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1 **Oxidative stress response in pulmonary cells exposed to different fractions of PM_{2.5-0.3}**
2 **from urban, traffic and industrial sites**

3
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15

16 **Abstract**

17 The aim of this work was to study the relationship between oxidative stress damages
18 and particulate matter (PM) chemical composition, sources, and PM fractions. PM_{2.5-0.3} (PM
19 with equivalent aerodynamic diameter between 2.5 and 0.3 µm) were collected at urban, road
20 traffic and industrial sites in the North of France, and were characterized for major and minor
21 chemical species. Four different fractions (whole PM_{2.5-0.3}, organic, water-soluble and non-
22 extractable matter) were considered for each of the PM_{2.5-0.3} samples from the three sites. After
23 exposure of BEAS-2B cells to the four different fractions, oxidative stress was studied in cells
24 by quantifying reactive oxygen species (ROS) accumulation, oxidative damage to proteins
25 (carbonylated proteins), membrane alteration (8-isoprostane) and DNA damages (8-OHdG).
26 Whole PM_{2.5-0.3} was capable of inducing ROS overproduction and caused damage to proteins
27 at higher levels than other fractions. Stronger cell membrane and DNA damages were found
28 associated with PM and organic fractions from the urban site. ROS overproduction was
29 correlated with level of expression of carbonylated proteins, DNA damages and membrane
30 alteration markers. The PM_{2.5-0.3} collected under industrial influence appears to be the less
31 linked to cell damages and ROS production in comparison with the other influences.

32

33 Keywords: Particulate matter; oxidative stress; reactive oxygen species; 8-isoprostane; 8-
34 OHdG; carbonylated proteins.

35 1. Introduction

36 Numerous studies have been conducted to study chemical atmospheric particulate matter (PM)
37 characteristics (Telesiński and Kiepas-Kokot, 2021), and to better understand the impact of PM
38 on human health (Broome et al., 2020; Fischer et al., 2020; Guan et al., 2021; Lin et al., 2020;
39 P. Wang et al., 2021; Zhang et al., 2021). Results have shown associations between PM and
40 cardiovascular disease (Aryal et al., 2021), fertility (L. Wang et al., 2021), cancer (Gogna et al.,
41 2019; Nagel et al., 2018), increased risk of autism spectrum disorders (Lam et al., 2016),
42 psychiatric disorders (Buoli et al., 2018; Chen et al., 2018; Oudin et al., 2016). PM has been
43 proven to cause damaging respiratory outcomes, especially through the triggering of oxidative
44 stress (Li et al., 2021; Mu et al., 2021; Quezada-Maldonado et al., 2021) which is one of the
45 first mechanism of stress appearing in the cell. Oxidative stress occurs in the cell when a break
46 in the redox homeostasis happens, due to a decrease in antioxidant capacity, or an
47 overproduction of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen
48 peroxide (H_2O_2), hydroxyl radical ($OH\cdot$). This phenomenon may lead to non-specific and
49 irreversible oxidation of DNA, proteins and cellular lipids (Badran et al., 2020a; Migdal and
50 Serres, 2011; Ndong Ba et al., 2019). Along with the direct measurement of ROS in the cells,
51 different cellular methods could be used for oxidative stress evaluation. Among the biomarkers
52 used for oxidative stress assessment (Ho et al., 2013): 8-hydroxy-2'-deoxyguanosine (8-OHdG)
53 is one of the predominant forms of ROS-induced oxidative DNA damage, isoprostanes are
54 generated by the peroxidation of a polyunsaturated fatty acid (Morrow et al., 1992), and
55 carbonylated proteins, are an indicator of oxidative alteration of proteins. The PM induced
56 oxidative stress has been linked to metals (Jan et al., 2020) and organic compounds (Badran et
57 al., 2020a, Win et al., 2018). In addition, the ability of the particles themselves (Dergham et al.,
58 2015, 2012; Leclercq et al., 2018; Moufarrej, 2021), their organic extract (OE) (Abbas et al.,
59 2019; Badran et al., 2020b; Billet et al., 2018) or their water soluble extract (WSE) (X. Liu et

60 al., 2020; Shuster-Meiseles et al., 2016; Xu et al., 2018; Yan et al., 2017) to induce oxidative
61 stress has been shown. Despite the numerous studies investigating the relationship between PM
62 physical-chemical properties and oxidative stress, a lack of information remains regarding the
63 fraction of PM that contributes the most in triggering of oxidative stress. This study aims to
64 investigate the dependence of oxidative stress to PM chemical composition, sources, and PM
65 fractions. In this work, different PM samples were collected under urban, industrial, and road
66 traffic influence in northern France, as the whole population is mainly exposed to such kind of
67 emission sources. The ROS, 8-isoprostane, carbonylated proteins and 8-OHdG were quantified
68 to assess the onset of oxidative stress. Correlations, principal component analysis, and
69 classifications were used to reveal the relationship between oxidative stress, different PM
70 fractions, and PM chemical composition.

71

72 2. **Material and methods**

73 2.1. PM sampling sites

74 PM with an equivalent aerodynamic diameter between 2.5 and 0.3 μm ($\text{PM}_{2.5-0.3}$) were collected
75 in different sites in northern France. Five samples were collected under urban influence, in
76 Dunkirk (51°01'35.8"N 2°22'20.0"E): one annual average sample (U-Y) March 19, 2014 to
77 March 18, 2015, and four seasonal samples: U-Sp during spring period (March 19, 2014 to June
78 21, 2014), U-Su during summer period (June 21 to September 26, 2014), U-A during autumn
79 period (September 27 to December 17, 2014) and U-W during winter period (December 18,
80 2014 to March 18, 2015). In addition to these five samples, one sample (I) was collected under
81 industrial influence at Grande-Synthe (51°01'29.0"N 2°18'11.7"E), near integrated steelworks
82 and chemical activities (steam cracking, oil stocks) between February 28, 2017 and June 02,
83 2017. Finally, another sample (T) was collected in a road traffic influenced site at Roubaix
84 (50°42'24.6"N 3°10'49.8"E) between March 02, 2017 and June 1, 2017. All these seven samples

85 were collected using 5 stages plus back up cascade impactor (Staplex[®] 235, TFIA, 68 m³/h) and
86 PM_{2.5-0.3} were retrieved from the 5 impaction plates (cut-off off the 5th plate was 0.33 μm) as
87 described elsewhere (Borgie et al. 2015).

88

89 2.2. Preparation of the different PM fractions

90 PM samples were studied by considering them as whole PM, water-soluble extract (WSE),
91 organic extract (OE) and non-extractable matter (dPM). In order to prepare the WSE for each
92 sample, 60 mg of PM_{2.5-0.3} were extracted with 3 mL of ultra-pure water in a glass conical tube,
93 using ultrasonic treatment for 20 minutes, and then centrifuged for 10 minutes at 4°C and 2500
94 g. The supernatant was then transferred in a polyethylene vial, and the particle pellet was again
95 suspended in ultrapure water, and the recovery steps were repeated twice (Landkocz et al.,
96 2017). Finally, the WSE was filtered through a nylon filter (0.22 μm) (and stored at -18 °C until
97 use. The same steps were followed to prepare the OE, using dichloromethane as a solvent.
98 Finally, the obtained extract was concentrated under nitrogen flow, and dimethyl sulfoxide was
99 added to the residue for solvent change. Finally, in order to prepare the dPM, the particulate
100 residue after successive extraction of both organic and water-soluble fractions was considered.

101

102 2.3. PM chemical characterization

103 PM_{2.5-0.3} were extensively characterized. Briefly, major and trace elements were quantified in
104 whole PM samples as well in WSE using Inductively Coupled Plasma coupled to Atomic
105 Emission Spectrometry or Mass spectrometry (ICP-AES and ICP-MS). Major water-soluble
106 ions were quantified after water leaching using ion chromatography. Total Carbon was
107 quantified using C, H, N, S microanalyzer. Polycyclic Aromatic Hydrocarbons (PAHs) were
108 quantified in the OE after Soxhlet extraction of PM_{2.5-0.3} using dichloromethane. Detailed
109 procedures used for chemical characterization can be found in Landkocz et al, 2017.

