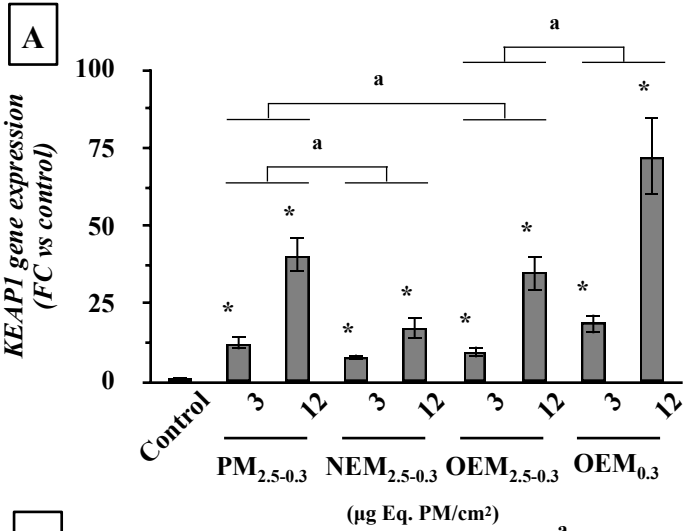
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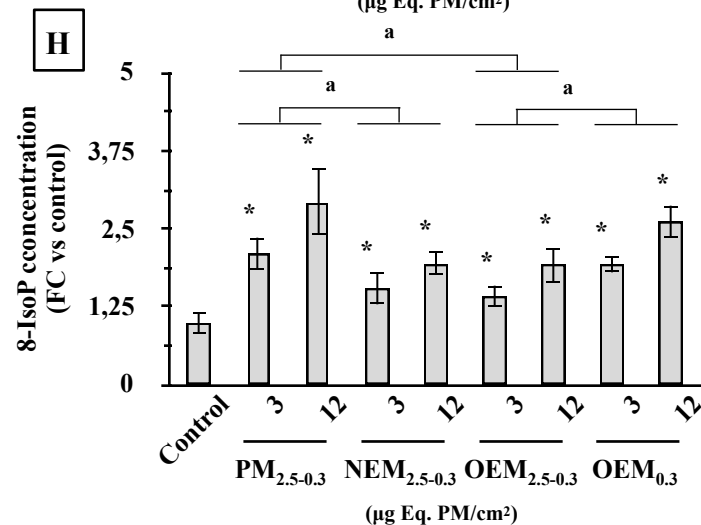
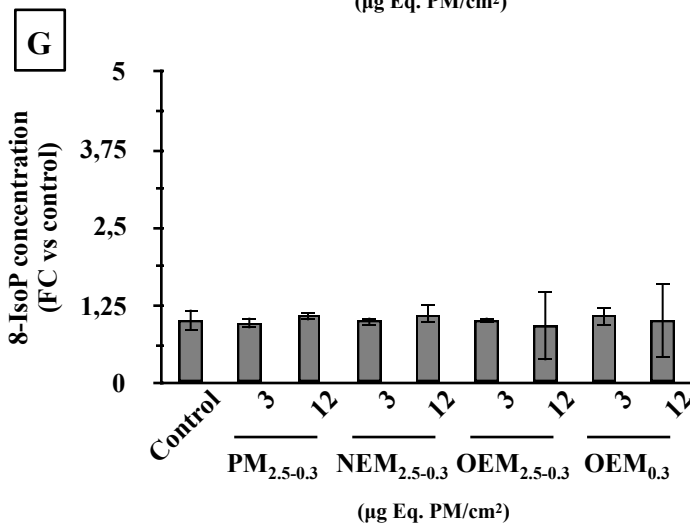
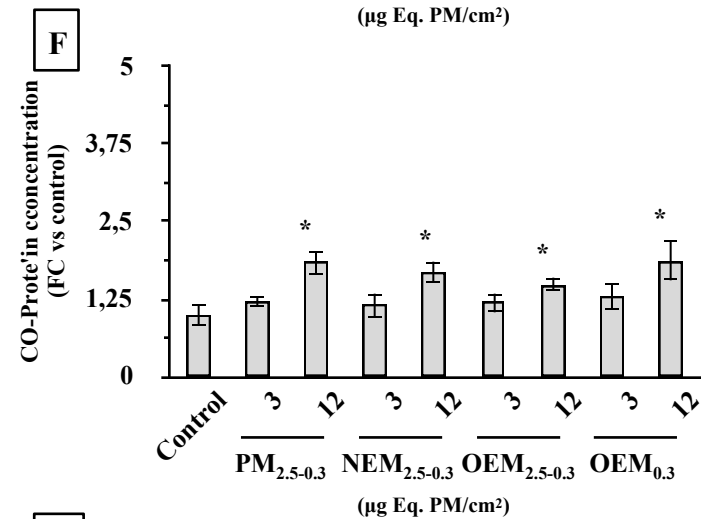
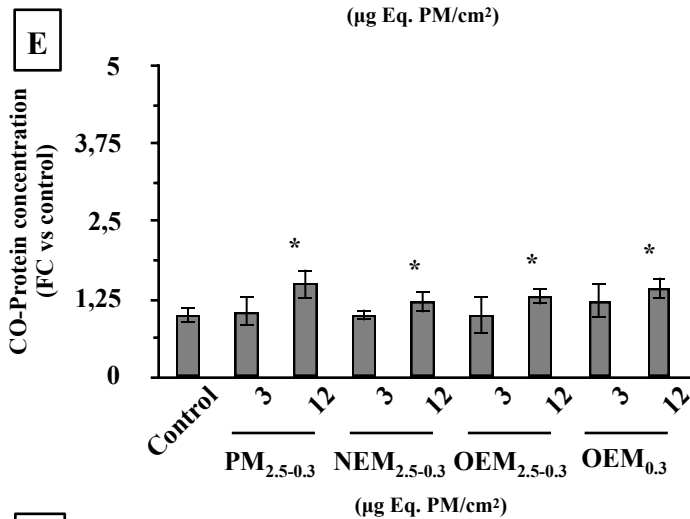
