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## **Lung anti-oxidant depletion: a predictive indicator of cellular stress induced by ambient fine particles**

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Running title: oxidative potential of PM<sub>2.5</sub>.

Abbreviations: OP: oxidative potential, PM<sub>2.5</sub>: particulate matter with an aerodynamic diameter < 2.5 μm; NCI-H292: human bronchial epithelial cell line; IL-8: interleukin 8; PAH: polycyclic aromatic hydrocarbons, HPAEC, human pulmonary artery endothelial cells, Traf: Traffic, BB: biomass burning, AA: ascorbic acid, GSH: glutathione.

## 2 **Abstract**

3 Regulations on ambient particulate matter (PM) are becoming more stringent due to adverse  
4 health effects arising from PM exposure. PM-induced oxidant production is a key mechanism  
5 behind the observed health effects and is heavily dependent on PM composition. Measurement  
6 of the intrinsic oxidative potential (OP) of PM could provide an integrated indicator of PM  
7 bioreactivity, and could serve as a better metric of PM hazard exposure than PM mass  
8 concentration. The OP of two chemically-contrasted PM<sub>2.5</sub> samples was compared through four  
9 acellular assays and OP predictive capability was evaluated in different cellular assays on two  
10 *in vitro* lung cell models. PM<sub>2.5</sub> collected in Paris at site close to the traffic exhibited a  
11 systematically higher OP in all assays compared to PM<sub>2.5</sub> enriched in particles from domestic  
12 wood burning. Similar results were obtained for oxidative stress, expression of anti-oxidant  
13 enzymes and pro-inflammatory chemokine in human bronchial epithelial and endothelial cells.  
14 The strongest correlations between OP assays and cellular responses were observed with the  
15 antioxidant (ascorbic acid and glutathione) depletion (OP<sup>AO</sup>) assay. Multivariate regression  
16 analysis from OP daily measurements suggested that OP<sup>AO</sup> was strongly correlated with PAH  
17 at traffic site while it was correlated with potassium for the domestic wood burning sample.

18 **Keywords:** PM<sub>2.5</sub>, lung, oxidative potential, oxidative stress, traffic, biomass burning

19

20

## 21 **Introduction**

22 Three decades of extensive epidemiological and experimental investigations have  
23 demonstrated the impact of air quality on health and the key role played by fine airborne  
24 particles<sup>1</sup>. These PM<sub>2.5</sub> (Particulate Matter with an aerodynamic diameter below 2.5µm), due to  
25 their small size and chemical composition, have been associated with a range of health effects  
26 both within the respiratory tract and in a broader systemic context<sup>2</sup>.

27 While policies have been implemented to reduce exposure to PM, the efficiency of these  
28 policies is hindered by a lack of knowledge regarding the involvement of individual particle  
29 components in specific health effects. Indeed, the World Health Organization, in the “Review  
30 of evidence on health aspects of air pollution” published in 2013, underlines that several  
31 components contribute to the health effects induced by PM<sub>2.5</sub>, but indications are still  
32 insufficient to differentiate individual chemical components (or sources) that are more strictly  
33 related to specific health outcomes<sup>3</sup>. Actually, regulation of particles is typically based on PM  
34 mass measurement and does not take into account the chemical composition that drives particle  
35 toxicity. Particle composition can significantly change as a function of location and period of  
36 the year, with some components exhibiting low toxicity such as inorganic ions (sulfate,  
37 ammonium, nitrate) but contributing a significant proportion of total PM mass. The chemical  
38 complexity of PM makes it difficult to thoroughly characterize PM composition and to assess  
39 the contribution of individual components to health effects. Alternative indicators based on the  
40 mechanisms of PM toxicity, such as the oxidation potential (OP) of particles have been  
41 proposed to overcome this limitation.

42 Inhaled PM can initiate the production of reactive oxygen species (ROS) in target cells  
43 leading to oxidative stress<sup>4</sup>. This stress promotes an inflammatory response that should  
44 normally be transient but may be persistent in case of chronic exposure, and could induce some  
45 pathologies like asthma, chronic obstructive pulmonary disease and cardiovascular disease<sup>5</sup>.  
46 ROS production by PM is complex and involves both aerosol surface properties and chemical  
47 composition. In particular, metals and some organic compounds have been shown to contribute  
48 substantially to PM-induced ROS generation<sup>6</sup>.

49 Generation of ROS by PM is responsible for the depletion of antioxidants present in the  
50 respiratory tract lining fluid (RTLFL) covering the airways<sup>7</sup>. Furthermore, PM is able to generate  
51 ROS indirectly in cells by activation of different pathways. The measurement of the OP of  
52 particulate samples, by its integrative nature, could represent a new unifying metric to assess  
53 PM exposure in a more realistic way<sup>8</sup>. Different methods have been developed to measure the  
54 OP of ambient and engineered particles by measuring the consumption of antioxidants or the

55 oxidation of biomolecules<sup>9-11</sup>. Some of them have been implemented in monitoring sites to  
56 characterize PM exposure in epidemiological studies. However, contradictory results have been  
57 obtained so far. Several studies reported an association between OP and health outcomes<sup>12-13</sup>  
58 whereas others reported a lack of association<sup>14</sup>. A recent review<sup>15</sup> provides a thorough analysis  
59 of the different reasons that could explain such inconsistencies.

60 A wide range of cell-free assays are available to measure the inherent OP of ambient  
61 particles, but systematic cross-comparisons have only been performed on a limited number of  
62 assays. Hence, the predictive value of oxidative and inflammatory responses in target cells  
63 using a set of fully chemically characterized PM samples has never been assessed. As a follow-  
64 up of preliminary studies addressing this issue<sup>6</sup>, the present study is performed on freshly  
65 sampled and chemically contrasted PM from traffic and domestic wood burning. We investigate  
66 here biological effects on two putative target cells: bronchial cells representing primary  
67 exposure targets and endothelial cells to mimic the cardiorespiratory outcome of PM<sub>2.5</sub>. Then  
68 we apply the most predictive OP assay to characterize the temporal variability of chemically  
69 resolved PM.

70 The overall aim of this study is to determine whether the measurement of the intrinsic  
71 OP by four different cell-free assays can be predictive of the oxidative and proinflammatory  
72 responses induced in lung epithelial and endothelial cells exposed to PM<sub>2.5</sub> sampled at locations  
73 with contrasted combustion sources (traffic vs domestic wood burning). Next we investigated  
74 the temporal variability of both the OP and PM chemical components at these locations. We  
75 compared here PM<sub>2.5</sub> sampled at an urban traffic site with PM<sub>2.5</sub> sampled at a suburban site  
76 impacted by domestic wood burning.