110

111 2.4. Cell culture

112 BEAS-2B cells are used in this work. They were initially generated from normal human
113 bronchial epithelial cells from non-cancerous subjects. BEAS-2B (ATCC® CRL-9609™,
114 ECACC, Wiltshire, UK) were cultured in Corning cellbind® plastic plates or flasks
115 (ThermoFisher, France) using LHC9 (ThermoFisher, France) as culture medium and incubated
116 at 37°C in a humidified atmosphere containing 5% CO₂. Medium was replaced every three days
117 and cells were passed when reaching 80% of confluence.

118

119 2.5. Cytotoxicity

120 The WST-1 assay allows the evaluation of mitochondrial activity and cell proliferation. WST-1
121 cytotoxicity test was performed in order to choose the dose of exposure allowing the
122 observation of a cellular response without massive cell mortality. The WST-1 colorimetric
123 assay kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). In the present
124 study, we would like to expose to a unique dose to be able to discriminate the 28 samples (7
125 sites and 4 different fractions). The heterogeneity of the 28 samples makes this dose more
126 difficult to define. Cells were exposed to PM, WSE, OE and dPM fractions of the different
127 samples using different doses (6, 12, 24, 48 and 96 µg/cm²), using four replicates for each
128 sample, and during 48 h. Exposure of BEAS-2B cells to the different PM_{2.5-0.3} samples showed
129 strong significant decrease in mitochondrial activity after 48 h of exposure, at 48 and 96 µg/cm².
130 For low doses (6 and 12 µg/cm²), very weak responses were recorded. For the 24 µg/m³ dose,
131 the samples showed medium decreases in mitochondrial activity. Considering all conditions of
132 exposure, the 24 µg/cm² dose appeared to be the most suitable dose for studying the oxidative
133 stress parameters, providing sufficient doses to cause certain adverse effects, without massive

134 cell death, and for comparing responses associated with the four fractions of the seven PM_{2.5-}
135 _{0.3} samples. It was therefore chosen as the exposure dose for the whole study.

136

137 2.6. Inflammation

138 The protein expression of the inflammatory cytokine IL-6 was quantified after 48 h of cell
139 exposure to the different PM fractions. The test was done using ELISA sandwich method, using
140 Human IL-6 kit (ELISA MAX™ Deluxe Set, BioLegend, San Diego, CA, USA) according to
141 manufacturer's protocol.

142

143 2.7. Oxidative stress

144 Oxidative stress was evaluated by the measurement of ROS overproduction, lipid membrane
145 alteration, and the measurement of protein and DNA damage.

146

147 ROS assay

148 Cells were cultivated in 96 well black plates according to the manufacturer's instructions. This
149 test was performed 48 h after the exposure (40,000 cells/well) using the DHE
150 (Dihydroethidium) Assay Kit—Reactive Ox Species (ab236206, Abcam China, Shanghai,
151 China), according to manufacturer's protocol. It allows the quantification of reactive oxygen
152 Species (Superoxide radical and hydrogen peroxide) in living cells, and uses dihydroethidium
153 DHE as a fluorescent probe for the detection of ROS.

154

155 *Assessment of oxidative DNA damage - quantification of 8-OHdG*

156 8-hydroxy-2'-deoxyguanosine (8-OHdG) is formed as a result of oxidative damage to DNA by
157 reactive oxygen and nitrogen species. The concentration of 8-OHdG was measured using
158 8-hydroxy-2'-deoxyguanosine ELISA Kit, (ab201734, Cayman, Ann Arbor, MI, USA),

159 according to manufacturer's protocol. The assay uses a plate coated with 8-hydroxy-2'-
160 deoxyguanosine, and an antibody, bound to the enzyme horseradish peroxidase (HRP) for
161 detection (Ndong Ba et al., 2019).

162

163 *Assessment of oxidative lipid damage - quantification of 8-isoprostane*

164 Isoprostanes are a family of eicosanoids of non-enzymatic origin, produced by the oxidation of
165 the phospholipids tissue by ROS. The 8-isoprostane competitive ELISA assay (Abcam
166 ab175819[®], Cambridge, UK) was used for the determination of 8-isoprostanes in cells,
167 according to manufacturer's protocol.

168

169 *Assessment of protein oxidative damage - quantification of carbonylated proteins*

170 Protein carbonylation is one of the irreversible oxidative modifications of proteins. The method
171 based on the reaction of 2,4-Dinitrophenylhydrazine (DNPH) with carbonylated proteins. The
172 DNP-hydrazones formed can be quantified by measuring its absorbance at 375 nm. Levels of
173 carbonyl groups were determined using Protein Carbonyl Content Assay kit (Abcam
174 ab126287[®], Cambridge, UK) according to manufacturer's protocol (Ndong Ba et al., 2019).

175

176 2.8. Statistical analysis

177 Toxicity test results are normalized to the control, and are presented as an average of the 4
178 replicates for cytotoxicity and inflammation tests, and as an average of the 3 replicates for
179 oxidative stress tests, considering the standard deviation. The nonparametric Mann-Whitney U
180 test was performed to compare the values obtained with the controls. Significant differences
181 are reported with values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

182 Correlations were studied using the Spearman coefficient as the normality of the distribution
183 was not verified (Shapiro-Wilk test, $p < 0.001$) for most of the variables. Two levels of

184 significance were considered, $p < 0.05$ and $p < 0.001$, allowing conclusions about correlation
185 with 95% and 99.9% confidence, respectively. In addition, the data were also examined using
186 the Principal Component Analysis (PCA), which was performed considering $n=84$ samples
187 including triplicates (7 PM samples x 4 fractions x 3 replicates).

188

189 3. Results

190 3.1. PM_{2.5-0.3} chemical composition

191 First of all, in relation with the procedure required to prepare samples for chemical analysis and
192 to prepare the four fractions of PM_{2.5-0.3}, it can be noticed that the ion composition was the same
193 in the WSE and the whole PM_{2.5-0.3}. As well, the PAHs composition was similar for the PM_{2.5-}
194 _{0.3} fraction and OE. No water-soluble and no PAH compound have been detected in the dPM
195 samples.

196 Table 1 gathers the average composition of the 7 PM_{2.5-0.3} samples. Total carbon (11.3 - 19.0%),
197 NO₃⁻ (8.1 - 14.3%), SO₄²⁻ (4.0 - 8.3%), Cl⁻ (0.4 - 8.6%), Ca²⁺ (3.7 - 5.8%), Na⁺ (0, 4 - 5.0 %),
198 NH₄⁺ (1.3 - 2.3 %), Fe (3.1 - 8.7 %) and Al (1.2 - 3.4 %) represented major compounds of the
199 particles. In the "Urban" samples (U-PM), these species explained between 52.6% (summer)
200 and 58.3% (winter) of the PM mass, compared to 51.7% in the "Traffic" PM (T-PM) and 57.5%
201 in the "Industrial" one (I-PM). Considering the urban site, NO₃⁻ ions were detected in higher
202 amount in spring than in other seasons, partly due to meteorological conditions favoring their
203 accumulation in the atmosphere (Favez et al., 2012;). NO₃⁻ ions were already shown to be the
204 preponderant species of secondary inorganic ions in the Region (Kfoury et al. 2016, Ledoux et
205 al. 2017). The highest proportion of NO₃⁻ is observed in the T-PM sample (14.3%), and this
206 could be related to the high concentration of NO_x observed at this site (Atmo Hauts-de-France
207 air quality network), which are well known to be converted in the atmosphere into nitrate
208 species (Seinfeld and Pandis, 2006). Na⁺ and Cl⁻ ions are known to be associated with marine

