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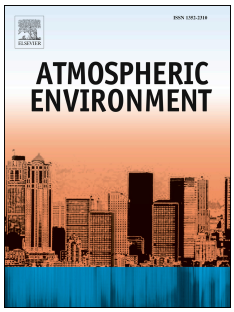

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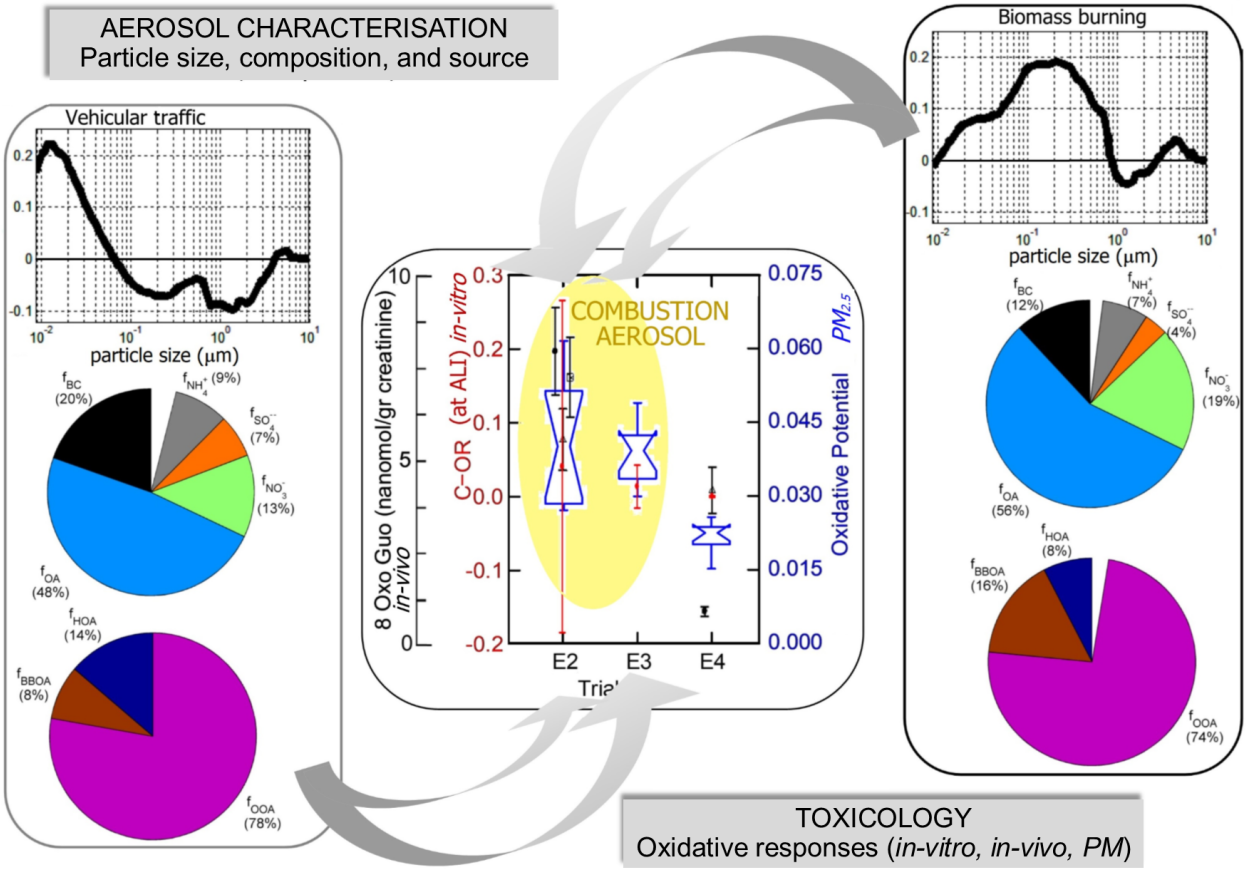

Evidence of association between aerosol properties and in-vitro cellular oxidative response to PM_1, oxidative potential of $PM_{2.5}$, a biomarker of RNA oxidation, and its dependency on combustion sources.

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Abstract

The causal link between ambient $PM_{2.5}$ and adverse health effects is still not clear enough, nor it is clear what factors (physical and/or chemical) contribute to $PM_{2.5}$ toxicity and by what mechanism(s). With a view on this, we launched the CARE experiment, during which we performed a comprehensive characterisation of the physicochemical properties of fine and ultrafine particles under exposure levels dominated by the urban combustion aerosol, and their toxicological assessment through in-vitro tests (lung epithelia cells cultured at the ALI) directly under ambient conditions, oxidative potential (determined through 2',7'-dichlorouorescin, OP^{DCFH}), and human biomonitoring. We already reported about aerosol characterisation, and in-vitro model results during CARE. Building upon these, in this work we assess the combustion aerosol oxidative response through the analysis of consistency between the three independent aerosol oxidative responses obtained, and the exploration of any causality link with the combustion aerosol. This is the first time to our knowledge that combustion related

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$PM_{2.5}$ physicochemical properties and its OP^{DCFH} are compared to the cellular-oxidative response (C-OR) obtained through the PM in-vitro test carried out (for the first time) directly under atmospheric ambient conditions, and to certain biomarkers of oxidative damage to DNA/RNA (8-oxo-7,8-dihydroguanine, 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-oxo-7,8 - dihydroguanosine). Our results provide a first evidence of a combustion-dependent association between the in vitro C-OR, the $PM_{2.5}$ OP^{DCFH}, and the urinary excretion of the 8-Oxo-7,8-dihydroguanosine. Yet this is not a substantial basis for drawing any cause-effect relationship. However, our findings support previous literature suggesting a link between combustion and oxidative response of $PM_{2.5}$. Moreover, we add a consistency across completely independent oxidative response measurements with a possible dependence on the combustion traffic-related aerosol. This is a piece of information that may have important implications in the understanding of how combustion sources contribute to oxidative response related diseases.

Keywords: Aerosol, UFPs, combustion, toxicity, biomarker, oxidative potential

1. INTRODUCTION

Urban air can contain several trace compounds blamed for posing serious risks to the human health, numerous evidences indicating that long-term exposure to the particulate matter fraction with aerodynamic diameter less than 2.5 μm ($PM_{2.5}$) accounts for the majority of health effects (Pope , 2000; Schwartz et al., 2002; COMEAP , 2010; Shindell et al., 2012; IARC , 2016; WHO , 2016; Cohen et al., 2017). A number of mechanisms for $PM_{2.5}$ health effects have been proposed, including inflammation, free-radical and reactive species production in the lungs, direct macro-molecules damage, modification and alteration of the immune system responses, cytotoxicity and genotoxicity in lungs (Salvi et al., 1999; Donaldson et al., 2001; Pope et al., 2002; Schwartz et al., 2002; Nel , 2005; Poschl , 2005; Dominici et al., 2006; Araujo et al., 2008; Valavanidis et al., 2008;

Lodovici and Bigagli , 2011; Heinzerling et al., 2016; Chen et al., 2017; Stone et al., 2017; Fu et al., 2018). However, the causal link between ambient $PM_{2.5}$ and its adverse health impact still needs to be clarified.

Yet it is not clear what $PM_{2.5}$ metrics (e.g., mass, number, surface area) better track the toxicological mechanisms, and in particular what aerosol properties (chemical composition, size distribution, mixing state, solubility) or combination of properties should be considered. Emerging evidence indicates the importance of both particle composition and size (Oberdörster et al., 2005; Valavanidis et al., 2008; Brown et al., 2013; Kim et al., 2015; Stone et al., 2017). Chemistry is especially important when considering components suspected to be toxic for humans, including organic and elemental (or black) carbon, nitrates, polycyclic aromatic hydrocarbons (PAHs) and their oxidation products (quinones, phenols, etc.), transition metals such as copper, vanadium, chromium, nickel, cobalt and iron. Particle size is equally important, especially when considering diameters smaller than 2.5 μm ($PM_{2.5}$, indeed). Particles between approx. 5 and 10 μm are most likely deposited in the upper respiratory tree; particles smaller than 1 μm (fine particles) may penetrate down to the alveoli; particles smaller than 0.1 μm (ultrafine particles, UFPs, or PM with diameter less than 0.1 μm, $PM_{0.1}$) can penetrate in the respiratory tree and, after translocation in the circulation system, can target other organs (such as heart, liver and brain) while the chemicals adsorbed on their large surface area can generate reactive oxygen species (ROS) (Nemmar et al., 2002; Li et al., 2003; Oberdörster et al., 2005; Geiser et al., 2005; Nel et al., 2006; Araujo et al., 2008; Samet et al., 2009; Delfino et al., 2010; Hertel et al., 2010; Li et al., 2016; Heinzerling et al., 2016; De Falco et al., 2017).