77

## 78 **Materials and Methods**

### 79 **PM collection and chemical characterization**

80 Ambient PM<sub>2.5</sub> were sampled continuously (every 24h, from midnight to midnight UTC)  
81 at two distinct locations of the region of Paris using low volume Leckel sequential samplers  
82 (Model SEQ47/50, Sven Leckel Ingenieurbüro GmbH, Germany) running in parallel at 2.3m<sup>3</sup>/h  
83 and equipped with 47-mm diameter Teflon (PTFE) and Quartz (TissuQuartz, Pallflex) filters.  
84 The first location (“Porte d’Auteuil”) is situated near the ring road surrounding Paris and was  
85 chosen to sample PM<sub>2.5</sub> related to traffic (filter samples called “Traf”). Samples were collected  
86 in fall in order to avoid other sources of combustion such as domestic wood burning. The second  
87 location (“SIRTA”; Site Instrumental de Recherche par Télédétection Atmosphérique) is

88 located 20 km southwest of Paris center and can be considered as a suburban background station  
89 surrounded by cultivated areas, forests, and small towns. Sampling at this location was  
90 performed in winter when domestic heating is widely used yielding an aerosol enriched in  
91 particles from biomass burning (filter samples called “BB”)<sup>16</sup>. For each site, samples were  
92 collected in three different periods: the first two consisting of batches of several (24-h sampled)  
93 filters that were merged in order to collect a sufficiently large mass of PM for chemical analysis  
94 and to realize both cell free assays and toxicological studies. The last sampling period consisted  
95 of daily sampling of PM<sub>2.5</sub> to study the temporal variability of the OP and link it to the day-to-  
96 day variability of the atmospheric concentration of individual PM chemical species (Table S1).

97 Chemical analyses of PM were performed on the Quartz filters running in parallel of the  
98 Teflon filters used for the different assays. They comprised: PM mass, major inorganic ions,  
99 carbonaceous aerosols: organic carbon (OC), elemental carbon (EC), carbohydrates including  
100 levoglucosan, a tracer of wood combustion, a total of 34 trace metals, a total of 16 Polyaromatic  
101 hydrocarbons (PAH) listed in SI. For each chemical analysis, standard operating procedures  
102 were performed as previously described<sup>17-19</sup>.

103 The four PM batches (Traf1; Traf2; BB1; BB2; Table S1) obtained from the first two  
104 sampling periods were extracted from Teflon filters by immersion in methanol and strong  
105 sonication (Branson Digital Sonifier equipped with a cup-horn, 50%, 200 W) for 2 x 30 s. The  
106 volume of methanol in particle suspension was reduced by evaporation (Concentrator plus,  
107 Eppendorf, Montesson, France) at 30 °C. Final evaporation was performed under a constant  
108 flow of nitrogen. Extracted (dried) PM were stored at -20 °C until use. These PM samples were  
109 then suspended at the concentration of 2 mg/mL in 15 mM HEPES buffer. Particle suspensions  
110 were homogenized by sonication for 5 min at 200 W (5 s ON; 0.5 s OFF) (Branson Digital  
111 sonifier).

112 For the last sampling period (2-week continuous sampling; “daily sampling” in Table  
113 S1), OP assays were performed directly on filters. For this purpose, filters were cut in 1/32  
114 portions, 3 of them being used for OP assay.

115 All chemicals for OP and biological assays were purchased from Sigma–Aldrich (Saint-  
116 Quentin Fallavier France) unless otherwise specified.

117

## 118 **Intrinsic oxidative potential of PM**

### 119 ***Dithiothreitol assay (DTT assay)***

120 This assay quantitatively determines the oxidation of DTT by particles by measuring  
121 the consumption of reduced DTT<sup>20</sup>. In this assay, the transfer of electrons from DTT to oxygen  
122 generates DD-disulfide and superoxide radicals (O<sub>2</sub><sup>-</sup>). The remaining non-oxidized DTT is then  
123 detected using the method described in<sup>6</sup>. The percentage of depletion is calculated as %PO<sup>DTT</sup>  
124 depletion = 100 - ([DTT] sample/[DTT]control)\*100).

### 125 ***Plasmid scission assay (Plasmid assay)***

126 The plasmid scission assay quantify particle induced strand breaks in supercoiled  
127 ϕX174RF1 plasmid, caused by generation of free radicals<sup>21</sup>. Single strand breaks induce a  
128 relaxed circular form which migrates less rapidly during gel electrophoresis than the  
129 supercoiled plasmid. Detailed methodology is provided in<sup>6</sup>. The proportion of circularized  
130 DNA was calculated as a percentage of the supercoiled intact DNA. This percentage was then  
131 expressed for each condition relatively to the control.

### 132 ***Antioxidant depletion assay (AO assay)***

133 This assay measures concentration variation of the antioxidants: ascorbic acid (AA),  
134 uric acid (UA) and glutathione (GSH), at physiological concentrations in presence of particle  
135 suspension<sup>9, 22</sup>. Particles from 10 to 100 µg/mL or 1/32 filters were incubated for 4 h at 37° C  
136 in a solution containing physiological concentrations (200 µM) of each AO in 0.9% NaCl at pH  
137 7.4 with constant mixing. After incubation, the final AO concentrations in samples were  
138 quantified by reversed-phase HPLC (Shimadzu HPLC system interfaced with the LabSolution  
139 software) according to the method detailed in<sup>6</sup>. Percentage of depletion was calculated as: 100  
140 - (AA or GSH concentration in the sample/ AA or GSH concentration in blank)\*100 and GSSG  
141 production as (100 + (GSSG concentration in the sample/ GSSG concentration in blank)\*100.  
142 For day-to-day samples results were expressed either per µg or per m<sup>3</sup> (OP/m<sup>3</sup> = OP/µg \* [PM]).

### 143 ***CM-H<sub>2</sub>DCF acellular assay***

144 In this assay ROS produced by the particles convert the non-fluorescent CM-H<sub>2</sub>DCF-  
145 DA ((5- (and-6) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate) probe (Fisher  
146 Scientific) into the fluorescent DCF (2', 7'-dichlorofluorescein). The detailed protocol is  
147 available in<sup>6</sup>. Briefly, CM-H<sub>2</sub>DCFDA probe is first activated by an incubation with 10 mM of  
148 NaOH solution and further neutralized with 10X phosphate buffered saline without calcium and  
149 magnesium (PBS; Life Technologies, Thermo-Fisher Scientific). Particles from 10 to 100

150  $\mu\text{g/mL}$  were incubated with  $16.5 \mu\text{M}$  CM- $\text{H}_2\text{DCF}$  in 96-well plate. Fluorescence generated by  
151 probe oxidation is read every hour for 4 h at 485 nm excitation and 520 nm emission, at  $37^\circ\text{C}$   
152 with FlexStation 3 (Molecular Devices). Results were normalized to the control (probe solution  
153 without particles).

#### 154 **Cell culture and particle treatment**

155 Human mucoepidermoid pulmonary carcinoma cell NCI-H292 (ATCC, Sigma), were  
156 grown as previously described<sup>6</sup>. PM were diluted in RPMI 1640 medium without phenol red  
157 and supplemented with 1% of penicillin-streptomycin and 1% of glutaMAX<sup>TM</sup> and NCI-H292  
158 cells were exposed to concentrations of PM ranging from 1 to  $10 \mu\text{g/cm}^2$ . For CM- $\text{H}_2\text{DCF}$   
159 assay, cells were grown in 96-well plates for fluorescence measurement at 4 and 24 h while for  
160 mRNA expression, cells were grown in 12-well plates during 24 h.

161 Human Pulmonary Artery Endothelial Cells (HPAEC) were isolated from the main  
162 branch of the pulmonary artery of a 23-year old male Caucasian donor. HPAEC were purchased  
163 from PromoCell® (France) and grown as described in<sup>23</sup>. HPAEC cells were exposed to  
164 concentrations from 1 to  $10 \mu\text{g/cm}^2$  of PM diluted in ECGM supplemented medium without  
165 phenol red. For mRNA expression, cells were grown in 12-well plates during 24 h while for  
166 CM- $\text{H}_2\text{DCF}$  assay, cells were grown in 24-well plates for fluorescence measurement at 4 h.