209 aerosols, whose contribution may be significant at the study area due to the proximity with the
210 English Channel-North Sea area. At the urban site, higher C content was detected in particles
211 sampled in autumn and winter. This could be explained by colder temperatures during these
212 seasons and therefore, a higher contribution of emissions related to residential heating using
213 fossil fuels. The metal element contents Al (1.2 - 3.4%) and Fe (3.1 - 8.7%) can be explained
214 by crustal and anthropogenic emissions, with however a significant contribution of industrial
215 emissions in samples collected at the industrial site (Kfoury et al., 2016). In particular, it should
216 be noticed that Fe and Mn contents were respectively 2.3 and 3.8 times higher in the I-PM
217 sample, compared to the U-PM urban samples. Fe, Mn, Zn, Pb, Cr, and Cd could be linked to
218 anthropogenic sources, especially to emissions from steelmaking and metallurgical activities
219 (Kfoury et al., 2016). Cu, Zn and Cr contents clearly reflect the influence of traffic emissions
220 (Gietl et al. 2010). In particular, the copper content at the T-PM site is 1.5 times higher than at
221 the urban site (U-Y-PM), and more than 3 times higher than at the I-PM site. Table S1 gathers
222 the chemical composition of the PM_{2.5-0.3} WSE. Among the considered metals, Mn appeared as
223 the most abundant element in the water-soluble fraction, far ahead of Sr and Ba, then Al, Cu,
224 Zn, Mo and Ni. Fe was only detected in the WSE of PM_{2.5-0.3} collected on the traffic and
225 industrial site (Table S1). Among the 16 priority PAHs listed by the United States
226 Environmental Protection Agency (USEPA), contents of dibenz[a,h]anthracene (5.9 - 11.6
227 µg/g), indeno[1,2,3-c,d]pyrene (3.2 - 12.2 µg/g), chrysene (2.2 - 13.2 µg/g),
228 benzo[b]fluoranthene (1.9 - 11.8 µg/g), benzo[g,h,i]perylene (2.1 - 9.6 µg/g) and
229 benzo[a]pyrene (2.0 - 9.4 µg/g) in PM were predominant. The other compounds (fluoranthene,
230 pyrene, benz[a]anthracene and benzo[k]fluoranthene) showed content levels below 5 µg/g,
231 while the 6 PAHs composed of 2 and 3 rings showed contents below 1 µg/g. For the urban site,
232 PAH contents were 3 to 4 times higher in autumn and winter than in spring and summer.
233 Between these two periods, the PAH profile (Figure S1) differed mainly by the content of

234 chrysene (contribution of wood combustion and coal distillation (Telesiński and Kiepas-Kokot,
 235 2021)) and benzo[b]fluoranthene (diesel vehicle emissions (Xing et al., 2022)). In parallel, the
 236 higher PAH contents were found for the industrial sample with a total content of 136.4 µg/g,
 237 which corresponds to a value between 2 and 8 times higher than that of the other sites. This
 238 observation was in agreement with other studies conducted in this urban site impacted by
 239 industrial emissions (Crenn et al., 2017; Landkocz et al., 2017). On the contrary, PAH contents
 240 were lower at the road-traffic site (17.8 µg/g).

241
 242 Table 1: Chemical composition (µg/g) of PM_{2.5-0.3} particles collected under urban influence –
 243 seasonal and yearly average (U-Sp, U-Su, U-A, U-W, U-Y), industrial influence (I), and traffic
 244 influence (T).

245

	U-Sp	U-Su	U-A	U-W	U-Y	T	I
Al	21810	27136	31719	34171	28026	11869	23800
As	12.2	12.1	17.3	16.1	13.9	10.0	10.0
Ba	272	343	399	339	331	932	285
Cd	6.8	6.2	5.6	6.2	6.3	10.0	10.0
Co	10.3	7.9	7.4	7.0	8.7	13.4	19.8
Cr	136	136	181	152	144	251	266
Cu	583	538	803	691	639	933	276
Fe	41179	40666	31331	43747	37530	38078	87549
Mn	1880	1984	1235	1922	1767	555	6692
Mo	24.0	30.5	40.2	30.7	30.3	35.9	22.3
Ni	137	111	105	95.9	116	59.5	467
P	1303	1882	1509	783	1345	979	1030
Pb	226	179	263	271	234	210	246
Sn	56.5	61.5	116	128	78.3	270	46.8
Sr	149	116	98.2	120	126	153.5	140.9
Ti	474	370	269	316	369	1631	1288
V	122	110	76	118	108	72.8	134
Zn	1259	1117	1335	1440	1293	1257	1841
Ca²⁺	52900	46200	34600	37800	44600	39667	37106
K⁺	4300	4500	4900	4700	4500	8053	6677
Mg²⁺	6800	6200	5200	6900	6500	2701	9307
Na⁺	49700	39600	29300	50100	43300	30620	41178

NH₄⁺	16800	15000	22900	21700	16400	14944	12497
Cl⁻	63200	45300	51000	86700	60900	38855	54882
NO₃⁻	126100	92900	80900	89900	100400	142872	116133
SO₄²⁻	70600	83400	72100	76600	75200	40013	53062
TC	113300	135700	190200	142500	142500	160000	149000
Naphthalene	0.2	0.3	0.4	0.4	0.2	0.2	1.1
Acenaphthylene	0.1	0.1	0.3	0.3	0.1	0.1	0.2
Acenaphthene	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fluorene	0.1	0.1	0.3	0.3	0.2	0.1	0.3
Phenanthrene	0.7	0.8	1.8	1.8	1.0	0.5	1.9
Anthracene	0.1	0.2	0.3	0.4	0.3	0.2	0.6
Fluoranthene	1.1	0.9	4.1	4.4	2.1	0.9	6.0
Pyrene	0.8	0.8	3.7	3.8	1.8	0.8	5.2
Benz[a]anthracene	0.6	0.4	0.4	5.5	1.9	0.7	10.1
Chrysene	2.2	2.2	11.7	13.2	5.4	2.6	20.5
Benzo[b]fluoranthene	2.8	1.9	11.0	11.8	5.3	2.3	20.0
Benzo[k]fluoranthene	0.7	0.5	3.2	3.5	1.7	0.6	7.1
Benzo[a]pyrene	2.6	2.0	9.0	9.4	3.9	1.7	15.5
Indeno[1,2,3-c,d]pyrene	3.9	3.3	12.2	10.2	6.1	4.2	16.9
Dibenz[a,h]anthracene	6.7	5.9	11.3	11.6	8.9	2.6	18.2
Benzo[g,h,i]perylene	2.6	2.1	9.6	8.6	4.4	3.0	12.8
TPAH	25.1	21.5	79.3	85.1	43.2	17.8	136.4

246

247

248 3.2. Inflammation

249 PM and dPM fractions were shown to induce an increase in IL-6 secretion, significantly higher
 250 than the OE fraction, while the WSE fraction seemed to have no effect (Figure S2). Indeed, a
 251 significant increase in IL-6 concentration was observed for all PM and dPM samples (Table
 252 S2). Concerning the organic extracts (OE), 5 samples caused significant increases in IL-6
 253 secretion while no effect was observed with the water-soluble extracts (WSE). These results
 254 are in line with numerous works that have previously demonstrated the development of an
 255 inflammatory response after exposure of lung cells to whole PM samples collected under
 256 different influences and in different locations (Cachon et al., 2014; Dieme et al., 2012; Dong et
 257 al., 2019; Xu et al., 2018).