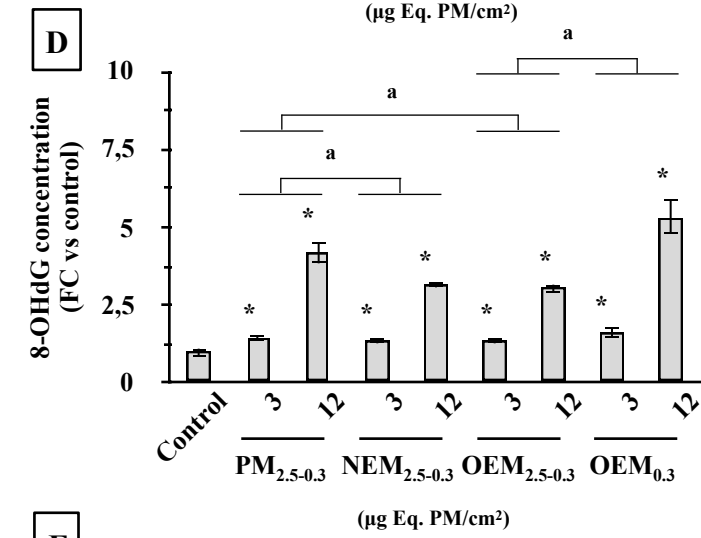
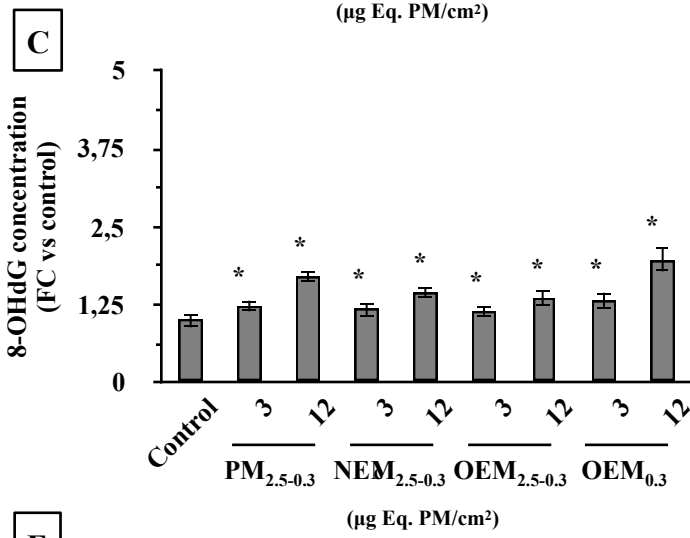
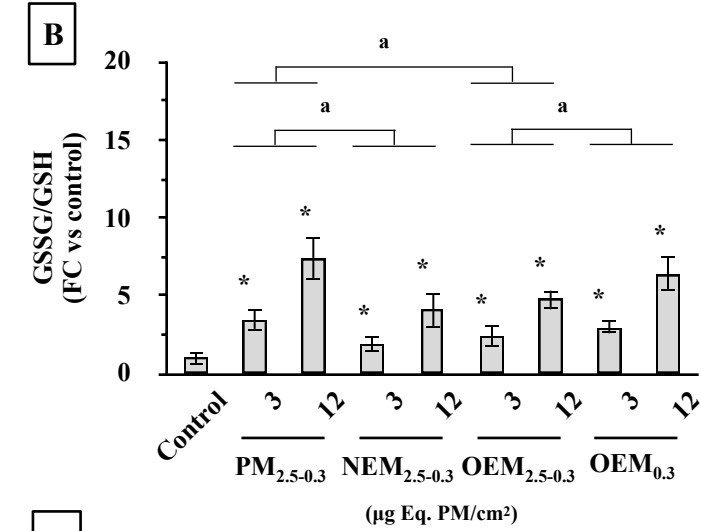
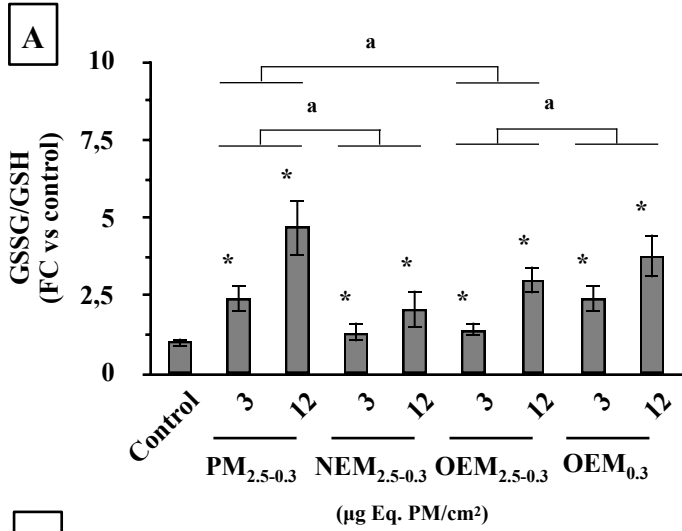
607 **Figure 5:** Protein expression of autophagy-related 5 (Figure 5-A), BECN1/Beclin 1 (Figure 5-B),  