#### 167 **CM- $\text{H}_2\text{DCFDA}$ cellular assay**

168 The probe used is the same as for the acellular assay, but without performing the  
169 activation step. CM- $\text{H}_2\text{DCFDA}$  was dissolved in dimethyl sulfoxide (DMSO) at 2 mM and  
170 diluted (to  $100 \mu\text{M}$  in RPMI 1640 medium for NCI-H292 and to  $20 \mu\text{M}$  in HBSS for HPAEC).  
171 After washing (with PBS for NCI6H292 or HBSS for HPAEC), cells were loaded with the  
172 probe for 45 min with NCI6H292 or 20 min with HPAEC at  $37^\circ\text{C}$  in 96-well or 24-well plates.  
173 Cells were then washed twice with PBS or HBSS and exposed to particles for 4 h. Fluorescence  
174 was read at 485 nm excitation and 520 nm emission, at  $37^\circ\text{C}$  with FlexStation 3 (Molecular  
175 Devices) for NCI-H292 and FLUOstar Omega 2.10 plate reader with analyses performed using  
176 the MARS Data Analysis Software 2.30 R3 (BMG Labtech®) for HPAEC.

#### 177 **mRNA expression by RT-qPCR**

178 TriReagent (TRI REAGENT®-RNA/DNA/Protein Isolation Reagent, Euromedex) was  
179 used for lysing cells after 24 h of treatment. The manufacturer's recommendations RNA were  
180 used to perform RNA extraction and their quantification was done using Nanodrop 2000  
181 (Thermo Scientific). 500 ng of oligo(dT) primers with M-MLV Reverse Transcriptase kit (Life  
182 Technologies, Thermo-Fisher Scientific) were added to  $1 \mu\text{g}$  of total RNA to perform the



183 reverse transcription (RT) using a thermocycler (UNO II, Biometra). A Roche LC480 using  
184 LightCycler®480 SYBR Green 1 Master mix (Roche Diagnostics, Mannheim, Germany) was  
185 used to perform the quantitative PCR following the manufacturer's recommendations. The  
186 cycle threshold (CT) values were determined and normalized to HSC70 expression by the  
187  $\Delta\Delta\text{CT}$  method, using LightCycler®480 software v1.5. The conditions of PCR and the list of  
188 primers are detailed in the supplementary materials (Table S2).

## 189 **Statistical analysis**

190 Values are expressed as means  $\pm$  standard error of the mean. Statistical analyses were  
191 conducted using either a one-way analysis of variance with Sidak's test for multiple  
192 comparisons or a two-way analysis of variance with Dunnett's test for multiple comparisons.  
193 For OP assays on daily samples, a Mann-Whitney test was performed. Bivariate correlations  
194 between OP and particle composition were quantified using non parametric Spearman  
195 correlation tests. GraphPad Prism v7.00 software was used to perform statistical analysis. The  
196 association between  $\text{OP}^{\text{AO}}$  and different particle concentrations was studied using multiple  
197 linear regression (MLR) analysis for both the BB and Traf sites according to the method  
198 described in SI.

## 199 **Results and discussion**

### 200 *PM<sub>2.5</sub> from traffic and biomass burning exhibited distinct chemical signature*

201 Both locations and periods of the year were selected to have compositionally distinct  
202 samples enriched for the specific chemical signature of the two main combustion sources  
203 (traffic, domestic wood burning). At the urban (Traffic, "Traf") site,  $\text{PM}_{2.5}$  were sampled during  
204 Fall (when domestic heating is largely still off) and close to the Parisian ring road in order to  
205 have an aerosol enriched in diesel particles. At the suburban background (Biomass Burning,  
206 "BB") site situated at 20 km from Paris, particles were sampled in winter in order to have an  
207 aerosol enriched in combustion particles from domestic wood burning<sup>16</sup>.

208 The average  $\text{PM}_{2.5}$  mass concentrations were  $15.01 \pm 7.48 \mu\text{g}/\text{m}^3$  and  $26.4 \pm 17.61 \mu\text{g}/\text{m}^3$   
209 at the Traf and BB sites respectively showing higher mass and higher daily variability at the  
210 BB site (Figure 1 and Figure S1). The high variability at the BB site was linked to 9 consecutive  
211 days with concentrations exceeding  $25 \mu\text{g}/\text{m}^3$  (03/14 to 03/21/2015) whereas this value was  
212 only exceeded two days at the Traf site (09/06 and 09/08/2013) (Figure S1). At the Traf site,  
213 the PM was dominated by carbonaceous compounds (OC + EC: 72.5%) whereas the BB site  
214 was characterized by a high inorganic ion content ( $\text{NH}_4^+ + \text{NO}_3^- + \text{SO}_4^{2-} = 73.85\%$ ) compared

215 to the carbonaceous content (OC + EC: 22.9%). The Traf site had an OC/EC ratio of  $0.293 \pm$   
216  $0.091$  whereas at the BB site this ratio was  $4.55 \pm 1.14$ , comparable to expected values for the  
217 respective sources of combustion particles (Figure S1)<sup>16</sup>. The Traf site had a higher metal  
218 content ( $683 \pm 316 \text{ ng/m}^3$ ) compared to the BB site ( $114 \pm 34 \text{ ng/m}^3$ ) both being dominated by  
219 Fe (Figure S2). At the Traf site, Cu, Mn, Mo, Sb, Sn, Fe were highly correlated ( $r < 0.915$ ,  
220  $p < 0.0001$ ), likely a result of the contribution of non-tailpipe emissions such as brake/tire wear  
221 (Figure S3). Such correlations were not observed for the BB site (Figure S4). The Traf sample  
222 also had a higher PAH content ( $1.8 \pm 0.515 \text{ ng/m}^3$  compared to  $0.362 \pm 0.116 \text{ ng/m}^3$  for BB site)  
223 with 2 PAHs (pyrene and Benzo(e)pyrene) representing 67% of the total PAHs at the Traf site  
224 (Figure S2). Correlations between PAHs were stronger at the BB site compared to the Traf site  
225 and most of the 17 analyzed PAHs were correlated to each other (Figure S5).

226

#### 227 *PM<sub>2.5</sub> from traffic and biomass burning sites exhibited distinct OP*

228 Four different assays (AO depletion assay, DCF assay, DTT assay, plasmid scission  
229 assay) were used to compare the intrinsic OP of PM<sub>2.5</sub> sampled at both sites (Traf1; Traf2; BB1;  
230 BB2; Table S1). Two samples were collected at each site to avoid sampling bias. In contrast to  
231 many other published studies, these assays were performed using particle suspensions instead  
232 of water-soluble fractions for both cellular and cell-free assays to better account for oxidative  
233 stress related to particle uptake, water insoluble organic (e.g. PAH) and trace metals in  
234 intracellular ROS production. A dose-dependent strategy was chosen to better cover the range  
235 of OP according to the sites. In the AO depletion assay (Figure 2A and 2B), depletion of AA  
236 and GSH tended to increase as a function of dose. No modification of uric acid concentration  
237 was observed (data not shown), as was already described elsewhere for other PM<sub>2.5</sub> samples<sup>6</sup>.  
238 The two Traf samples (Traf1, Traf2) showed a 50% AA depletion from 25  $\mu\text{g/mL}$  with nearly  
239 a total depletion at 100  $\mu\text{g/mL}$  whereas for the samples at the BB site, the 50 % depletion was  
240 reached only at 100  $\mu\text{g/mL}$  (Figure 2A). For all the concentrations, a clear difference was  
241 observed in AA depletion between Traf and BB samples. GSH depletion was less pronounced  
242 than AA depletion although samples showed a similar dose-dependent response. (Figure 2B).  
243 In the GSH assay, BB2 exhibited a higher response than BB1, closer to that of the Traf samples  
244 (Figure 2B). A corresponding difference between the two BB samples was also observed in  
245 GSSG production (Figure 2C). A high correlation was observed between GSH depletion and  
246 GSSG production ensuring that GSH depletion was the result of oxidation and not of a covalent  
247 binding to organic compounds ( $r = 0.965$ ). Moreover, the AA, GSH and GSSG data exhibited a