258

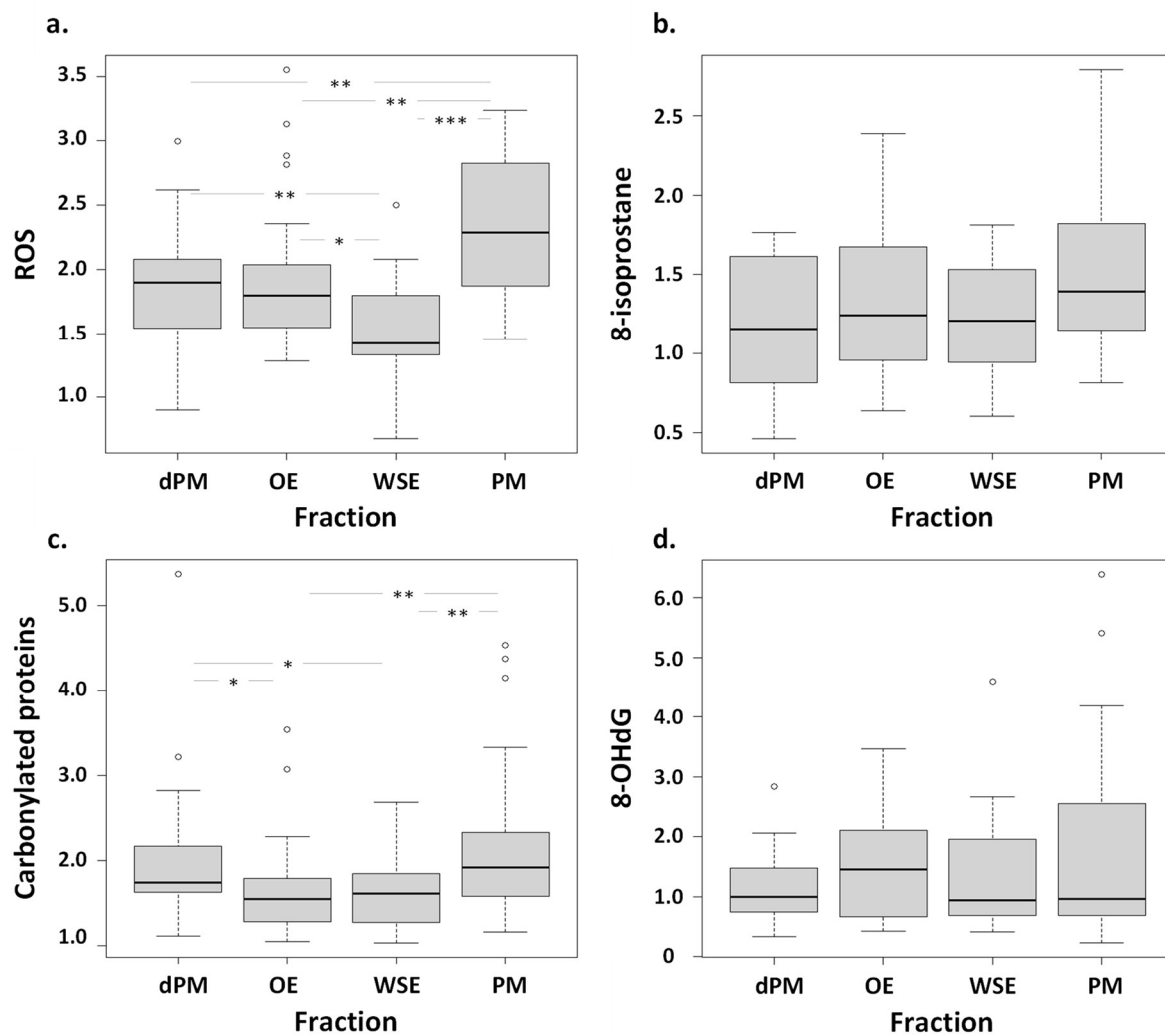
259 3.3. Oxidative stress

260 3.3.1. Reactive oxygen species

261 Regardless the site typology, considering the average response associated to the 4 fractions,
262 PM_{2.5-0.3} have significantly induced ROS accumulation in cells at higher levels than the other
263 fractions (Figure 1-a). Looking into details, ROS production significantly increased ($p < 0.05$)
264 following BEAS-2B exposure to all fractions of the PM_{2.5-0.3} samples, except for some water-
265 soluble extracts (U-A, U-Y and U-Sp) (Figure 2-a). The ROS overproduction was more
266 pronounced for PM collected under urban and road traffic influences, and for the organic
267 fraction of urban samples corresponding to cold seasons that appeared to be more concentrated
268 in PAH (79 and 85 $\mu\text{g/g}$ PM for U-A-OE and U-W-OE respectively).