Aerosol sources are another key factor to consider when assessing health effects, the source determining those aerosol properties and thus their toxicity. Combustion, one of the largest UFPs sources in the atmosphere, has been studied for years, traffic-related combustion aerosols (especially diesel engine exhausts) being defined carcinogenic to humans (group 1) (Avakian et al., 2002; Yim et al., 2012; Nel , 2005; IARC , 2016). De Falco et al. (2017) showed that

concentrations of pg/ml of combustion-generated soot UFPs produced under controlled conditions in laboratory can induce an inflammatory process responsible for the IL-1-like cytokine release that, together with other pulmonary insults, may underlie lung cancer. We found that soot UFPs enriched in PAHs may activate xenobiotic responsive genes (Gualtieri et al., 2018). Although evidences are increasing, however, the understanding is still uncomplete.

To investigate these toxicity action mechanisms, both in-vitro and in-vivo toxicological studies have been used. The generally adopted procedure for in-vitro model exposure requires the collection of particles on filters, their detachment from the substrate, and resuspension in an appropriate medium (particles are generally analysed off-line); the human cells cultured are treated in conventional submerged or ALI conditions (Gualtieri et al., 2011; Schwarze et al., 2013; Dergham et al., 2015; Borgie et al., 2015; Yan et al., 2016; Longhin et al., 2016; Leclercq et al., 2017). Unfortunately, this approach is prone to introduce biases in the particle size distribution and chemical composition. We reported the possibility to treat cells directly under environmental conditions and at human relevant exposure doses, a methodology providing a novel approach to evaluate the potential adverse health impact of $PM_{2.5}$ (Gualtieri et al., 2018).

Human bio-monitoring, namely the determination of biomarkers in human specimens (e.g. urine, blood, saliva, hair), has been used for understanding the magnitude of integrated exposure to a toxic compound from all pathways and sources (regardless of the route of exposure) (Angerer et al., 2007). Since PM generated ROS may induce oxidative modifications to DNA and RNA, several biomonitoring studies have focused on guanine oxidation (Sekiguchi , 2006; Valavanidis et al., 2009; Lettieri Barbato et al., 2010; Broedbaek et al., 2011; Kjær et al., 2017; Gan et al., 2018). Guanine is a purine (molecular formula $C_5H_5N_5O$) found within RNA in the form of a ribonucleotidyl residue and in DNA in the form of a deoxynucleotidyl residue, and its alteration is known to be mutagenic (Kasai et al., 1997). A number of studies have used the urinary excretion of 8-oxo-7,8-dihydro-2-deoxyguanosine as a biomarker of response to evaluate pollutants' pro-oxidant effects on DNA (Sekiguchi , 2006; Valavanidis

et al., 2009; Lettieri Barbato et al., 2010). To date, a very limited number of studies have focused on the RNA adduct, the 8-Oxo-7,8-dihydroguanosine (8-oxoGuo), despite the increasing evidences linking this biomarker to the human ageing process (Broedbaek et al., 2011; Kjær et al., 2017; Gan et al., 2018; Tranfo et al., 2019).

As evidences suggest that factors contributing to $PM_{2.5}$ mass are not necessarily the same contributing to $PM_{2.5}$ toxicity, additional metrics are necessary to represent $PM_{2.5}$ adverse health effects (e.g., Ayres et al., 2008; Stone et al., 2017). Considering that PM-induced inflammatory effects are linked to ROS generation and consequent oxidative stress, the PM-induced oxidative potential (OP) has been suggested as a new metric (e.g., Møller et al., 2008; Bates et al., 2019). OP is a relatively simple acellular metric intended to represent PM intrinsic capacity to oxidize target molecules, generate ROS, and induce oxidative stress (Borm et al., 2007; Bates et al., 2019). Several acellular assays have been proposed to quantify the OP, including the use of ascorbic acid (OP^{AA}), dithiothreitol (OP^{DTT}), 2',7'-dichlorouorescin (OP^{DCFH}), electron spin resonance (OP^{ESR}), respiratory tract lining fluid (OP^{RTLF}), and the profluorescent nitroxide probe (PFN) (Ayres et al., 2008; Miljevic et al., 2010; Janssen et al., 2014; Huang et al., 2016; Stevanovic et al., 2017; Weber et al., 2018; Simonetti et al., 2018a,b; Calas et al., 2018; Bates et al., 2019). Yet it has to be elucidated what health effect can be predicted by what OP assays, what association can be found between PM-induced OP and quantifiable biological endpoints, and what link exists between PM-induced OP and key aerosol sources such as combustion (Delfino et al., 2013; Janssen et al., 2014; Bates et al., 2015; Fang et al., 2016; Stevanovic et al., 2017; Crobeddu et al., 2017; Weber et al., 2018; Bates et al., 2019). Therefore, the literature remains inconclusive and $PM_{2.5}$ related health impact is still assessed through $PM_{2.5}$ mass (WHO , 2016).

Starting from these considerations, we launched in 2017 the "Carbonaceous Aerosol in Rome and Environs" (CARE) experiment (Costabile et al., 2017b; Gualtieri et al., 2018; Alas et al., 2019). We performed a comprehensive characterisation of the physicochemical properties of fine and ultrafine particles under

exposure levels dominated by the urban combustion aerosol, and their toxicological assessment through in-vitro tests (lung epithelia cells cultured at the ALI) directly under ambient conditions, the assay of $PM_{2.5}$ OP^{DCFH} (2 hour time resolution), aerosol dosimetry, and human biomonitoring. A preliminary analysis (considering only 2.5 days) showed the $PM_{2.5}$ OP^{DCFH} to be particle composition and size dependent, with higher values associated to smaller traffic-generated particles that typically occurred at low mass concentrations (Costabile et al., 2017b). The toxicological results showed (Gualtieri et al., 2018) : (i) a correlation between secondary aerosol increase and genes representative of the activation of the antioxidant responsive element related to $PM_{1.5}$-induced oxidative stress (heme oxygenase gene, HO-1), (ii) oxidative responses in cells determined by secondary and aged PM, and (iii) cytochrome 1B1 gene (CYP1B1) activation with primary and PAHs enriched UFPs.

Building upon these results, in this work we extend our analysis. The major objective is to assess the combustion aerosol oxidative response during the CARE experiment through the analysis of consistency between the independent aerosol oxidative responses obtained, and the exploration of any causality link with the combustion aerosol. We first characterise $PM_{2.5}$ physicochemical properties to apportion the combustion source contributions; then, evaluate (and compare to) the oxidative responses from the in-vitro model, the $PM_{2.5}$ OP^{DCFH}, and selected oxidative stress biomarkers (8-oxo-7,8-dihydroguanine, 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-oxo-7,8 - dihydroguanosine). This is the first time to our knowledge that combustion related $PM_{2.5}$ physicochemical properties and its oxidative potential are compared to the cellular-oxidative response obtained through the PM in-vitro test carried out (for the first time) directly under atmospheric ambient conditions, and to the biomarkers of oxidative damage to DNA/RNA.

The remaining of the paper is organized as follows. Section 2 describes materials and methods, separately for aerosol (sect.2.1), and oxidative damage (sect.2.2). Section 2.1 refers to aerosol physicochemical properties (Sect.2.1.1), and combustion aerosol source apportionment (Sect.2.1.2). Section 2.2 refers

to in vitro effects (Sect.2.2.1), PM-induced oxidative potential (Sect.2.2.2), and biomarkers of oxidative stress (Sect.2.2.3). Section 3 presents results for the combustion aerosol source apportionment (Sect.3.1), and the related aerosol oxidative responses (Sect.3.2). Finally, Section 4 puts forward a joint discussion, in an attempt to tackle the overall topic as a whole.