248 significant correlation between them (AA-GSH: 0.769, AA-GSSG: 0.664) (Table S3). The  
249 lower GSH depletion induced by PM compared to AA depletion and the correlation between  
250 GSH depletion and GSSG production confirmed previous results with other PM<sub>2.5</sub> samples<sup>6</sup>.  
251 With the DCF assay, BB samples never triggered an oxidation of the DCF probe whereas a  
252 similar dose-dependent effect was shown with the two Traf samples with a significant effect  
253 from 50 µg/mL (Figure 2D). The sensitivity of the DCF assay was previously observed with  
254 other Paris traffic samples<sup>6, 23</sup>. For the DTT assay, Traf samples exhibited a higher significant  
255 effect than BB samples at 10 µg/mL and DTT depletion was not increased at 100 µg/mL (Figure  
256 2E) illustrating the non-linear DTT response to particle concentrations<sup>24</sup>. The DTT assay did  
257 not show significant correlation with OP<sup>AO</sup> and OP<sup>DCF</sup> assays (Table S3). For the semi-  
258 quantitative plasmid scission assay, a dose-dependent increase in DNA breaks was observed  
259 with a trend of a higher effect for Traf samples that is weak due to the high variability (Figure  
260 2F). This assay provides the strongest correlations with other assays (Table S3). Correlations  
261 are weaker between other assays suggesting that each one, taken separately, could bring  
262 additional information on the PM chemical components involved in the assay. Therefore,  
263 several assays may have to be combined in order to provide a full picture of the ROS  
264 sources/mechanisms of generation. In a previous study, better correlations were observed  
265 between these assays although these correlations were derived from a higher number of PM  
266 samples<sup>6</sup>. Similarly, Calas et al., studying the seasonal variability of c.a.100 PM samples  
267 collected in the same city, observed a significant correlation between the different assays  
268 including DTT and AO (AA and GSH)<sup>25</sup>.

269 Due to technical limitations, the availability of chemical data for these Traf and BB  
270 continuous samples was limited to trace metals. However, trace metal concentrations exhibited  
271 a strong correlation between continuous and daily samplings ( $r=0.975$  and  $r=0.98$  for the Traf  
272 and BB sites respectively) showing the consistency in particle composition over the whole  
273 sampling campaigns. Spearman correlation coefficients between trace metal concentrations and  
274 the different OP assays are shown in Table S4. Trace metals correlating positively with 5 or 6  
275 OP assays were ranked according to decreasing sum correlation values:  
276 Mo>Mn>V>Ba>Zn>Fe>Sb>Cu>Sn. Among these, Cu, Mn, Mo, Sb, Sn and Fe were found to  
277 be co-correlated in the Traffic site and are markers of non-tailpipe emissions. This is consistent  
278 with the previously mentioned study, where Cu was the “unifying factor” among the OP assays  
279 that were used<sup>25</sup> as well as with data reported in a review on OP measurements across Italy<sup>26</sup>.

280 *PM<sub>2.5</sub> from traffic and domestic biomass burning sites produced distinct oxidative stress*  
281 *in both epithelial and endothelial cells*

282 In addition to measurement of their inherent oxidative capacity, the same samples were  
283 evaluated for oxidative and pro-inflammatory responses on human lung epithelial and  
284 endothelial cells. Bronchial epithelial cells were selected as they are among the first cells  
285 encountered by inhaled particles. Endothelial cells are also directly influenced by inhaled  
286 particles as well as by particle-derived soluble compounds that translocate through the epithelial  
287 barrier.

288 The effect of PM<sub>2.5</sub> on the intracellular production of ROS was measured in epithelial  
289 and endothelial cells using the CM-H<sub>2</sub>DCFDA probe. As shown in Figure 3 A and D, PM<sub>2.5</sub>  
290 induced an increase of the DCF fluorescence in both cell lines with increasing concentrations.  
291 Samples from the same site induced consistent effects and a clear difference was observed  
292 between Traf and BB samples in epithelial cells at the highest concentration (Figure 3A). By  
293 contrast no significant difference was seen between Traf and BB samples in exposed endothelial  
294 cells that otherwise showed a higher DCF oxidation (Figure 3D). No significant correlation was  
295 observed in DCF oxidation between the 2 cell types ( $r=0.524$ ,  $p>0.05$ ).

296 In order to determine whether this intracellular increase in ROS production was  
297 associated with an adaptive response to oxidative stress, heme-oxygenase-1 (HO-1) and  
298 superoxide dismutase-2 (SOD-2) expression were measured. The expression of these two anti-  
299 oxidant enzymes is driven by the activation of the transcription factor Nrf2 involved in the  
300 oxidative stress pathway. As shown Figure 3B and 3E, PM<sub>2.5</sub> induced a dose-dependent increase  
301 of the HO-1 expression in both cell lines, with the induction being higher in endothelial cells  
302 likely related to the higher ROS production. A clear difference in HO-1 expression is observed  
303 between Traf and BB samples at the highest tested concentrations in epithelial cells (Figure 3B)  
304 whereas for endothelial cells it was significant at 1  $\mu\text{g}/\text{cm}^2$  (Figure 3E). For SOD-2, no  
305 significant induction was shown in epithelial cells irrespective of the concentration and the type  
306 of PM<sub>2.5</sub> (Figure 3C). By contrast in endothelial cells, Traf 1 induced a significant upregulation  
307 of SOD-2 expression at 10  $\mu\text{g}/\text{cm}^2$  compared to BB2 PM<sub>2.5</sub> (Figure 3F). HO-1 and SOD-2  
308 expression were significantly correlated in each cell types ( $r=0.65$ ,  $p<0.026$  in epithelial cells  
309 and  $r=0.902$ ,  $p<0.0001$  in endothelial cells). In epithelial cells, HO-1 and SOD-2 expression  
310 were significantly correlated to the intracellular DCF oxidation ( $r=0.678$ ,  $p<0.02$  for HO-1 and  
311  $r=0.769$ ,  $p<0.005$  for SOD-2) whereas for endothelial cells a correlation with DCF was only  
312 observed with SOD-2 ( $r=0.833$ ,  $p<0.015$ ). Overall, it appears that endothelial cells exhibit a

313 more pronounced response than bronchial cells, which may imply a different sensitivity to  
314 oxidative stress. Correlations with trace metal content showed numerous significant  
315 correlations but the metals for which a significant correlation was observed for all 3 cellular  
316 endpoints and in both cell types were Bi, Ce, Cu, Mo, Pb, Sb, Se, Sn and V (Table S4).