269

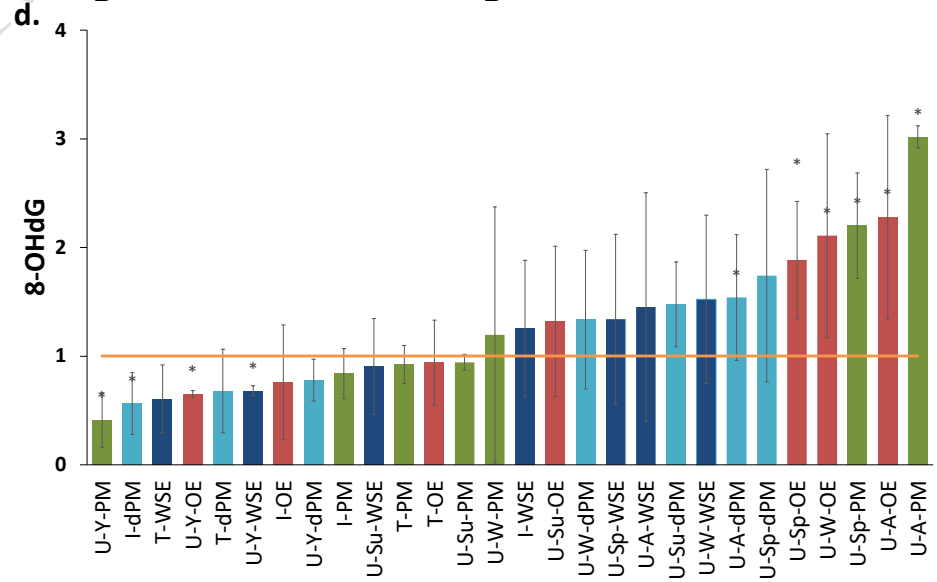
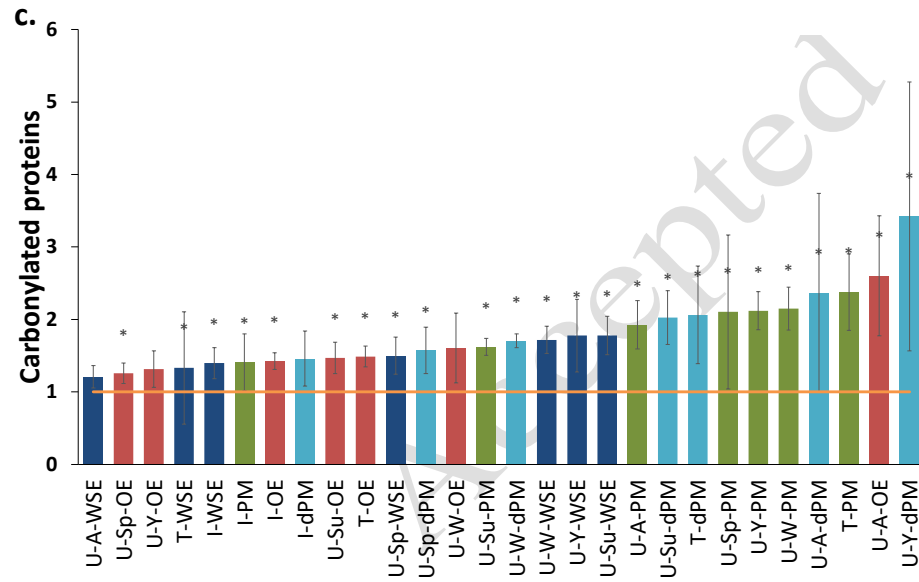
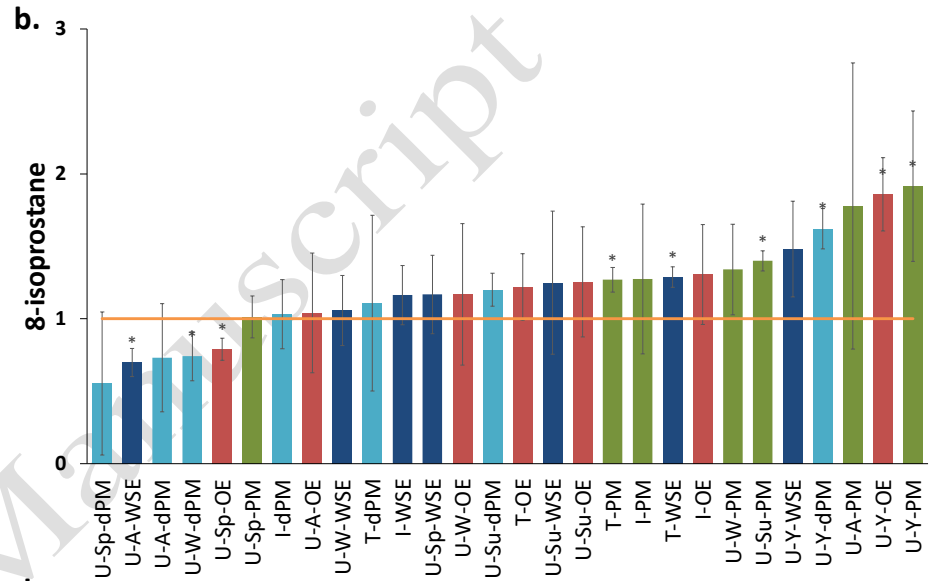
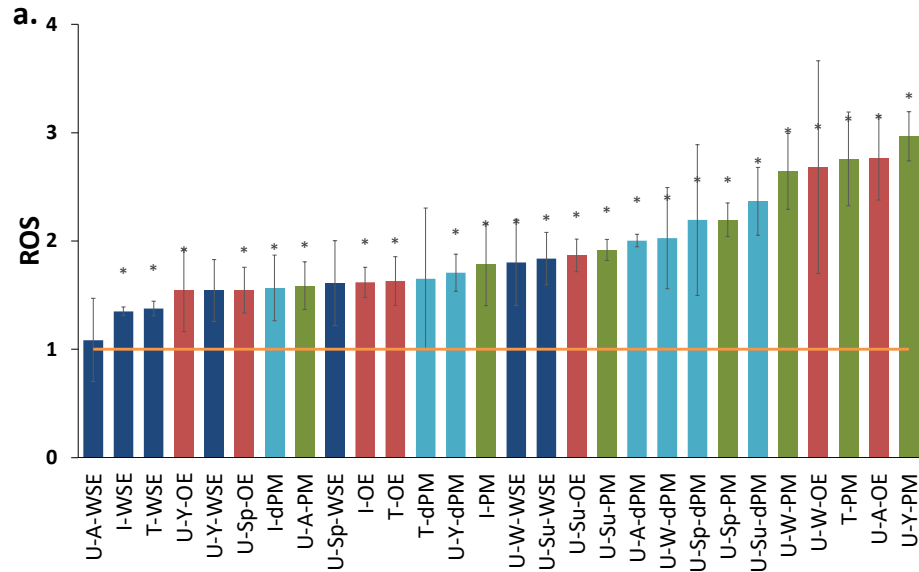
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270

271 Figure 1: Concentration of (a) ROS, (b) 8-isoprostane, (c) carbonylated proteins and (d) 8-
 272 OHdG, reported to total protein and control in BEAS-2B cells exposed to different fractions of
 273 PM (dPM, OE, WSE, PM) at 24 µg/cm². Results are represented by median and interquartile
 274 ranges (n=21). Mann-Whitney test: **p* <0.05; ***p* <0.01; ****p* <0.001.

275



277 Figure 2: Relative concentration of (a) ROS, (b) 8-isoprostane, (c) carbonylated proteins and
278 (d) 8-OHdG, reported to total protein and control in BEAS-2B cells exposed to different
279 fractions of PM collected under urban influence–seasonal and yearly average (U-Sp, U-Su, U-
280 -A, U-W, U-Y), industrial influence (I), and traffic influence (T), at the dose 24 $\mu\text{g}/\text{cm}^2$,
281 represented by means and standard deviations over three biological replicates. Mann-Whitney
282 U-test vs. controls; * $p < 0.05$ (Color code: green =PM; red=OE; light blue=dPM, dark
283 blue=WSE).

284

285 3.3.2. 8-isoprostane

286 Whatever the fraction considered, there was no significant difference in the 8-isoprostane
287 response after cell exposure (Figure 1-b). It is to note that U-Y was the sample inducing higher
288 concentration of 8-isoprostane, followed by T-PM sample and its water-soluble extract (T-
289 WSE). Whole $\text{PM}_{2.5-0.3}$ appeared to be the fraction that is the most involved in the increase in
290 8-isoprostane. The industrial sample (I-PM) did not appear to have any effect on the 8-
291 isoprostane generation (Figure 2-b).

292

293 3.3.3. Carbonylated Proteins

294 PM and dPM fractions induced once again the stronger oxidative damages compared to OE and
295 WSE (Figure 1-c). The concentration of carbonylated proteins significantly increased ($p < 0.05$)
296 after exposure of BEAS-2B cells to the majority of the PM fractions. Indeed, only 4 samples
297 (U-A-WSE, U-Y-OE, I-dPM and U-W-OE) did not induce a significant increase of
298 carbonylated proteins concentration (Figure 2-c).

299

300 3.3.4. 8-OHdG

301 In average, there was no significant difference between the 8-OHdG concentration after cell
 302 exposure to the different fractions (Figure 1-d). However, the PM fractions and OE would
 303 appear to be the more involved in DNA damages: U-A-PM, U-Sp-PM and U-A-OE, U-W-OE,
 304 and U-Sp-OE caused a significant increase in 8-OHdG formation (Figure 2-d). The samples
 305 collected under the influence of road traffic and industrial emissions did not exhibit any
 306 response whatever the fraction considered, and the same observation was made for the urban
 307 samples collected in spring and summer.

308

309 3.4. Correlation analysis

310 The ROS concentrations showed weak correlations with the concentration data of 8-OHdG
 311 ($r=0.28$), 8-isoprostane ($r=0.26$), and IL-6 ($r=0.33$) (Table 2). On the other hand, ROS
 312 concentrations were strongly correlated with carbonylated protein concentrations ($r=0.67$).
 313 Carbonylated proteins also showed weak correlations with IL-6 ($r=0.29$) and moderate
 314 correlations with 8-isoprostane ($r=0.44$). Concerning correlation with the chemical composition
 315 (Table S3), positive correlations were found between ROS accumulation and several elements
 316 (Cd: $r=0.43$, $p < 0.05$; Sr: $r=0.55$, $p < 0.01$), protein oxidation (Al: $r=0.42$, $p < 0.05$; As: $r=$
 317 0.50 , $p < 0.05$; Cu: $r=0.45$, $p < 0.05$; Pb: $r=0.43$, $p < 0.05$). Positive correlations were also
 318 evidenced between cell membrane alteration and some elements (Al: $r=0.54$, $p < 0.01$; As:
 319 $r=0.48$, $p < 0.05$).

320

321 Table 2: Correlation coefficient (Spearman) between cell parameters (n=96). Only correlations
 322 with $p < 0.05$ are reported (** $p < 0.01$; *** $p < 0.001$).