2. MATERIAL AND METHODS

Measurements were carried out in the city centre of Rome (Italy), from 27 January to 28 February 2017, at a site representative of the urban background (Figure S1 of the supplementary material). Instrumentation used to measure aerosol properties and toxicological endpoints, protocols adopted and set-up, are partly described in Costabile et al. (2017b) and Gualtieri et al. (2018), and are summarised in this section. Although data from the entire field campaign are presented, the focus of this work is on periods selected for the in-vitro experiment described in Gualtieri et al. (2018): 16–17, 20–21, and 23–24 of February 2017, from 12:00 p.m. to 12:00 p.m. (E2, E3, E4, respectively), all working days of the week.

2.1. Aerosol

2.1.1. Measurement equipment

Aerosol chemical, microphysical and optical properties were measured by different techniques, under dry conditions (RH<30%), with the highest time resolution available (from seconds to 1 hour to 24 hours), and values inter-compared to reduce measurement biases. Micro-meteorological and remotely sensed data were also taken. Microphysical and optical data were collected from the same sampling line equipped with a PM_{10} sampling head, while chemical data were collected with either PM_1 or $PM_{2.5}$ sampling heads.

The continuous particle number size distribution from 0.008 to 20 μm, with 5-min time resolution, was obtained by merging data of a mobility particle size spectrometer (TROPOS SMPS) equipped with butanol-based condensation particle counter (CPC, TSI model 3772) and a commercial aerodynamic

particle sizer (APS, TSI). Spectral optical properties with 1 min time resolution were obtained by an Aethalometer (model A33, Magee Scientific, AE33), a Multi Angle Absorption Photometer (MAAP, Thermo Scientific, Waltham, MA, USA), and a Nephelometer (Model Aurora 3000, Ecotech, Australia). Chemical components were obtained from a number of instruments. The Aerodyne Aerosol Chemical Speciation Monitor (ACSM) was used to measure the major non-refractory PM_1 chemical components, namely organic aerosol, nitrate, sulfate, ammonium, and chloride, with 30-minute time resolution. The Particle Induced X-ray Emission (PIXE) analysis was used for the quantification of elemental components in $PM_{2.5}$ (Calzolai et al., 2015). The EcoChem Photoelectric Aerosol Sensor (PAS 2000 CE, EcoChem Analytics, Texas, and Matter Engineering AG, Switzerland) was used for the surface-associated total polycyclic aromatic hydrocarbons (PAHs) concentration.

2.1.2. Source apportionment

The measurement site is affected by local urban combustion sources, namely vehicular traffic and biomass burning (heating, cooking), as well as by transport aerosol sources (sulfates, dust and marine aerosols). To better investigate how these impacted the periods selected in this study, two different methods of source apportionment were applied. The non-refractory PM_1 components were analysed by positive matrix factorization (PMF) to find major sources of the organic aerosol (OA), as already presented in Costabile et al. (2017b). The particle number size distribution (PNSD, from 0.008 to 10 μm) was analysed by principal component analysis (PCA) to find relevant aerosol components, and is here presented for the first time. The methodology adopted is already described in previous studies (Costabile et al., 2009, 2010, 2017a; Brines et al., 2015). In short, principal components (PCs) retained in the analysis are arranged in decreasing order of variance explained, PC1 being the component explaining the largest variance. The k^{th} eigenvector is composed of scalar coefficients describing the new PC_k as a linear combination of the original variables (the original variables are the time series of the PNSD expressed as dN/dlogdp). Factor

loadings of PC_k represent the relative weight of the original variables in the PC_k re-scaled. Loadings thus show the 'mode' of the PNSD associated with the PCs. Factor scores of PCk represent the time series of PC_k values in the new coordinates of the space defined by the PCs. Scores thus represent the PC_k values in the time series of the original variables.

2.2. Toxicological endpoints

The equipment used to measure toxicological endpoints was istalled at the same aerosol measurement site under environmental conditions (i.e. not dry). The ALI system (Sect.2.2.1) used a very short sampling line (less than 1 m) equipped with PM_1.5 sampling head, and was located less than 1 m from the aerosol line. The system to measure the oxidative potential (Sect.2.2.2) used a sampling line equipped with $PM_{2.5}$ sampling head, and located approx. 3 meters from the aerosol sampling line. Both systems together with the biomarker study (Sect.2.2.2) are described in more detail in the followings.

2.2.1. In-vitro lung cell culture and exposure

The detailed protocol used to treat cells under environmental conditions is reported in Gualtieri et al. (2018). Briefly, BEAS-2B cells were cultured at the ALI on collagen coated inserts and 24 hours before exposure they were differentiated by removing the culture medium on the apical section of the insert. The inserts were then transferred to the CULTEX RFS Compact module and then exposed directly at the atmospheric aerosol. Control cells were exposed to the environmental air, filtered from particulate matter and volatile organic compounds. Exposure lasted for 24 hours and then cells were submitted for gene expression analysis.

The oxidative response in human cells is a complex process orchestrated by several signal transduction molecules. In our study we considered three genes, namely heme-oxygenase (HO-1), cytochrome 1B1 (Cyp1B1) and NAD(P)H-quinone-dehydrogenase (NQO1), as representative of the main processes related to air pollution that can induce reactive (oxygen) species R(O)S in exposed cells.

HO-1 is usually considered a defensive protein against oxidative damages and its up-regulation, at gene and protein level, is frequently reported in air pollution toxicological studies, NQO1, reducing quinones to hydroquinones, functions to end the redox cycling of quinones that determines the formation of ROS, finally Cyp1B1, as member representative of cytochrome proteins family, is involved in the response pathway activated to metabolise PAHs that in turn may promote the formation of reactive species and/or quinones.

Given the complexity of the biological responses, we evaluated a composite indicator (defined as cellular-oxidative response, C-OR) that takes into account the relative variation of the expression of the selected genes. Briefly, for each period of exposure the mean value and standard deviation of the genes expression was calculated for control and exposed cells. The variation of expression over the relative control group was then calculated for each gene and for each exposure experiments. The data were then considered together in order to identify the maximal variation for each gene among the three period of exposure. The maximum variation for each gene was then used to calculate the ratio of variation of that gene during the experiments: this ratio was equal to 1, for the experiment with the maximal variation of gene expression, and lower than 1 in the other experiments. C-OR was then calculated by multiplying, for each period of exposure, the relative variations of expression of the three genes previously defined. The variations were taken as absolute values in order to avoid negative values related to under expression of genes relative to the controls. R-OC may vary between 1, if during that specific exposure period all the genes were maximally expressed over the relative control, and values lower than 1, according to ratio of the expression of the genes in a specific exposure period over the maximal expression identified among all the exposure experiments. The standard deviation of C-OR was calculated starting from the standard deviation of the expression of each gene during the different exposure periods.

2.2.2. Oxidative potential (OP^{DCFH}) of $PM_{2.5}$

The aerosol oxidative potential (OP) was determined by the 2' 7'- dichlorofluorescein (DCFH) assay (OP^{DCFH}) with 2-hour time resolution in the soluble fraction of $PM_{2.5}$. Methods and protocols are already described in (Costabile et al., 2017b), together with a preliminary analysis of 2.5 days (14-17 February, E2). A particle-into-liquid sampler (PILS) was used. The sampling line was equipped with a $PM_{2.5}$ head and with a denuder line to exclude acid and basic gases from the sampling. Inside the PILS, aerosol particles were grown in a saturated water vapour chamber to form droplets. These were collected by inertial impact on a collection plate, continuously washed by deionized water. This sampler allows for a continuous collection of the PM as a diluted solution of soluble species in which insoluble particles are suspended. It is worth noting that a complete recovery of small and hydrophobic particles is not ensured by the system, as the particle growing is obtained by water condensation.