317

318 *PM<sub>2.5</sub> from traffic and domestic biomass burning sites induced pro-inflammatory*  
319 *response in epithelial and endothelial cells*

320 IL-8 mRNA expression was used in both cell types to characterize the pro-inflammatory  
321 response. An increase of IL-8 mRNA expression was observed with exposure to both Traf and  
322 BB samples in both cell types (Figure S6). In endothelial cells a clear dose-response is observed  
323 for Traf and BB samples, with consistent results between samples of the same site. There was  
324 a significant difference between Traf and BB samples even at the lowest concentrations, BB  
325 samples being less reactive (Figure S6B). With epithelial cells, similar tendencies were  
326 observed but high within-site variability means that this data is not independently statistically  
327 significant (Figure S6A). However, a significant correlation is observed between the 2 cell types  
328 ( $r=0.748$ ,  $p<0.007$ ). The pro-inflammatory response is often considered to be related to  
329 oxidative stress, which is supported in this experiment by the strong correlation between IL-8  
330 expression and HO-1 and SOD-2 expression in each cell type ( $r=0.573$ ,  $p<0.056$  (HO-1),  
331  $r=0.916$ ,  $p<0.0001$  (SOD-2) for epithelial cells, and  $r=0.846$ ,  $p<0.001$  (HO-1) and  $r=0.881$ ,  
332  $p<0.0001$  (SOD-2) for endothelial cells).

333

334 *Lung antioxidant depletion assay is the most predictive OP assay of cell responses*

335 In order to determine which OP assays were better predictors of oxidative cell responses,  
336 correlation studies were performed (Table S5). The OP<sup>plasmid</sup> assay provides the best correlation  
337 with other cell-free and cell assays but due to its semi-quantitative and time-consuming nature,  
338 it was not considered for further experiments. OP<sup>AA</sup> and OP<sup>DCF</sup> also give the most significant  
339 correlations for the 2 cell types. The OP<sup>AO</sup> includes the simultaneous detection of depletion or  
340 oxidation of two antioxidants, and by combining these measurements it covers several possible  
341 cellular responses. Especially noteworthy is the high degree of correlation between OP<sup>AO</sup>  
342 endpoints and HO-1 expression in both cell types.

343 Taking into consideration the correlation between OP<sup>AO</sup> and cellular responses as well  
344 as the opportunity to measure different anti-oxidants and the formation of an oxidation product

345 in the same assay, we focused on the  $OP^{AO}$  assay to realize a daily characterization of OP of  
346  $PM_{2.5}$  sampled at the 2 sites over 3 weeks (Table S1). Furthermore,  $OP^{AO}$  assays could be  
347 validated using filters instead of particle suspensions whereas this is not possible for the plasmid  
348 assay (Figure S7).

349 *Daily analysis of  $OP^{AO}$  always revealed a higher OP for  $PM_{2.5}$  from traffic site*  
350 *compared to biomass burning site*

351  $OP^{AO}$  was measured on  $PM_{2.5}$  sampled for 24h during n=19 and n=22 consecutive days  
352 at the Traf and BB sites respectively. Compared to blank filters, all daily samples exhibited, a  
353 clear temporal variability with a depletion of AA (Figure 4) and GSH (Figure S8) and a  
354 production of GSSG (Figure S9). For both sites, GSH depletion correlated well to GSSG  
355 production ( $r=0.945$  for Traf site and  $r=0.978$  for BB site,  $p<0.0001$ ). Independently of the site,  
356 the depletion was systematically larger for AA than for GSH (Figure 4, and Figure S8  
357 respectively) but a significant correlation was observed between  $OP^{AA}$  and  $OP^{GSH}$  ( $r=0.784$ ,  
358  $p<0.0001$  for  $OP/m^3$  and  $r=0.914$  for  $OP/\mu g$ ,  $p<0.0001$ ).  $OP^{AA}$  when expressed per  $m^3$ , showed  
359 a temporal variability directly related to ambient  $PM_{2.5}$  concentrations.  $OP^{AA}$  measurements  
360 from Traf site samples were significantly higher ( $1.39\pm 0.25$ ) than  $OP^{AA}$  from BB site samples  
361 ( $0.910\pm 0.252$ ) with a ratio Traf/BB of 1.52 ( $p<0.001$ , Figure 4A, B, C). When  $OP^{AA}$  was  
362 expressed per  $\mu g$  of PM, a temporal variability was again observed reflecting the contribution  
363 of PM components to OP, and  $OP^{AA}$  measurements from Traf site samples were significantly  
364 higher ( $0.098\pm 0.036$ ) than  $OP^{AA}$  from BB site samples ( $0.044\pm 0.021$ ) with a ratio Traf/BB of  
365 2.22 ( $p<0.0001$ , Figure 4D, E, F). At the BB site, the  $OP^{AA}/\mu g$  was particularly low during a  
366 period when  $PM_{2.5}$  mass was high due to the increase of secondary pollutants such as ions and  
367 organics in PM, which are considered not to contribute significantly to OP (Figure 1).

368 No correlation was observed between  $OP^{AA}/m^3$  and  $OP^{AA}/\mu g$  suggesting that OP was not  
369 directly proportional to ambient PM concentrations. The same conclusions were obtained with  
370  $OP^{GSH}$  and  $OP^{GSSH}$  with significant differences between the two sites considering results  
371 expressed per  $m^3$  or  $\mu g$  but with less magnitude (Figure S8, S9). Altogether these data clearly  
372 underline the major role of PM composition in OP.

373 To further explore site-to-site differences in OP, we examined multivariable linear regression  
374 (MLR) models in order to characterize the relationships between PM chemical composition and  
375  $OP^{AA}$ . The MLR analyses were performed using volume based data and PAH, trace metals or  
376 ion descriptor sets, separately or together. We selected descriptors that contributed more than  
377 0.9 in terms of  $R^2$  when possible at Traf and BB sites (Table 1).

378 For the Traf site, OP<sup>AA</sup> is very well explained by a panel of 8 trace metal descriptors ( $R^2=0.856$ )  
379 with Ti and Mo being the strongest contributors and well explained by PAH ( $R^2_{\text{adj}}=0.646$ ) but  
380 not by ions ( $R^2_{\text{adj}}=0.075$ ) (Table 1 and figure S10). Considering all components measured  
381 (PAH, trace metals, ions) including OC and EC, the combination of descriptors BaA, Al, Pb,  
382 As, BbF, NO<sub>3</sub>, Sr and Ti improves the model ( $R^2_{\text{adj}}=0.923$ ). For the BB site, OP<sup>AA</sup> is similarly  
383 very well explained by trace metals ( $R^2_{\text{adj}}=0.907$ , 13 descriptors) and well explained by PAH  
384 ( $R^2_{\text{adj}}=0.559$ , 1 descriptor). In addition, OP<sup>AA</sup> is well explained by ions ( $R^2_{\text{adj}}=0.857$ , 4  
385 descriptors). When all components are considered (including OC and EC), 4 descriptors (Cu,  
386 K, SO<sub>4</sub><sup>2-</sup> and Mo) were sufficient to obtain a model with  $R^2_{\text{adj}}=0.906$ . Next important descriptor  
387 to improve the  $R^2_{\text{adj}}$  is OC from 0.906 to 0.920. Levoglucosan data is available for this site and  
388 its addition in the model increases the correlation to  $R^2_{\text{adj}}=0.914$  whereas substituting it for K  
389 decreases the correlation to  $R^2_{\text{adj}}=0.873$ . The strong contribution to the regression by tracers of  
390 biomass burning supports the contribution of this source to the overall OP<sup>AA</sup> of the BB samples.  
391 The similar values of  $R^2_{\text{adj}}$  and  $R^2_{\text{LOO}}$  obtained confirm the stability of the regression models  
392 for both Traf and BB sites.