	IL-6	ROS	Carbonylated proteins	8-OHdG	8-isoprostane
IL-6		0.33**	0.29**		
ROS	0.33**		0.67***	0.28**	0.26

Carbonylated proteins	0.29**	0.67***	0.30**	0.44***
8-OHdG		0.28**	0.30**	
8-isoprostane		0.26	0.44***	

323

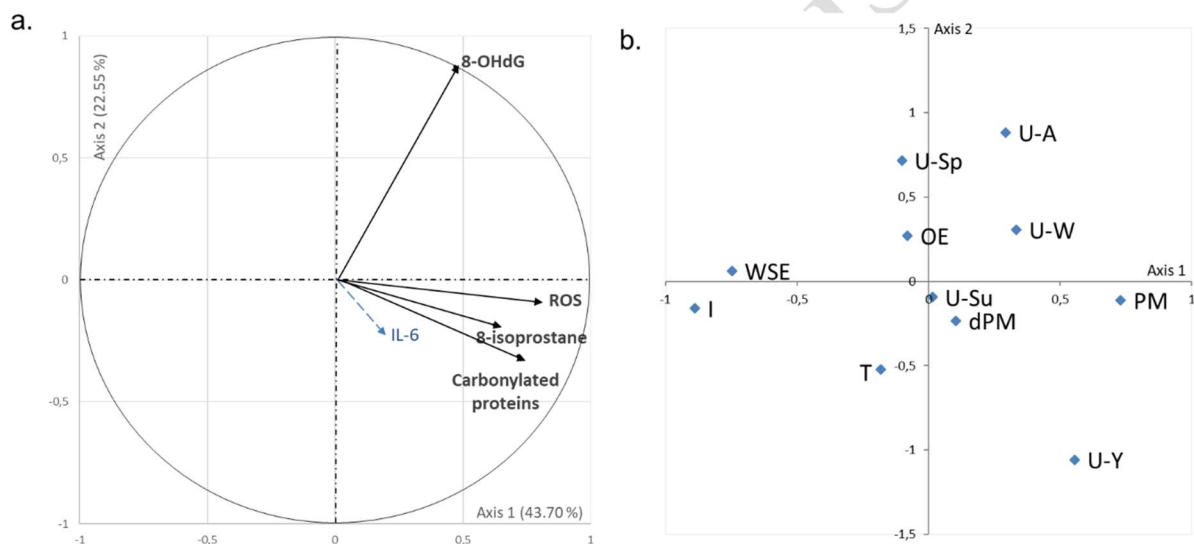
324 3.5. Principal component analysis

325 PCA was performed considering 84 samples including triplicates. The first axis explained about
326 44% of the variability of the data, and appeared to be strongly related to the amount of ROS, 8-
327 isoprostane and carbonylated proteins (Figure 3-a). Thus, this axis is clearly related to the
328 capacity of the samples to induce damage to the cell membrane and proteins. Axis 2 explained
329 about 22% of the variability of the data, and appeared to be related to DNA damages. Note that
330 the 8-OHdG variable appeared to be totally uncorrelated with the other oxidative stress
331 parameters. Similarly, IL6 considered as supplementary variable, showed poor relationship
332 with the oxidative damage parameters in this plan (1,2) explaining 66% of the variability in the
333 data. The 84 samples were categorized according to their influence and the fractions they belong
334 to. The barycenters relative to the 4 fractions of the same influence (T, I, U-Sp U-Su, U-A U-
335 W and U-Y) and those relative to all the influences of the same fraction (PM, dPM, OE and
336 WSE) were projected in the (1,2) plane (Figure 3-b) in order to highlight links between the
337 latter and the 4 oxidative stress parameters. The axis 1 which is associated to damage to the
338 membrane and proteins and ROS overproduction, shows a ranking WSE < OE and dPM < PM
339 evidencing the capacity of the latter to induce the more cell damages and WSE to induce less
340 cell damages. It appears in agreement with the previous observations made on each of the
341 parameters (Figures 1 and 2).

342 If we adopt the same reasoning, focusing now on the influences, it appears that the industrial
343 influence appears to be the less linked to cell damages and ROS production in comparison with
344 the other influences.

345 Another interesting observation can be made. In this work, we considered 4 seasonal samples
346 and an average annual sample (made up of 25% of each seasonal sample) for the Urban site. It
347 appears that the projection of the characteristics of this average sample (U-Y) is not in the center
348 of those of the seasonal samples. This illustrates that the response of cells exposed to particles
349 from the annual sample is not an average of the ones observed after cell exposure to particles
350 from seasonal samples. This tends to show and prove that complex interactions could take place
351 between the compounds of these samples, and that these interactions would be able to modulate
352 the cell responses to oxidative stress, and the resulting damages.

353



354

355

356 Figure 3: (a) PCA performed considering the 4 parameters ROS, 8-OHdG, carbonylated
357 proteins, and 8-isoprostane, on n=84 samples - (b) Representation of the barycenters of the
358 different fractions or influences in the (1,2) plane.

359

360 To go further, a hierarchical classification on the principal components was performed in order
361 to statistically evidence group samples and highlight their specificity (depending on sampling
362 sites, fractions and cellular parameters) (Table 3). Three groups are highlighted (the

363 associations mentioned below are statistically significant at $p < 0.05$): The first group ($n=28$) is
364 mostly related to the industrial influence ($p < 0.01$) and to the water-soluble fractions of the
365 different PM samples. It is characterized by low values for ROS, carbonylated proteins and 8-
366 isoprostane parameters ($p < 0.001$ for these three parameters). With a lower statistical weight,
367 low values of parameters 8-OHdG ($p=0.06$) and IL-6 ($p=0.1$) seem to be associated with this
368 group. The second group ($n=45$) is characterized, by high values of ROS ($p < 0.01$) and 8-
369 isoprostane ($p < 0.01$) and low values for 8-OHdG. This class is strongly represented by the
370 urban influence, especially U-Su ($p < 0.01$). The industrial influence do not statistically belong
371 to this group ($p < 0.05$). The third and last group ($n=11$) is characterized by high ROS values (p
372 < 0.001) and damage parameters (8-isoprostane and especially 8-OHdG ($p < 0.001$)). The
373 samples associated with this third group are responsible for more damages compared to the
374 other samples and cause a more marked disruption of cell function. In particular, the U-A
375 influence stands out. The WSE fraction is statistically less associated with this 3rd group (p
376 < 0.05) showing that WSE is not the most involved fraction of $PM_{2.5-0.3}$ in ROS overproduction.
377 The test value for the PM fraction also seems to show that this 3rd group would be related to
378 the PM fraction ($p = 0.13$). The study of the barycentres of the fractions (Figure 3-b) tends to
379 support this hypothesis.

380

381 Table 3: Test values obtained for the "Fraction" and "Influence" categories and for the
382 quantitative variables associated to each of the classes identified after Principal Component
383 Analysis followed by Hierarchical Cluster Analysis. The test value allows to highlight the
384 characteristics that differ significantly in the class compared to the whole samples. Only the
385 most remarkable test values have been reported with their significance level ($*p < 0.05$; $**p$
386 < 0.01 ; $***p < 0.001$). Values in italic are close to -2 and +2 corresponding to $p < 0.05$.