The OP^{DCFH} assay is a method trying to reproduce the NAD/NADH balance. This method was used here with a view on its frequent use in in vitro trials assessing the oxidative stress at intracellular level. The OP^{DCFH} assay was indeed formerly developed for the in vitro determination of ROS in biological cells (e.g., (Halliwell et al., 2004)). It has been applied for years, a fair amount of relevant work done on DCFH real time measurements (Venkatachari and Hopke , 2008; Huang et al., 2016; Wragg et al., 2016). It is a highly sensitive method applicable to the highly diluted PILS solutions. In this assay, the non-fluorescent DCFH is oxidized to the fluorescent dichlorofluorescein (DCF) in the presence of horseradish peroxidase (HRP).

There are other methods described in the literature to determine the OP, and each of these is sensitive to different target molecules and thus expected to lead to different OP values (Ayres et al., 2008; Janssen et al., 2014; Huang et al., 2016; Stevanovic et al., 2017; Calas et al., 2018; Weber et al., 2018; Simonetti et al., 2018a,b; Bates et al., 2019). Calas et al. (2018) showed that the OP^{DTT}, OP^{AA}, OP^{ESR}, and OP^{RTLF} assays were strongly inter-correlated,

but with different correlations to PM properties and sources, and suggested to use a combination of these assays. Weber et al. (2018) assessed the OP^{AA} and OP^{DTT} assays, and highlighted the importance of both biomass burning and vehicular sources to explain the OP for both assays, the relative importance of these sources differing from one test to the other. Fang et al. (2016) suggested a strong contribution from traffic emissions and secondary processes (e.g., organic aerosol oxidation or metals mobilization by secondary acids) to both OP^{AA} and OP^{DTT} assays in urban Atlanta. Simonetti et al. (2018a,b) showed that the OP^{AA}, OP^{DTT}, and OP^{DCHF} have a very different sensitivity towards dusts generated by difference sources and towards PM size fraction (cut in nine sizes from 0.18 to 18 μm). The OP^{DCFH} and OP^{DTT} resulted less sensitive than OP^{AA} and OP^{DTT} toward the soluble fraction of pellet ashes, on the other hand, the OP^{DCFH} and OP^{DTT} size distributions were similar (but not identical) to each other with a broad maximum in the 0.32-1.8 μm, while the OP^{AA} was sensitive almost exclusively to coarse mode particles.

2.2.3. Biomarkers of oxidative stress (DNA and RNA oxidation)

Three nucleic acid oxidation biomarkers were determined: the 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) as a biomarker of the oxidative damage to DNA, the 8-Oxo-7,8-dihydroguanosine (8-oxoGuo) as a biomarker of the oxidative damage to RNA, and (iii) the 8-Oxo-7,8-dihydroguanine (8-oxoGua) as a biomarker from both, but predominantly from DNA (Valavanidis et al., 2009).

These were determined in the human urine, the protocol to take urine samples already described in Costabile et al. (2017b). Samples were collected in sterile polypropylene containers, divided into three aliquots and frozen at -20^oC in polypropylene screw-cap tubes until analysis. The protocol was intended to allow for the collection of an elevated number of samples (approx. two samples every 12 hours collected for several consecutive days) in a relatively short period (one month, i.e. the duration of the CARE experiment) aimed at the comparison of values between two subjects with similar exposure levels. The

two subjects were a man and a woman, aged 25 and 40 years respectively, both researchers daily involved in the daily field measurements (and living nearby), with no contamination from tobacco, food and drink, and therefore exposed to similar aerosol concentrations for almost 24 hours a day. Conversely, most of the previous studies used protocols comparing two subjects groups (such as smokers vs non-smokers, patients vs controls, workers vs general population) not necessarily having a similar exposure for 24 h a day, the sample time resolution larger than the 12 hours used here. Recently, Tranfo et al. (2017) showed that the inter-individual variability in a large number of subjects (131 subjects resident in central Italy, aged 35-69 years, including smokers) is large. With a view on the inter-individual variability values obtained by Tranfo et al. (2017), in the present study we focus on the intra-individual variability of the subjects, and on its determinants, namely the aerosol exposure levels in an urban environment.

The 8-oxodGuo, 8-oxoGuo, and 8-oxoGua were determined on an aliquot of the urine samples by isotopic dilution LC-MS/MS using a AB-Sciex API 4000 triple-quadrupole mass spectrometer, according to the method described by Andreoli et al. (2010) with some modifications that involve the use of commercial standards for 8-oxodGuo and 8-oxoGuo, of a Synergi 4U Polar RP C18 column and acetic acid (instead of formic acid) for the mobile phase acidification. Under these conditions the LODs, calculated using the approach based on the Standard Deviation of the Response and the Slope, and expressed as 3.3 /S, were 2.99 nmol/L for 8oxoGua, 1.69 nmol/L for 8-oxodGuo and 2.34 nmol/L of 8-oxoGuo. The variability of the method expressed as % CV was in the range 2 -17% for intra-day and between 1 -21% for inter-day determinations.

The urinary excretion of 8-oxodGuo has been largely used as a biomarker of response to evaluate the prooxidant effects on DNA (Sekiguchi , 2006; Valavanidis et al., 2009; Lettieri Barbato et al., 2010). The rationale is that guanine residues in DNA are uniquely susceptible to alteration by ROS, and when guanine in DNA undergoes oxidation to 8-oxoguanine, its base-pairing properties do change: most cells have an active repair system removing 8-oxoguanine residues from DNA, thereby avoiding the potentially hazardous creation of a transver-

sion mutation. Conversely, the 8-oxoGuo has found limited application as a biomarker, and only recent studies have underlined its importance (Valavanidis et al., 2009; Broedbaek et al., 2011; Kjær et al., 2017; Gan et al., 2018). Broedbaek et al. (2011) showed that high urinary excretion of the 8-oxoGuo may be associated with increased mortality. Kjær et al. (2017) associated the urinary excretion of 8-oxoGuo with both all-cause and specific cardiovascular mortality risk (in patients with diabetes). (Gan et al., 2018) analysed both the 8-oxodGuo and 8-oxoGuo as the two most promising aging molecular biomarkers of physiologic age, and concluded that the urinary 8-oxoGuo may be a potential biomarker of aging (better than the 8-oxodGuo) to determine a persons physiologic age and identify individuals at high risk of developing age-associated disease.

3. RESULTS

3.1. Combustion aerosol source apportionment

The PMF performed on PM_1 non-refractory components identified three OA sources, namely vehicular traffic (hydrocarbon-like OA, HOA), biomass burning (BBOA), and oxygenated secondary aerosol (OOA). To characterize the particle number size distribution (PNSD) of these sources, a PCA was carried out on the PNSDs (Sect.2.1.2). The PCA retained three principal components (PC1PC3, explaining more than 80% of the variance), interpreted as biomass burning (PC1), fresh traffic related aerosol (PC2) and dust (PC3). This interpretation is based on the PCs particle size ranges (relevant factor loadings illustrated in Figure 1a) and diurnal cycles of scores (illustrated in Figure 1b), and on the comparison with the HOA and BBOA components identified by the PMF (illustrated in Figure 2).