393 The same analysis was performed for OP<sup>GSH</sup> (table S6 and figure S10). It provides consistent  
394 quality of regression models but involves different descriptors suggesting that these two OP do  
395 not react with the same components of the sample. Like OP<sup>AA</sup>, OP<sup>GSH</sup> is very well explained by  
396 a panel of several metal descriptors ( $R^2_{\text{adj}}=0.825$  for Traf and 0.932 for BB sites) and that  
397 regression models are improved by combining different types of descriptors ( $R^2_{\text{adj}}=0.913$  for  
398 Traf and 0.919 for BB sites). In summary, adequate explanation of the measured OP requires a  
399 site-specific regression model which accounts for differences in chemical components arising  
400 from different emission sources. Similar conclusions were recently drawn by Weichenthal,  
401 concerning within-city variations of PM<sub>2.5</sub> OP<sup>AO</sup> in Toronto<sup>27</sup>.

402 In this study we provided evidence that OP measurement is predictive of cellular oxidative and  
403 pro-inflammatory responses strengthening the idea that OP of PM could be used as a  
404 biologically-relevant exposure metric for studies of air pollution and health. Modelling  
405 approaches using source apportionment are currently being developed to determine the  
406 contribution of natural and anthropogenic sources to PM-OP and to estimate OP over a large  
407 spatio-temporal scale<sup>28-29</sup>. Whether OP predicts health effects better than currently regulated  
408 PM characteristics, including mass and composition is not yet fully supported in  
409 epidemiological studies exhibiting either the absence or positive association<sup>12-14, 30-31</sup>.

410 Contradictory results obtained in such studies could result from the present lack of standardized  
411 protocols for particle extraction and OP assays<sup>25</sup>.

412

### 413 **Associated content**

414 Supporting Information

415 Supporting information consists of details of methodology, 11 figures, and 6 tables.

416

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418

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429



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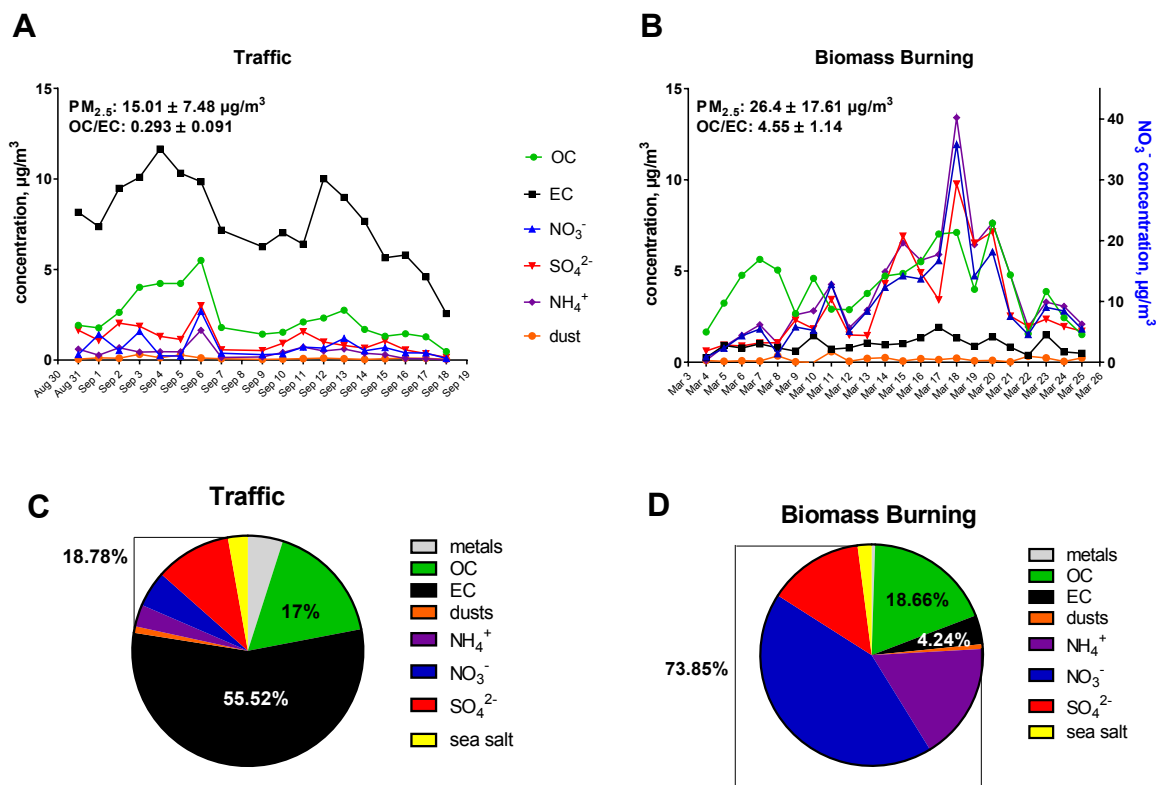
565 Table 1: **Results of multivariate linear regression analysis between PM chemical**  
 566 **composition and OP<sup>AA</sup>** with  $R^2_{\text{adj}}$ ,  $R^2_{\text{LOO}}$  indicated

	<b>Traffic (n=19)</b>	<b>Biomass Burning (n=22)</b>
<b>PAH</b>	BaA, CHR, BbF, BeP $R^2_{\text{adj}} = 0.646$ , $R^2_{\text{LOO}} = 0.647 (\pm 0.019)$	BeP $R^2_{\text{adj}} = 0.559$ , $R^2_{\text{LOO}} = 0.559 (\pm 0.039)$
<b>Trace metals</b>	Ti, Mo, Pb, As, Co, Al, Ba, Sb $R^2_{\text{adj}} = 0.856$ , $R^2_{\text{LOO}} = 0.856 (\pm 0.021)$	Cu, Mo, Hg, Zn, Rb, As, Sr, Ti, Ce, Li, V, Al, Se $R^2_{\text{adj}} = 0.907$ , $R^2_{\text{LOO}} = 0.908 (\pm 0.017)$
<b>Ions</b>	Na $R^2_{\text{adj}} = 0.075$ , $R^2_{\text{LOO}} = 0.074 (\pm 0.029)$	K, Cl, $\text{Mg}^{2+}$ , Na, $\text{Ca}^{2+}$ $R^2_{\text{adj}} = 0.857$ , $R^2_{\text{LOO}} = 0.857 (\pm 0.021)$
<b>All components</b>	BaA, Al, Pb, As, BbF, $\text{NO}_3^-$ , Sr, Ti $R^2_{\text{adj}} = 0.923$ , $R^2_{\text{LOO}} = 0.893 (\pm 0.029)$	Cu, K, $\text{SO}_4^{2-}$ , Mo $R^2_{\text{adj}} = 0.906$ , $R^2_{\text{LOO}} = 0.842 (\pm 0.017)$