387

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Category		Class 1	Class 2	Class 3
Fraction	WSE	2.50*		-2,15*
	PM	-2.18*		1,51 (<i>p</i> =0,13)
Influence	I	3.05**	-2.02*	
	U-A		-1.75 (<i>p</i> =0.08)	2,05*
	U-Su	-2.02*	2.92**	
	U-Y		1.59 (<i>p</i> =0.11)	
cellular parameter	ROS	-6.04***	2.93**	4,11***
	carbonylated proteins	-4.70***		4,82***
	8-isoprostane	-4.34***	2.62**	2,20*
	8-OHdG	-1.89 (<i>p</i> =0.06)	-2.08*	5,71***
	IL-6	-1.66 (<i>p</i> =0.10)		

392

393

394 4. Discussion

395 Regardless the site typology and the season, cell exposure to particles as a whole (PM) and
396 dPM caused a significant increase (*p* <0.001; Figure S2) in the secretion of the pro-
397 inflammatory cytokine interleukin-6. To a lesser extent, organic extracts gave the same effect,
398 whereas exposure to water-soluble extracts showed no effect. Several studies have already
399 reported the greater ability of particulate samples to induce an inflammatory response compared
400 to their water-soluble (Shang et al., 2020) or organic extracts (Badran et al., 2020b; Verdin et
401 al., 2017). As observed in this work, the literature also reported no inflammatory response
402 following exposure of BEAS-2B cells to water-soluble and/or organic extracts (Chowdhury et
403 al., 2018; Honda et al., 2017). A recent study showed that particles deprived of their organic or
404 water-soluble fractions were able to induce inflammation (TNF- α) to a greater degree than
405 organic and water-soluble fractions (Gali et al., 2019). Our observations are in line with these
406 previous findings, pointing out the important contribution of the particulate skeleton in

407 triggering the inflammatory response. The latter has been widely described in the literature as
408 intimately linked to the initiation of cellular oxidative stress, including a strong link between
409 the secretion of pro-inflammatory interleukins and the accumulation of ROS (Dergham et al.,
410 2015; Jiang et al., 2020; Symon et al., 2020; Xu et al., 2018).

411
412 In this study, attention was also paid on the possible development of a cellular oxidative stress.
413 It appeared that BEAS-2B cells exposed to the different PM_{2.5-0.3} samples/fractions exhibited
414 ROS accumulation under all exposure conditions, whatever the fraction considered (PM, OE,
415 WSE, dPM). In the literature, several studies had already demonstrated the ability of airborne
416 particulate extracts to generate ROS accumulation in cells, either after exposure to their organic
417 (Libalova et al., 2018; Rodríguez-Cotto et al., 2015; Yan et al., 2017) or water-soluble fractions
418 (Q. Liu et al., 2020; Shuster-Meiseles et al., 2016; Xu et al., 2018; Yan et al., 2017). Several
419 recent studies have highlighted the significant contribution of metals in ROS generation,
420 especially the elements As, Bi, Ce, Cd, Cr, Co, Cu, Mo, Ni, Pb, Sb, Se, Sn, V and Zn (Crobeddu
421 et al., 2020; Das et al., 2021; Fang et al., 2020). The relatively high contents of PAHs, water
422 soluble ions and elements (Cr, Co, Cu, Sn, NO₃⁻, Al, Cu, Pb, Sn and V) in the "Traffic" and the
423 different "Urban" samples could also explain their strong capacity to generate ROS in cells.
424 This is also the case for the sample collected under industrial influence (I), particularly rich in
425 PAH (136 µg/g) and in Co, Cr, Mn, Ni and V (Table 1). It is to note that a preponderant effect
426 in ROS accumulation was shown for the particulate fraction comparing to the moderate effect
427 obtained with water-soluble and organic extracts.

428
429 Concerning the cell membrane damages assessed by the 8-isoprostane quantification, only a
430 trend could be revealed for PM_{2.5-0.3} samples. However, recent studies have demonstrated the
431 ability of airborne particles to alter the cell membrane. Badran et al, (2020) showed that

432 particulate samples induced a more pronounced membrane alteration than their organic
433 extracts, which also appeared to be the trend here, although the difference was not significant.

434

435 Focusing on the DNA damages, 8-OHdG did not show significant difference between most of
436 the different PM_{2.5-0.3} fractions. However, the effect of PM and OE fractions seemed to be
437 predominant. This result is confirmed by several statistical analyses, in particular the PCA and
438 classification (Figure 3; Figure S3), which showed a clear discrimination between the effect of
439 the particulate fractions and the organic and then water-soluble extracts. Results are in
440 agreement with works that have demonstrated the ability of the particles themselves (Dergham
441 et al., 2015, 2012; Leclercq et al., 2018) or their organic extracts (Abbas et al., 2019; Badran et
442 al., 2020b; Billet et al., 2018) to induce oxidative DNA damages, by 8-OHdG determination.
443 Similarly, other authors have also highlighted the genotoxic effects triggered by atmospheric
444 fine particles, notably by the phospho-H2AX assay (Borgie et al., 2015), and the assessment of
445 DNA damage via the comet (Gualtieri et al., 2011; Platel et al., 2020).

446

447 Concerning carbonylated proteins, our observations are in agreement with Ndong Ba et al,
448 (2019) who demonstrated, the ability of atmospheric particles to trigger cellular protein
449 oxidation on the BEAS-2B cell line. Recently, Badran et al, (2020) also found that PM could
450 induce more oxidative damage to proteins than their organic extracts did.

451

452 The correlation analysis has revealed a weak positive correlation ($r=0.33$, $p < 0.01$) between
453 cellular ROS concentration and IL-6 secretion. This hyper accumulation of ROS in the cell is
454 often described as being associated with alterations in biological macromolecules (Cachon et
455 al., 2014; Cui et al., 2020; Dergham et al., 2012; Dieme et al., 2012; Van Den Heuvel et al.,
456 2016; Yan et al., 2017). In our work, positive correlations between intracellular ROS

457 accumulation and cell membrane alterations ($r=0.26$, $p < 0.0128$), protein oxidation ($r=0.67$, p
458 < 0.001) and oxidative DNA adduct formation ($r=0.28$, $p < 0.006$) were found. Indeed, the
459 predominant impact of the particulate fraction (as a whole or free of organic and water-soluble
460 fractions) compared to the other fractions was observed on the irreversible oxidation of proteins
461 by the determination of carbonylated proteins ($p < 0.01$).

462
463 In this study, positive correlations were found between several elements and oxidative stress
464 biomarkers. Numerous elements such as Al, As, Cd, Cr, Co, Cu, Pb, Sr, V, Zn, etc are known
465 to play a major role in ROS generation and oxidative damages (Crobbeddu et al., 2017; Das et
466 al., 2021; Shuster-Meiseles et al., 2016). Indeed, Al and Pb have already been described to be
467 associated with redox reactions (Verstraeten et al., 2008) while Cr, As and Cd, carcinogenic to
468 human elements (IARC, 2012) can induce the overproduction of ROS in cells and thus
469 contribute to plasma membrane alterations as well as to protein and DNA damages (Chen and
470 Lippmann, 2009; Fortoul et al., 2015). Exposure of BEAS-2B cells to TiO_2 has been shown to
471 induce ROS generation, increased 8-OHdG concentrations, lipid peroxidation (Bhattacharya et
472 al., 2009; Gurr et al., 2005). ZnO has been previously described to induce ROS overproduction,
473 protein oxidation, DNA damages, lipid peroxidation (Huang et al., 2010; Saliani et al., 2016;
474 Singh et al., 2020; Syama et al., 2013; Zoufan et al., 2020).