608 SQSTM1/p62 protein (Figure 5-C), and microtubule-associated protein 1 light chain 3 b  
609 (MAP1LC3B/LC3B) (Figure 5-D) in BEAS-2B cells 24 and 48 h after BEAS-2B cell exposure to  
610 fine particles ( $PM_{2.5-0.3}$ ) and their extractable ( $OEM_{2.5-0.3}$ ) and non-extractable ( $NEM_{2.5-0.3}$ )

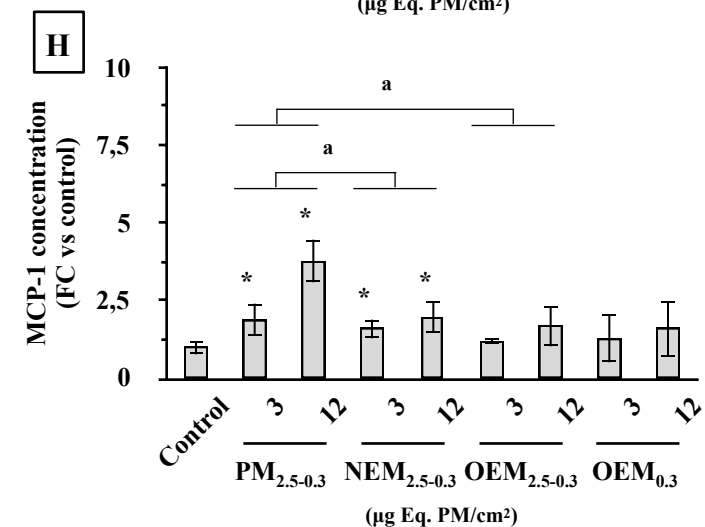
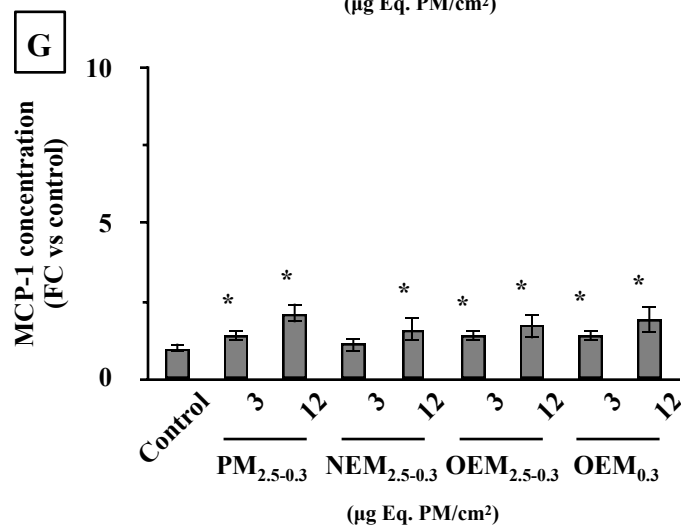
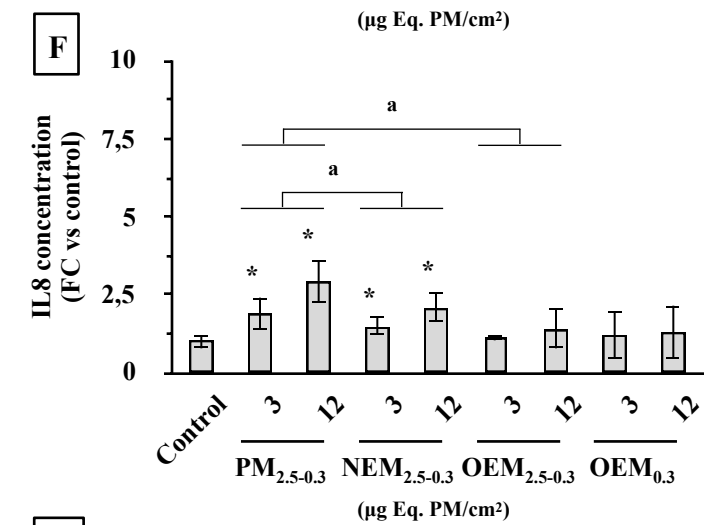
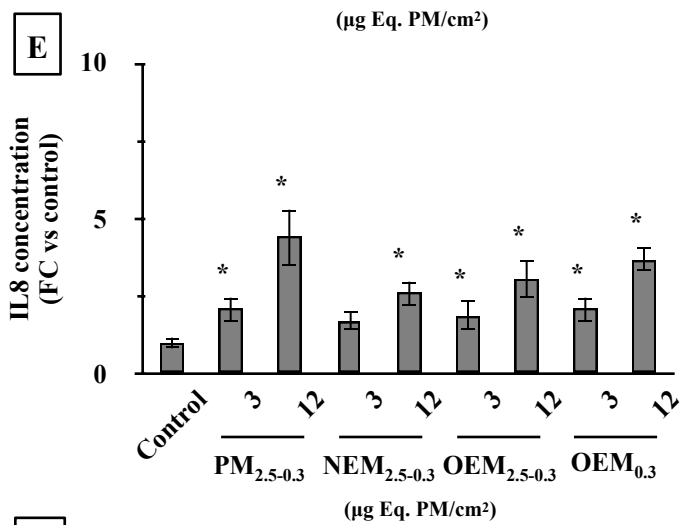
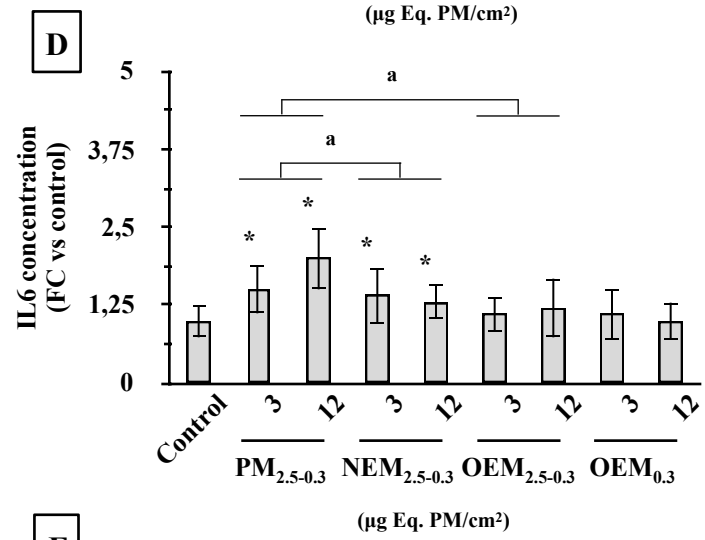