The BBOA component significantly correlates with PC1 (r^2=0.8, p<0.001, Fig.2a). The HOA component significantly correlates with the traffic-related component, PC2 (r^2=0.7, p<0.001, Fig. 2b). Also, the biomass burning component significantly correlates with the eBC mass concentration (r=0.9, p<0.0001),

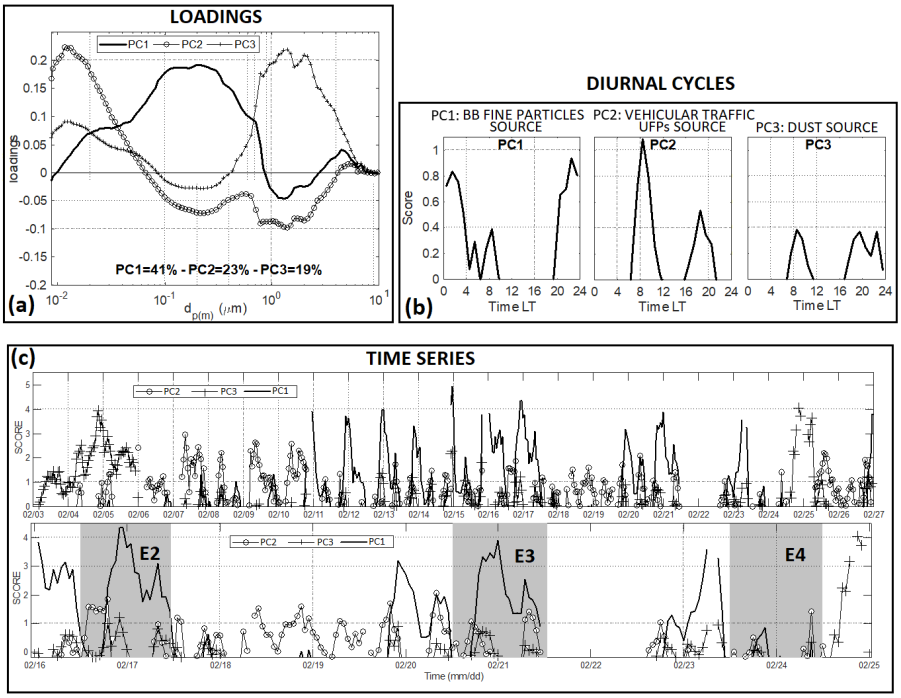

Figure 1: Source apportionment of the combustion aerosol: particle number size distributions of the major aerosol components. Three principal components (PC1 - PC3) were retained by PCA (Sect.2.1.2) explaining 41%, 23% and 19% of the temporal variance. These were interpreted as: (PC1) a biomass burning (BB) fine particle source, (PC2) a fresh vehicular traffic-related UFPs source, and (PC3) a dust source. Relevant loadings (panel a), diurnal cycles (panel b), and time series of scores (panel c) during the entire CARE experiment (with a zoom of scores during the E2-E4 periods in panel c, bottom) of PC1- PC3 are indicated.

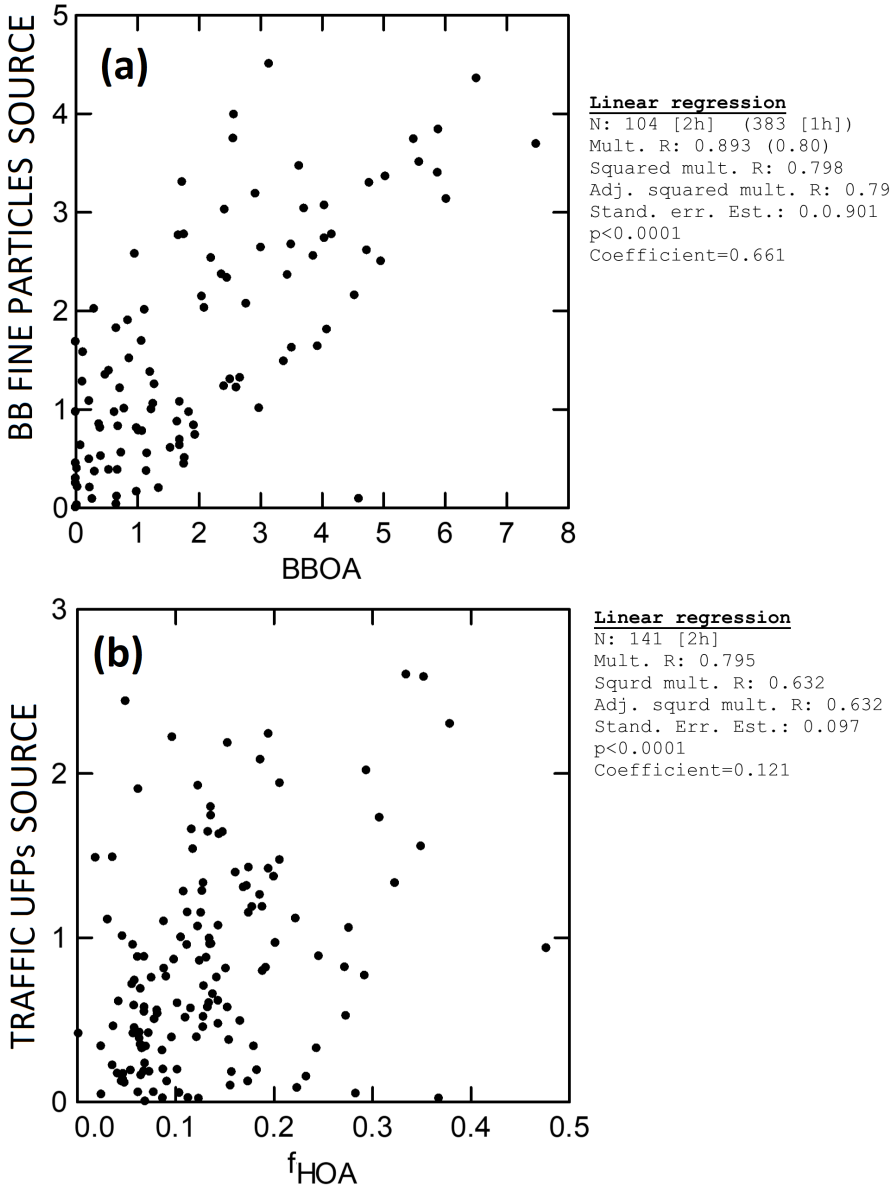

Figure 2: Source apportionment of the combustion aerosol : comparison between aerosol sources determined by the PCA of particle number size distribution and presented in Fig. 1, and aerosol sources determined by the PMF of OA. Scatter plots of: (a) BBOA component (as mass concentration) and BB fine-mode particle component (as PCA score), (b) HOA component (as mass fraction, f_{HOA}) and the traffic related UFPs component (as PCA score). Relevant linear regression data are indicated, including the number of points (N), the multiple correlation coefficient R, and the Bonferroni probability (p).

while the traffic related component correlates with the median particle diameter (inverse correlation, r=-0.73, p¡0.0001). Figure 3 shows the major PM_1 chemical components for the highest scores of these two PCs (panels a and b).

Contrary to the biomass burning component, the traffic-related component PC2 shows a mono-modal PNSD peaking in the UFP size range (below 20 nm, Fig. 1a), with high scores during the rush hours only (Fig. 1b), both arguments supporting the interpretation that it represents fresh traffic related UFPs. When we look at chemical components (Fig. 3), PC2 shows - in addition to the higher HOA content - lower OA mass fraction (48% vs 56%), and higher BC mass fraction (20% vs 12%) than the biomass burning component. These results were confronted with the preliminary results of the statistical analysis (PMF) performed on the elemental components in $PM_{2.5}$ obtained by PIXE. This was preliminarily presented in Costabile et al. (2017b) and Nava et al. (2018), and will be presented in more details in a dedicated paper. Nava et al. (2018) identified five sources, namely traffic, biomass burning, crustal, sulphate rich, and marine aerosol, with the EC mainly associated to the traffic source, and the OC to the biomass burning source.

3.2. Aerosol and oxidative responses

Time series of the aerosol physicochemical properties measured during the entire CARE experiment, already presented in Costabile et al. (2017b), are summarised for clarity in Figures S2 and S3 of the supplementary material.

Figure 4 shows the temporal patterns of the urinary excretion of the 8-Oxo-7,8-dihydroguanosine (8-oxoGuo) measured during the entire CARE experiment.

Descriptive statistics of these data together with data of the 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), and 8-Oxo-7,8-dihydroguanine (8-oxoGua) are presented in Table S1 of the supplementary material, in comparison with values reported by Tranfo et al (2017) in 131 subjects. Figure 5 shows the temporal patterns of the $PM_{2.5}$ OP^{DCFH} measured during the entire CARE experiment.