475
476 In this work, correlations were found between the contents of some light PAHs in $PM_{2.5-0.3}$ and
477 oxidative damage to proteins (acy: $r=0.33$, $p < 0.05$; ace: $r=0.42$, $p < 0.01$), cell membranes
478 (ace: $r=0.56$, $p < 0.001$; phe: $r=0.42$, $p < 0.001$; ant: $r=0.37$, $p < 0.01$; fla: $r=0.36$, $p < 0.05$;
479 pyr: $r=0.35$, $p < 0.05$) as well as to DNA (nap: $r=0.37$, $p < 0.05$; ace: $r=0.46$, $p < 0.01$). It has
480 already been shown in the literature that these PAHs are capable of inducing ROS
481 overproduction, lipid peroxidation and DNA damage (Ma et al., 2019; Machado et al., 2014;

482 Vuchetich et al., 1996; Wang and Zheng, 2008). In addition, PAHs in PM_{2.5} have previously
483 been associated with the induction of oxidative stress and inflammation in human bronchial cell
484 models (Boublil et al., 2013; Dergham et al., 2015, 2012; Leclercq et al., 2018). Similarly, the
485 particulate and organic fractions of the traffic-influenced sample (T) and the urban samples
486 collected during cold seasons (U-A and U-W) were found to be among the most toxic across
487 all tests performed. For the urban samples, the total PAH content of these samples (79 and 85
488 µg/g for U-A and U-W respectively) and trace elements such as Al, As, Cd, Cr, Co, Cu, Pb, Sr,
489 and Zn could be involved in the level of cell response. Indeed, PAH contents were 3 to 4 times
490 higher in particles collected during cold seasons compared to warm seasons (U-W=85; U-
491 A=79; U-Sp=25 and U-Su=21 µg/g PM).

492
493 On the basis of the contents of organic and inorganic compounds, it appeared surprising that
494 the sample collected under industrial influence (I), whether for its particulate fraction or its
495 water-soluble and organic extracts, was found to show a cell response positioned among the
496 least toxic samples. This is the case for all the parameters of oxidative stress evaluated (also
497 highlighted through the PCA/classification). Indeed, this I-PM sample exhibits the highest
498 contents in PAHs (136 µg/g) (Table 1; Figure S1) and in some trace elements as Cd, Cr, Co,
499 Pb, Sr and Zn (Table 1), known for exerting toxic effects (Guo et al., 2022; Liu et al., 2022).
500 However, the same observation had already been made by Dergham et al, (2012), who had also
501 collected a sample under industrial influence at the same site. Although significantly higher in
502 organic compounds and metallic elements, the PM_{2.5-0.3} sample collected under the same
503 conditions was found to induce a lower cell response than traffic and urban influence to
504 oxidative stress parameters. This finding could be explained by the richness of these industrial
505 samples in Fe (more than twice as concentrated as the other samples) and in particular by the
506 presence of the Fe₃O₄ form in the collected PM (Heliot, 2018; Kfoury et al., 2016; Laversin,

507 2006) which is related due to emissions from the nearby steel complex (Hleis et al., 2013).
508 Indeed, it was shown that the Fe₃O₄ form was found in particles emitted at the blast furnace and
509 steel mill. Complementary tests on BEAS-2B cells were carried out by considering reference
510 samples, and results showed that Fe₃O₄ sample, when considered pure or in mixture with other
511 metal oxide compounds as Mn₂O₃, ZnO and Fe₂O₃, induced oxidative damages in a lesser extent
512 than other metal oxides tested. Fe₃O₄ is a compound formed at high temperature during in the
513 industrial process. It associates Fe(II) and Fe(III) and is less oxidized than Fe(III) oxide. This
514 observation tends to show that Fe₃O₄ would exert an inhibiting effect regarding oxidative stress.
515 A recent study conducted on A549 cells showed that following exposure to the Mn/Pb mixture,
516 cell viability was 45%, and was higher in the case of the Fe/Mn/Pb mixture (69%), which would
517 mean that the presence of iron would inhibit the cytotoxicity (Yuan et al., 2019). We also
518 showed that the PM_{2.5-0.3} sample collected under industrial influence (I-PM) have a high
519 manganese content (6 692 µg/g) almost 4 times higher than that of the urban sample (Table 1).
520 However, it was shown through tests performed on the synthetic samples that Mn₂O₃ could
521 cause high ROS production and significant increases in other oxidative stress parameters.
522 Studies have proven the role of Mn₂O₃ in inducing oxidative stress through protein oxidation
523 and lipid peroxidation (Ognik et al., 2019; Sun et al., 2013). Another recent study showed that
524 exposure of A549 cells to 79 µg/L Mn²⁺ could decrease their cell viability (Yuan et al., 2019).
525 In our case, and considering the oxidative stress results for the industrial sample (I-PM), it can
526 be suggested that the effect of manganese is limited due to the presence of Fe₃O₄ in the PM
527 sample.

528

529 Interactions between organic compounds, such as PAHs or even HULIS-type organic
530 compounds (although not analyzed in our work) with metallic elements cannot be excluded. It
531 can be suggested that synergistic interactions between metals and organic compounds

532 contribute to the oxidative power of organic compounds such as quinones, which oxidize
533 cellular antioxidants and subsequently generate H₂O₂. The latter can then be converted to OH
534 radical by metal species, through the Fenton reaction (Wang et al., 2018). This result is in
535 agreement with those obtained in other recent studies (Al Hanai et al., 2019; Xu et al., 2020).
536 This type of interaction between PM compounds could also be considered to explain the higher
537 level of oxidative stress for PM_{2.5-0.3} samples at the urban site in the cold season (U-A-PM and
538 U-W-PM), which are richer in PAHs than the samples collected in spring and summer (U-Sp-
539 PM and U-Su-PM).
540 Finally, the four PM fractions and the three studied influences were able to induce oxidative
541 stress damages, with different levels of magnitude and different target (membrane DNA,
542 proteins). Oxidative stress is considered as the first mechanism involved in the PM toxicity on
543 cells and consequently each PM fraction can be an inducer of the whole PM toxicity.

544

545 **5. Conclusion**

546 The main objective of this study was to discriminate the effect of different PM_{2.5-0.3} fractions
547 from various origins on the induction of oxidative stress and related damages after exposure of
548 BEAS-2B cells. The results showed that all fractions tested induced oxidative stress. The PM<sub>2.5-
549 0.3</sub> particles were able to trigger the overproduction of ROS and cause protein damage at higher
550 levels than the other fractions. The predominant effect of the particulate fraction on these
551 parameters (ROS and carbonylated proteins) is confirmed by the results obtained on the dPM
552 sample, whose response was higher than that measured with the organic and water-soluble
553 extracts. Correlation analysis showed that ROS concentrations were correlated with
554 inflammation (IL-6; r=0.33) and with the three oxidative stress-related injury parameters: weak
555 correlations with 8-OHdG (r=0.28) and 8-isoprostane (r=0.26) concentrations, but strong
556 correlations with carbonylated protein concentration (r=0.67). On the other hand, oxidative

557 stress parameters showed weak or moderate correlations with some chemical compounds,
558 whether major and trace metal elements or PAHs. This tends to illustrate the complexity of
559 interactions between PM constituents, and potentially the existence of additive, synergistic and
560 antagonistic effects, which may explain the observed results. Principal component analysis
561 (PCA) showed a correlation between the amount of ROS detected and the 8-isoprostane and
562 carbonylated protein parameters, whereas 8-OHdG appeared to be completely uncorrelated
563 with the other oxidative stress damage parameters. Sample collected under industrial influence
564 appears to be the less linked to cell damages and ROS production in comparison with the other
565 influences. The classification results confirmed the ability of the particulate fractions to induce
566 the most damage at the protein and cell membrane level.

567 In order to better understand the synergistic and antagonistic effects between metals and/or
568 organic compounds, it would be interesting to repeat the same experiments on synthetic
569 samples, by preparing combinations of metals, carbon black, and by including individual
570 organic compounds in these mixtures, especially quinones. To go further, it could be relevant
571 to also study the effect of dose, by preparing synthetic samples with different mass proportions
572 of the compounds.

573

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579 (PMCO) for the funding of her PhD. PM_{2.5-0.3} from the urban site, and from the industrial and
580 traffic sites were collected and characterized within the framework of the National Research
581 Program for Environmental and Occupational Health: Projects "Biomarkers of toxicity of

582 particulate air pollution influenced by age, PACATOX" (ANSES, 2014) and "Impact of PM_{2.5}
583 varying according to their source (Road traffic - Industries) on respiratory health. In vitro
584 approach (TRIPLER)" (ANSES, 2016).

585

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