567 benzo(a)anthracene (BaA), chrysene (CHR), benzo(e)pyrene (BeP), benzo(b)fluoranthene (BbF)

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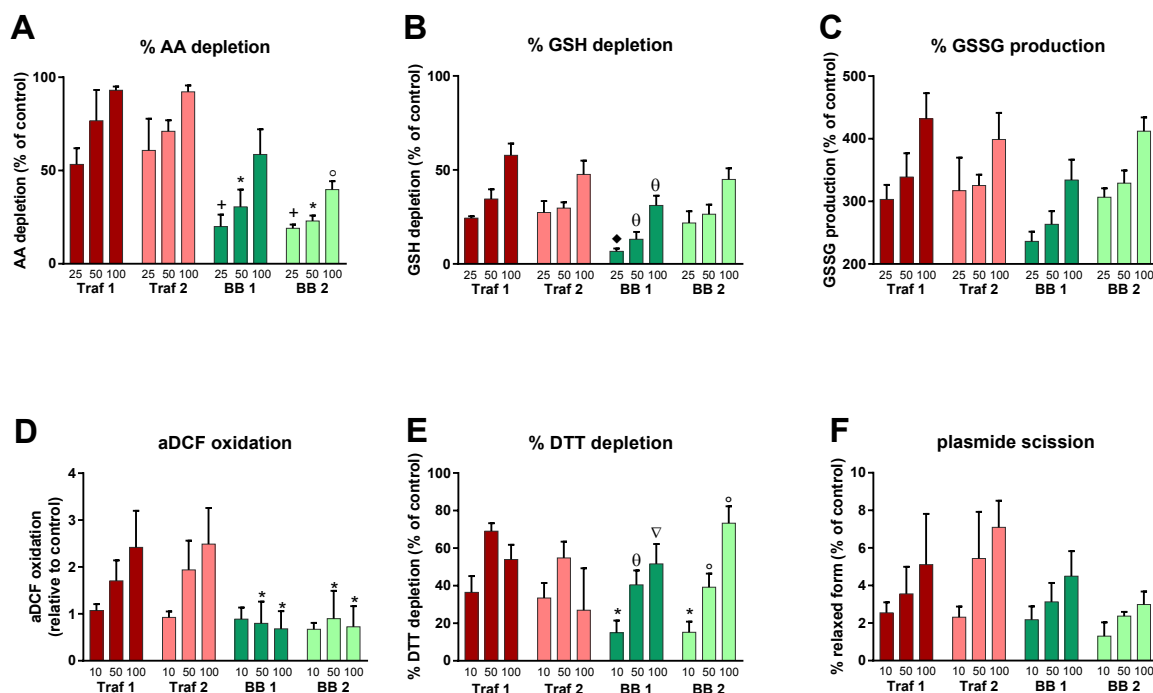
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570

571 **Figure 1: Main  $PM_{2.5}$  chemical components at the Traffic (A-C) and Biomass Burning (B-**  
 572 **D) sites.** Daily concentrations ( $\mu\text{g}/\text{m}^3$ ) of carbonaceous (organic carbon (OC) and elemental  
 573 (EC)) compounds, inorganic ions ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ) and dusts for the Traffic (A) and Biomass  
 574 Burning (B) sites. Mass distribution of the main chemical components in  $PM_{2.5}$  from the Traffic  
 575 (C) and Biomass burning (D) sites. The numbers on the left side of the pie chart represent the  
 576 proportion of inorganic ion content ( $\text{NH}_4^+ + \text{NO}_3^- + \text{SO}_4^{2-}$ ).