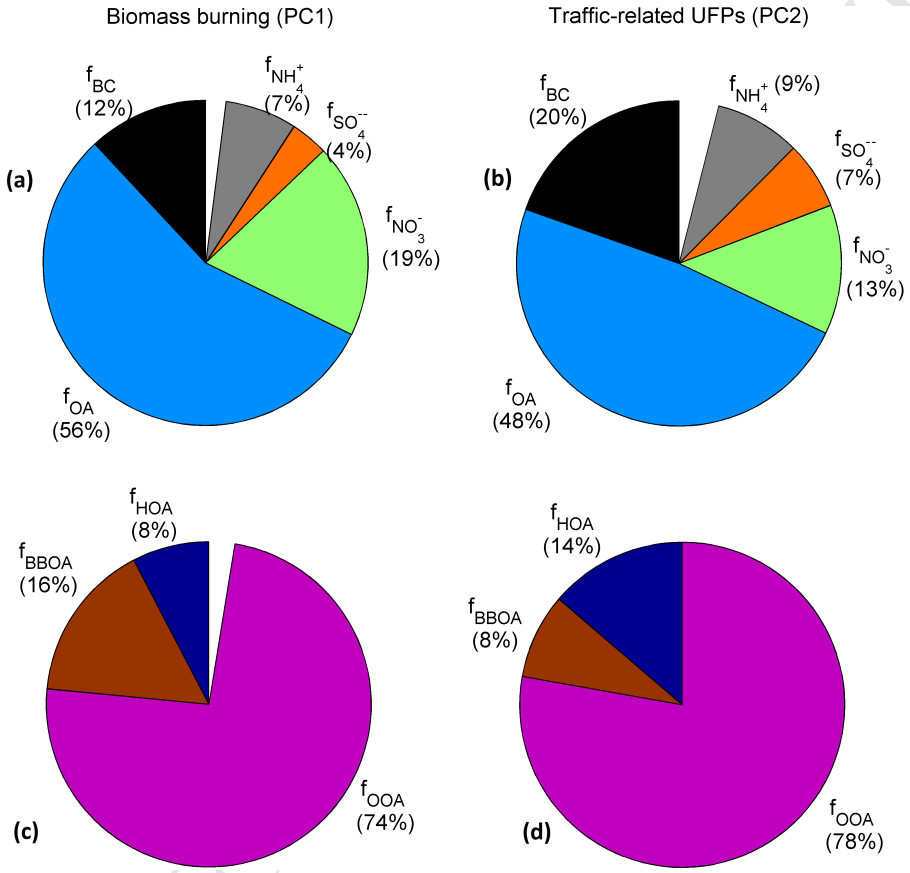

Figure 3: Source apportionment of the combustion aerosol: major chemical components of the combustion aerosol (PM_1). Pie charts of: (a) biomass burning source (as PM_1 major components), (b) traffic related UFP source (as PM_1 major components), (c) biomass burning source (as PM_1 OA major components), (b) traffic related UFP source (as PM_1 OA major components). All chemical components are expressed as mass fractions (f).

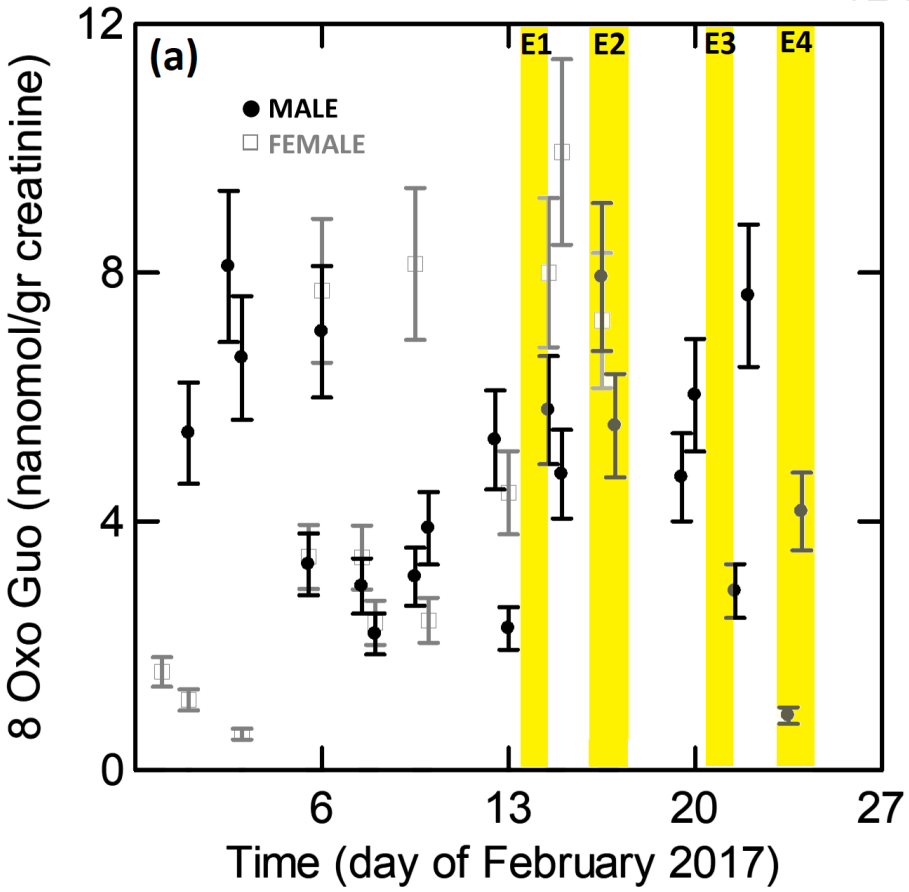

Figure 4: The biomarker of oxidative stress (RNA oxidation) measured during the entire CARE experiment: temporal patterns of the urinary excretion of the 8-Oxo-7,8-dihydroguanosine (8-oxoGuo). Data is represented by error bar graphs (median ± uncertainty), separately for male (black) and female (grey) subjects. Areas shaded yellow show the E1–E4 periods.

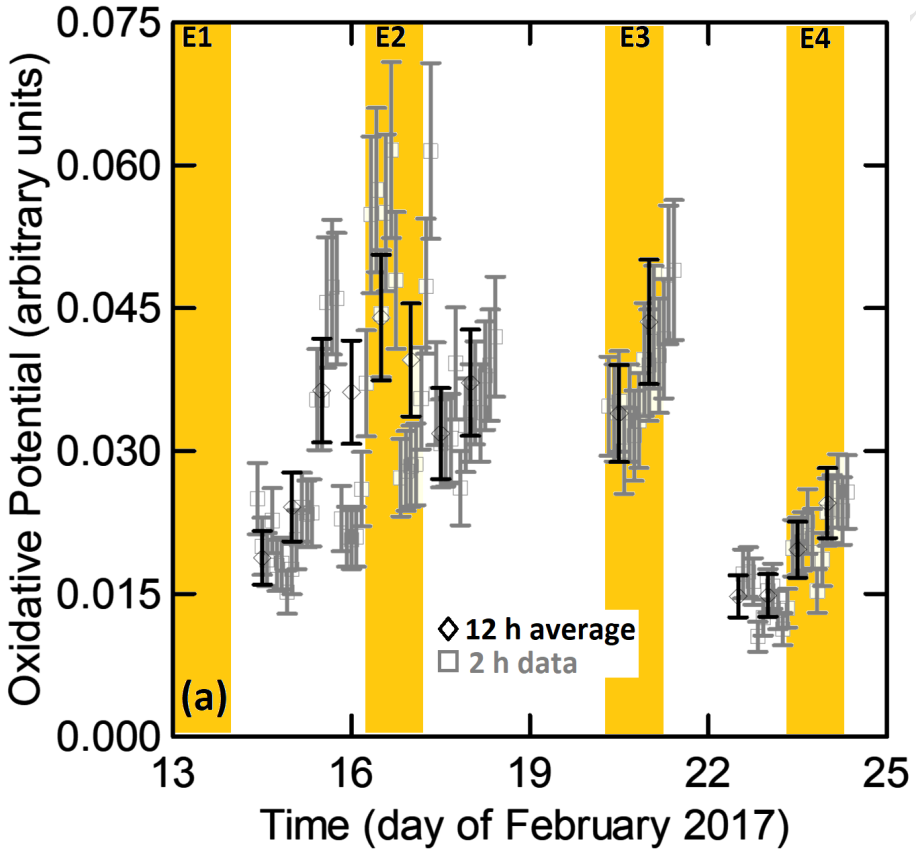

Figure 5: The $PM_{2.5}$ OP^{DCFH} measured during the entire CARE experiment. Data is represented by error-bar graphs (median ± uncertainty) for both 2-hour values, and 12-h average values (for comparison to 12-h data of the 8-oxoGuo in Fig.4). Areas shaded yellow show the E1–E4 periods.