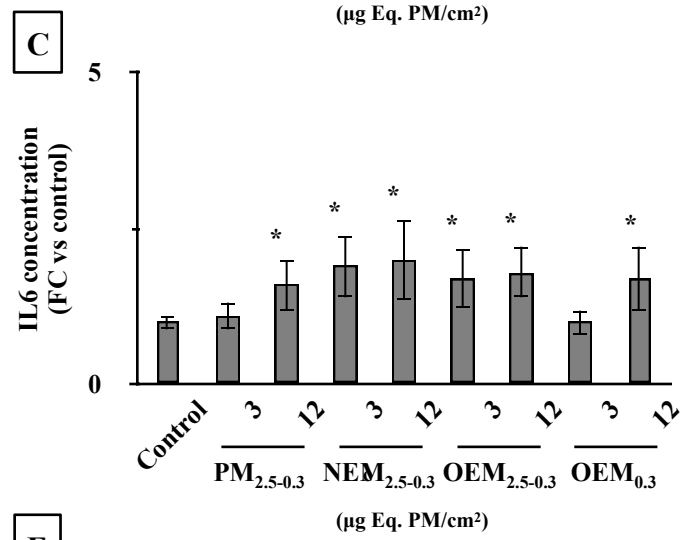
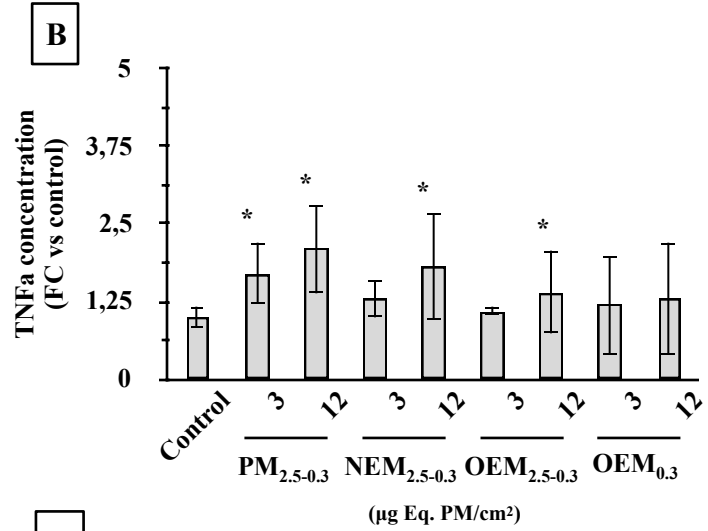
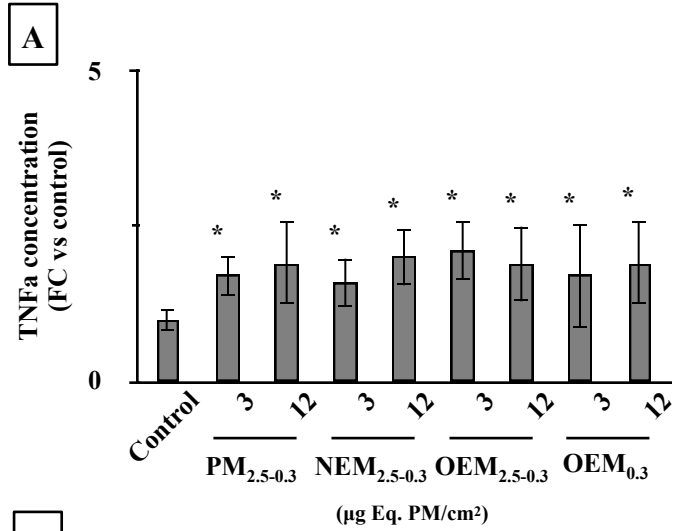
611 fractions, and to the extractable fraction (OEM<sub>0.3</sub>) of the ultrafine particles (PM<sub>0.3</sub>) at 12 µg Eq.  
612 PM/cm<sup>2</sup>. Results are normalized to controls and presented as means and standard deviations of 3  
613 replicates from three independent experiments. (Mann-Whitney U-test; vs. controls: \* =  $p < 0.05$ ;  
614 vs. PM<sub>2.5-0.3</sub> or vs OEM<sub>0.3</sub>:  $c = p < 0.05$ ).