577

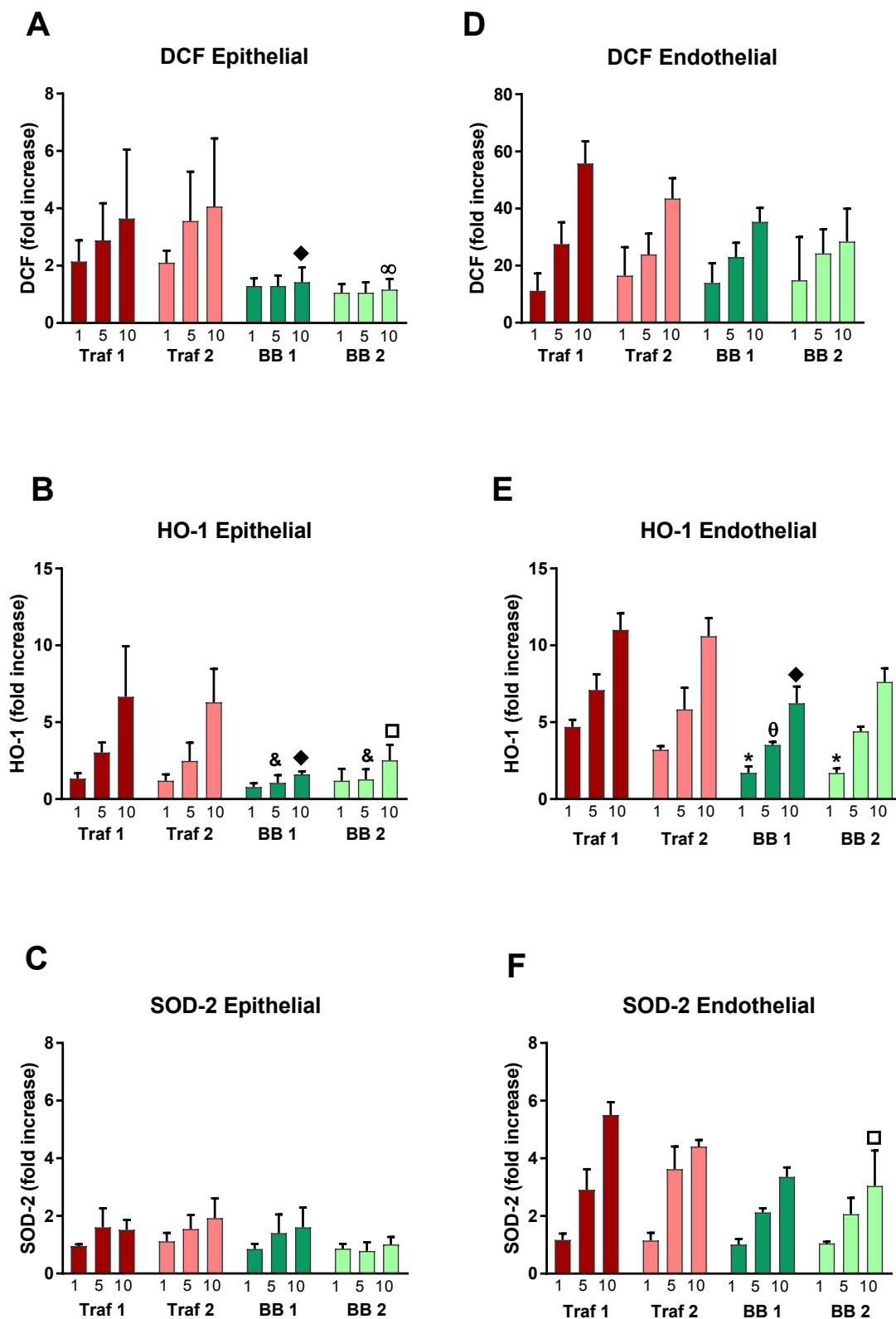


578  
 579 **Figure 2: Intrinsic oxidative capacity of PM<sub>2.5</sub> sampled at Traffic and Biomass Burning**  
 580 **sites characterized through different oxidative potential assays.** Measurement of A) acid  
 581 ascorbic (AA) depletion B) reduced glutathione (GSH) depletion C) oxidized glutathione  
 582 (GSSG) production in a simplified synthetic respiratory tract lining fluid after a 4 h incubation  
 583 at 37°C in presence of 25 to 100  $\mu\text{g/mL}$  of PM<sub>2.5</sub> (2 different traffic sites (Traf 1 and Traf2) and  
 584 biomass burning sites (BB 1 and BB 2). Results are expressed as the percentage of initial AO  
 585 concentrations (200  $\mu\text{M}$ ) in the sRTLFL and data are represented as mean  $\pm$  SEM of 2  
 586 independent experiments with 3 replicates. D) The fluorescence of the oxidized CM-DCF was  
 587 measured after 2 h of incubation at 37°C in presence of 10 to 100  $\mu\text{g/mL}$  of PM<sub>2.5</sub>. Results were  
 588 normalized to the control and data are represented as mean  $\pm$  SEM of 3 independent experiments  
 589 of 4 replicates. Measurement of reduced DTT remaining after incubation in the presence of 10  
 590 to 100  $\mu\text{g/mL}$  of PM<sub>2.5</sub> during 1 h at 37°C. Results are expressed as percentage of reduced DTT  
 591 depletion relatively to control and data are represented as mean  $\pm$  SEM of 3 independent  
 592 experiments with 3 replicates. F) Quantification of relaxed circular form of DNA produced after  
 593 4 h of incubation at 37°C in the presence of H<sub>2</sub>O<sub>2</sub> or 10 to 100  $\mu\text{g/mL}$  of PM<sub>2.5</sub>. Results are  
 594 expressed as the percentage of relaxed circular form relative to the supercoiled intact DNA and  
 595 data are represented as mean  $\pm$  SEM of 3 independent experiments with 3 replicates. \*  $p < 0.05$   
 596 between Traf and BB samples at the same dose, +  $p < 0.05$  between Traf 2 and BB samples at  
 597 the same dose, °  $p < 0.05$  between Traf and BB2 sample at the same dose, Θ  $p < 0.05$  between



598 Traf 1 and BB1 sample at the same dose,  $\nabla$   $p < 0.05$  between Traf 2 and BB1 sample at the same  
599 dose,  $\blacklozenge$   $p < 0.05$  between Traf and BB1 sample at the same dose.

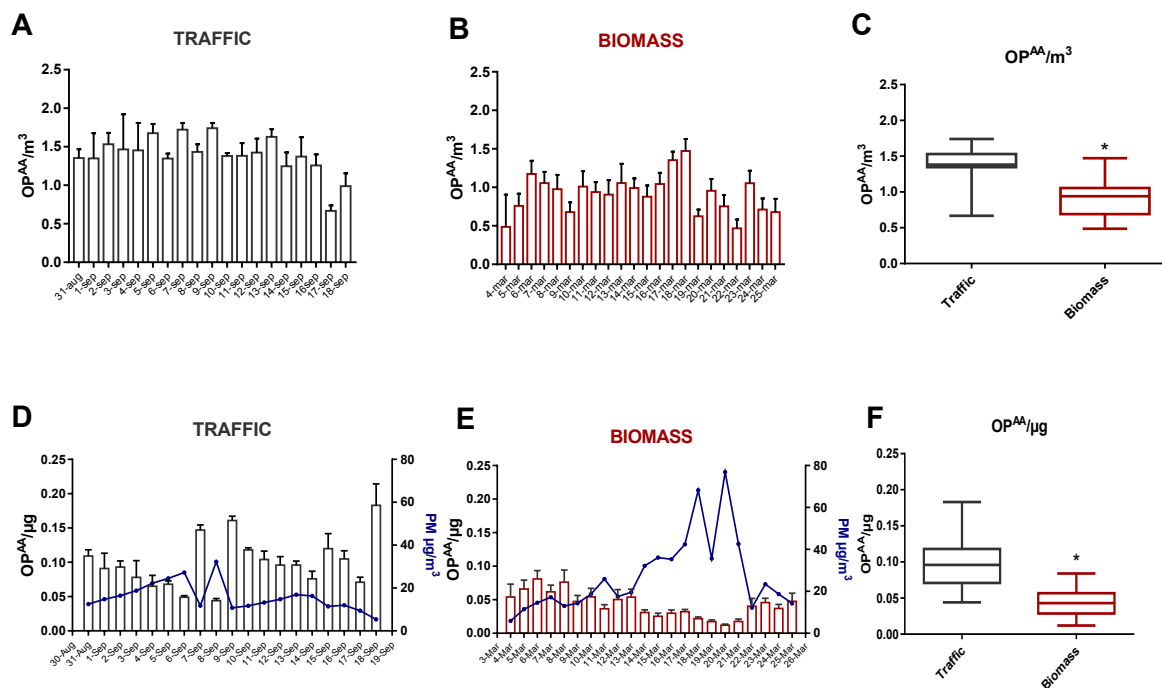
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601  
 602 **Figure 3: ROS production and antioxidant responses in bronchial epithelial (A, B, C) and**  
 603 **pulmonary endothelial (D, E, F) cells exposed to PM<sub>2.5</sub> sampled at Traf and BB sites.**  
 604 Measurement of A-D) DCF fluorescence to assess the intracellular ROS production in cells  
 605 exposed to PM<sub>2.5</sub> for 4 h, B-E) HO-1 (Heme-oxygenase-1) mRNA expression in cells exposed

606 to 1 to 10  $\mu\text{g}/\text{cm}^2$  of  $\text{PM}_{2.5}$  for 24 h, C-F) SOD-2 (superoxide dismutase-2) mRNA expression  
607 in cells exposed to 1 to 10  $\mu\text{g}/\text{cm}^2$  of  $\text{PM}_{2.5}$  for 24 h. Results are normalized to control and data  
608 are presented as mean  $\pm$  SEM of 2 independent experiments with 3 replicates. \*  $p < 0.05$  between  
609 Traf and BB samples at the same dose,  $\infty$   $p < 0.05$  between Traf 2 and BB2 sample at the same  
610 dose,  $\Theta$   $p < 0.05$  between Traf 1 and BB1 sample at the same dose,  $\blacklozenge$   $p < 0.05$  between Traf and  
611 BB1 sample at the same dose, &  $p < 0.05$  between Traf 1 and BB samples at the same dose,  $\square$   
612  $p < 0.05$  between Traf 1 and BB2 sample at the same dose.

613



614

615 **Figure 4: Ascorbic acid depletion (OP<sup>AA</sup>) of daily PM samples collected at the Traffic sites**  
 616 **Traffic, A and D) or at the Biomass Burning sites (Biomass, B and E).** Results are expressed  
 617 per m<sup>3</sup> (A and B) or per μg (D and E). In addition to OP<sup>AA</sup>, PM<sub>2.5</sub> concentrations expressed as  
 618 μg/m<sup>3</sup> are shown on D and E (blue curve). C and F box plots representation showing the median,  
 619 25th and 75th percentile for the OP<sup>AA</sup> in each site expressed per m<sup>3</sup> (C) or per/μg (F). Mann  
 620 Whitney \*p<0.0001

621