Figure 6 compares, for the first time to our knowledge, data of the cellular-oxidative responses (C-OR) induced in the epithelial cell line of the in vitro experiment in Gualtieri et al. (2018) to data of the 8-oxoGuo and data of the $PM_{2.5}$ OP^{DCFH} presented in Fig.4 and Fig.5, respectively.

Table 1 summarizes these results, together with previous outcomes in Gualtieri et al. (2018), Costabile et al. (2017b), and Tranfo et al. (2017).

Findings in Fig.6 and Tab.1 both demonstrate a consistency between the three independent oxidative responses observed, and suggest a link between these and the aerosol properties. In contrast to E2 and E3, the E4 period was characterised by weather conditions with stronger winds (from E-SE), lower pressure, and some rain. Accordingly, we observed a lower combustion aerosol source contribution (both from traffic and biomass burning, Fig.1c, and Fig.S3 e, f, i, l) with larger mass fractions of secondary components (OOA, sulphates and ammonium, Fig.S3m, p, q). All oxidative responses during the E4 period were consistently lower. In contrast to E1, E2 and E3, there was no pro-inflammatory proteins release (IL-6 and IL-8) in the exposed cells Gualtieri et al. (2018). C-OR was low in E4. Although the C-OR values do not statistically vary among the experiments analysed, mainly due the high standard deviations, it is noteworthy the clear trend of this parameter higher in E2 and lower in E4. Lower values of both the $PM_{2.5}$ OP^{DCFH} (Fig. 6), and the 8-oxoGuo were measured (Fig. 5). Note that values of both the $PM_{2.5}$ OP^{DCFH} and the 8-oxoGuo were lower during the first part than during the second part of E4 (lower from h 12:00 to h 23:59 of 23/02 than from h 0:00 to h 12:00 of 24/02). It is worth a note that 8-oxoGuo values presented here are in the range of the inter-individual variability determined in 131 subjects by Tranfo et al. (2017) (Tab. S1 of the Supplementary material), although they have lower absolute values (subjects considered here were younger and do not include smokers).

Weather conditions during both the E2 and E3 periods were more stable than during E4. These likely favoured the increase of locally emitted aerosol concentrations (mass, number and surface, Fig.S3a,b,c and Fig.S2 of the Supplementary material), and accordingly of the combustion aerosol contribution

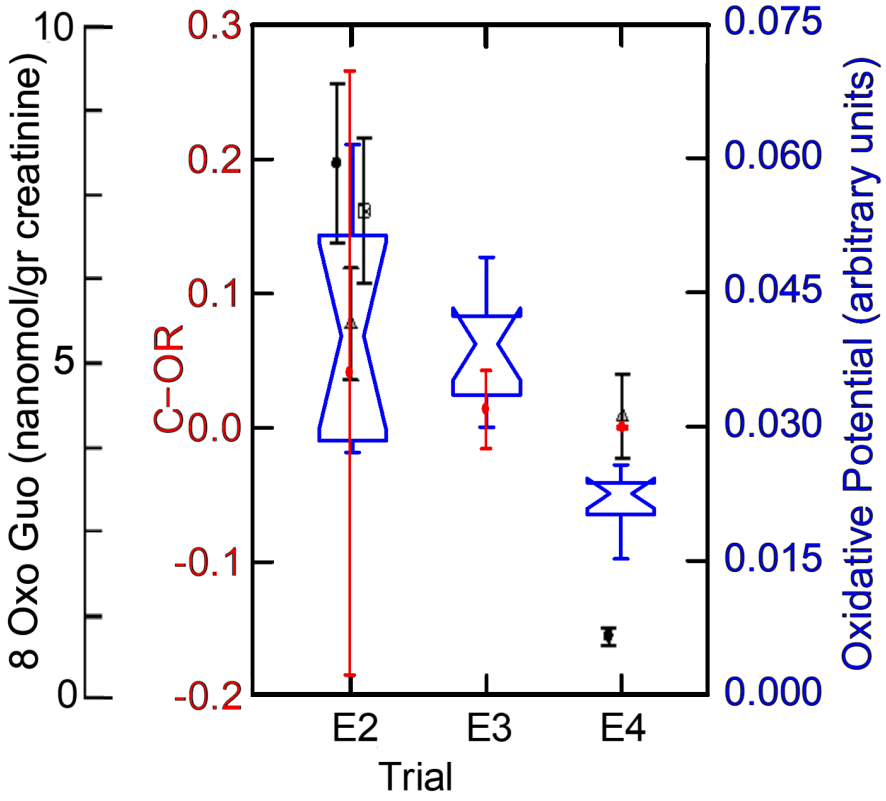

Figure 6: The aerosol-induced oxidative responses measured during the E2-E4 periods. Comparisons between the cellular-oxidative responses induced in the epithelial cell line of the in-vitro experiment (C-OR), the 8-oxoGuo (Fig.4) and the $PM_{2.5}$ OP^{DCFH} (Fig.5). The error bar graph of the C-OR data shows mean $\pm$ standard deviation. The OP^{DCFH} data is presented as box-notch (horizontal lines showing minimum observation, 25^{th} percentile, 75^{th} percentile, and maximum observation; oblique lines identifying interquartile ranges and median values). The error bar graph of the 8-oxoGuo shows median $\pm$ uncertainty.

Table 1: Summary of aerosol and biological results observed during the E2, E3 and E4 periods.

	E2	E3	E4	reference
Aerosol source	Combustion aerosol (both traffic and biomass burning) mixed to transport aerosol sources (sulf.)	Combustion aerosol (both traffic and biomass burning)	Transport aerosol source (sulfates) dominant	Costabile et al. (2017b) This work
Weather conditions	Highly stable conditions	Good weather	- Strong winds (E-SE) - low pressure - some rain	Costabile et al. (2017b)
Particle physico-chemical properties	- mass, number, surface conc. higher than in E4 -PM_1 NO_3^- mass fraction higher than in E4 - traffic related UFPs higher than in E4, but values far more dispersed around the median than in E3 - particle median diameter larger than in E3 and E4	- number, surface conc. higher than in E4 - PM_1 OA (both HOA and BBOA), PAH, and NO_3^- mass fraction higher than in E4 - traffic related UFPs higher than in E4, with values far more centred around the median than in E2 - particle median diameter smaller than in E2	- mass, number, surface conc. lower than in E2 and E3 -PM_1 SO_4^{--}, OOA, NH_4^+ mass fraction higher than in E2 and E3 - combustion related components lower than in E2 and E3	Costabile et al. (2017b) This work
In-vitro biological responses	Significant expression of genes belonging to the antioxidant responsive element (HO1 and NQO1)	Soot UFPs enriched in PAHs associated to the possible activation of the xenobiotic responsive genes (Cyp1B1)	No significant biological response	Gualtieri et al. (2018)
In-vitro cellular-oxidative response (C-OR)	- Higher C-OR than in E4 - values highly dispersed around the median	- Higher C-OR than in E4 - values centred around the median	Lower C-OR than in E2 and E3	This work
$PM_{2.5}$ OP_{DCFH}	- Higher OP_{DCFH} than in E4 - values highly dispersed around the median	- Higher OP_{DCFH} than in E4 - values centred around the median	Lower OP_{DCFH} than in E2 and E3	This work
Biomarker of RNA oxidation (8OxoGuo)	8-oxoGuo mean value: - higher than in E4 (test T, p=0.09) - in the range of the lowest values observed in 131 subjects (aged 35-69, and including smokers)		8-oxoGuo mean value: - lower than in E2 (test T, p=0.09) - in the range of the lowest values observed in 131 subjects aged 35-69, and including smokers)	Tranfo et al. (2017) This work

(Fig.1c and Fig.S3e, f, i, l). In contrast to E3, there was during E2 a larger contribution of secondary aerosol components (OOA, sulfates and ammonium, Fig.S2b of the Supplementary material, Fig.S3m, p, q). The oxidative response observed during the E2 period was important. The highest values of the $PM_{2.5}$ OP^{DCFH} were observed (Fig.5). A significant expression of genes belonging to the antioxidant responsive element (HO1 and NQO1) occurred Gualtieri et al. (2018). The mean value of 8-oxoGuo was higher (test T, p=0.09) in both subjects (Fig.4).