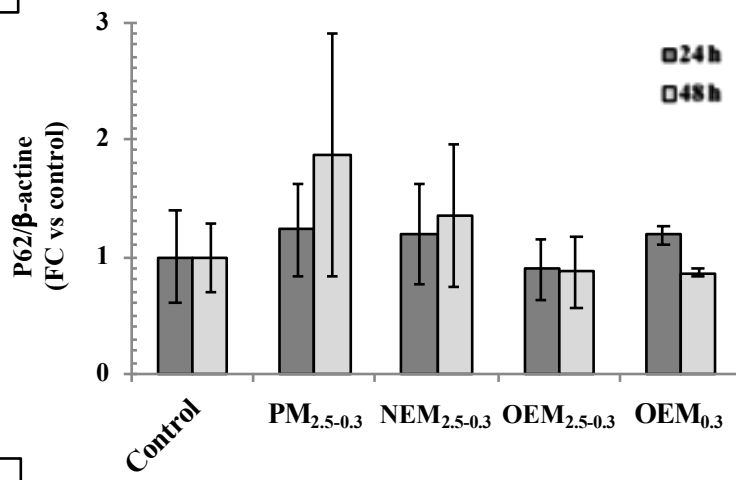
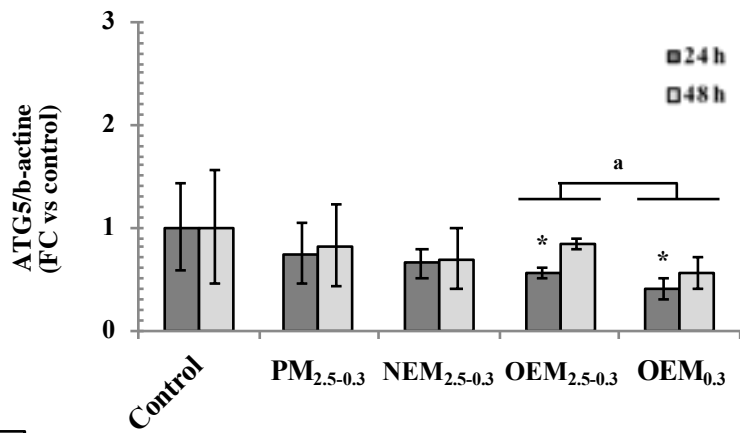
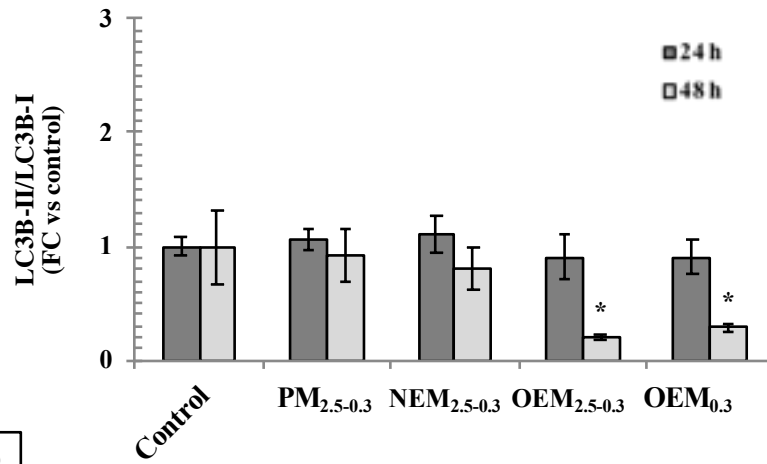
615  
616 **Figure 6:** Activities of caspases 3/7, 8, and 9 (Figure 6-A, B, C, respectively),  
617 immunofluorescence labelling of apoptotic cells using the TdT-mediated dUTP Nick-End  
618 Labeling (TUNEL) method (Figure 6-D), detection of apoptotic cells using the Single-channel  
619 dead cell apoptosis kit with Annexin V Alexa Fluor™ 488 and SYTOX™ green dyes (Figure 6-  
620 F) and cell cycle analysis using Click-iT™ Plus EdU Alexa Fluor™ 647 flow cytometry assay kit  
621 (Figure 6-G) in BEAS-2B cells, 24 and 48 h after BEAS-2B cell exposure to fine particles (PM<sub>2.5-  
622 0.3</sub>) and their extractable (OEM<sub>2.5-0.3</sub>) and non-extractable (NEM<sub>2.5-0.3</sub>) fractions, and to the  
623 extractable fraction (OEM<sub>0.3</sub>) of the ultrafine particles (PM<sub>0.3</sub>) at 12 µg Eq. PM/cm<sup>2</sup>. Staurosporine  
624 (2.5 µM, 24 h) has been used as positive control. Results are normalized to controls and presented  
625 as means and standard deviations of 3 replicates from three independent experiments. (Mann-  
626 Whitney U-test; vs. controls; \* =  $p < 0.05$ ).









**A****B****C****D**