The oxidative response observed during the E3 period was important, as well. Larger values of the $PM_{2.5}$ OP^{DCFH} were observed, especially during the second part of the E3 period (Fig.5). The results obtained by the in vitro test were associated in Gualtieri et al. (2018) to the possible activation of xenobiotic responsive genes (Cyp1B1). In contrast to E2, during the E3 period we observed lower values of the biomass burning components (Fig.1c) and higher values of the traffic-related aerosol components (larger HOA, PAH, and eBC mass fractions, Fig.S3i, n, h and Fig. S2b of the Supplementary material), with accordingly lower OOA, sulfates and ammonium contents (Fig.S2b, Fig.4m, p, q). Note that, in contrast to E2, the scores of the traffic-related UFPs component during E3 are well centred around the median value (Fig.S3f), and are as high as those of the biomass burning component during the second part of the E3 period (Fig.1c).

4. DISCUSSION

We are aware that the present work presents some limitations, i.e. that the number of experiments during which all the three independent parameters were recorded is limited (only three days), that the number of genes considered to calculate the C-OR is limited and that other genes may be more representative (such as the variation in Nrf2, ALDH3A1, AKR1B3, etc.), and finally that the number of persons involved in the biomarker evaluation is very limited and cannot account for a proper representativeness of an exposed population.

Nonetheless, results of this work (Sect.3) provide an evidence of association between three completely independent oxidative responses, and suggest a dependency between these and the combustion aerosol. Larger values of the in vitro cellular-oxidative response in an epithelial cell line directly exposed to the ambient $PM_{1.5}$, and of the OP^{DCFH} assay induced by the soluble fraction of $PM_{2.5}$, and of the urinary excretion of 8-Oxo-7,8-dihydroguanosine, a biomarker of RNA oxidation, were all associated in a consistent manner to periods with larger contributions from combustion aerosols. Why this is observed is something that we can't explain at this point. In addition, although very far from being clarified, these results put forth many open questions. We reckon worthy to define at least four possible main fields of investigation that can shed light on the associations here reported. These are further discussed in this section.

First, it should be clarified how differently certain combustion aerosol types (namely, vehicular traffic and biomass burning) contribute to generate the $PM_{2.5}$ induced oxidative response. Here, we found different contributions from the traffic-related aerosol and the biomass burning aerosol. Three completely independent measures indicated in a consistent manner that (Fig.6 and Tab.1): (i) the higher the combustion aerosol exposure levels, the higher the oxidative response (higher during the E2 and E3 periods than during the E4 period), and (ii) the more the aerosol is dominated by combustion sources, in particular from the fresh traffic-related aerosol (higher during the E3 period than during the E2 period), the more the oxidative response is statistically centred around the median value. The latter point (ii) is hard to explain here, but calls for a further speculation on the role of the traffic-related aerosol. On the one hand, we may explain it with an inverse association between the oxidative response and the secondary aerosol: the higher the aerosol sulfates, ammonium and OOA content (higher during E4 than during E2 than during E3, Fig.S3m, p, q), the lower the oxidative response (lower during E4 than during E2 and E3, Fig.6). This result is consistent with Weber et al. (2018), which found that the sulphate-rich aerosol was slightly anti-correlated to the OP^{AA}. This is in contrast, however, with other works having showed a correlation between markers of SOA and OP

(McWhinney et al., 2010; Stevanovic et al., 2013). McWhinney et al. (2010) demonstrated increasing levels of oxidation of engine exhaust correlated to increased redox-cycling activity. Stevanovic et al. (2013) showed that the PM oxidative potential had a much higher correlation with the oxygenated organic fraction. On the other hand, we may thus explain the result with a link between the oxidative response and the fresh traffic-related aerosol. This is supported by data of Figure S4 of the Supplementary material, showing that the $PM_{2.5}$ OP^{DCFH} weakly, but significantly, increased with increasing the traffic-related UFPs component (r^2=0.4, p<0.001) during the entire CARE experiment (83 points), while it did not correlate with the biomass burning component. Figure S5, Figure S6 and Figure S7 of the supplementary material show that data follow the same pattern if we look at the E2, E3, and E4 periods separately. This result is consistent with our findings in Gualtieri et al. (2018), which have showed that soot UFPs enriched in PAHs (higher for the traffic related aerosol) could have activate xenobiotic responsive genes (CYP1B1 cytochrome activation). This result is consistent with previous studies, which have highlighted the importance of the vehicular traffic source to explain the OP^{AA} and OP^{DTT} (Verma et al., 2014; Bates et al., 2015; Fang et al., 2016; Weber et al., 2018). Fang et al. (2016) suggested a larger contribution from traffic emissions to both OP^{AA} and OP^{DTT} activities in urban Atlanta (they found a weaker activity for the biomass burning source in the OP^{AA} test). Weber et al. (2018) found values of the intrinsic OP^{DTT} test higher for the vehicular source than for the biomass burning source (the opposite was found for the OP^{AA} test). However, this is still a contrasting issue, and some authors (e.g., Simonetti et al. (2018b) suggest a larger activity for the biomass burning source in the OP^{DTT} and OP^{DCHF}. It is noteworthy, also, that Fuller et al. (2014)'s results (obtained with the DCFH/HRP assay) suggested that ROS in oxidized organic particles can be divided into a short-lived fraction (half-life of only a few minutes), and a long-lived ROS fraction (stable for hours or days), the short-lived one dominating the total ROS concentration (5 times larger than the long-lived ROS components). The higher correlation found with the traffic source could depend

on the detection of the short-lived species (explained by the relatively short distance of our measurement site from the traffic-related source). Future studies should confirm, or reject, our hypothesis of association between fresh traffic related aerosol and oxidative response. It is worthy of note that this speculation is based on the aerosol source contribution taken as a whole, and not on a specific particle type, chemical component or physical property, and includes accurate data of UFPs.

Secondly, it should be clarified how differently certain particle properties contribute to generate the $PM_{2.5}$ induced oxidative response. We argue if particle size (as indicated by the traffic-related UFPs), or particle composition (as indicated by the PM_1 HOA), or particle solubility, or particle physical state (solid, liquid, glassy), are relevant metrics to explain our findings. Particularly challenging is to understand the role of particle solubility and physical state, both properties being in turn linked to particle size, and composition. The OP^{DCHF} assay here was performed on the water-soluble $PM_{2.5}$ fraction plus the entire suspended particle fraction passing through a 0.45 μm filter (i.e., including the insoluble non agglomerated UFPs). The in vitro cell experiment at the ALI here was performed on the total (soluble and insoluble, liquid and solid) airborne $PM_{1.5}$. The biomarkers of RNA oxidation in the urine suitably tracked here the total inhalable PM fraction. There may be many completely unknown mechanisms by which combustion aerosol by-products enter the circulation system (e.g., translocation of insoluble UFPs, dissolution of water-soluble compounds) or by which the oxidative response triggered in the lung is transferred to other tissues in the body (translocation of biochemical mediators of response and/or damage), and affects other targets in the body (namely, the brain). We here intend only to present these responses altogether (Tab. 1), and open the discussion. Particularly, we argue if, and via what mechanisms, either soluble (water-soluble or not) combustion aerosol by-products, or insoluble (UFPs or not, solid or not) particles can be associated, directly or indirectly, to the oxidative responses observed. Also, we especially argue what is the link to the biomarkers of RNA oxidation here determined. So far toxicological studies,

both in vitro and in vivo, reported PM responses considering all the particles as solid (i.e., without arguing if all, or part of them, may be actually liquid or glassy). Studies on lipid nanoparticles, intended for biomedical application, clearly showed that the physical state of the particles, solid or not, may induce different responses in cells (Sharma et al., 2012; Knudsen et al., 2015). We argue if this is relevant here to interpret our results and in general fine PM induced health effects.

Thirdly, it should be clarified if and how combustion sources contribute to the association observed here in a consistent manner between completely independent measures of the aerosol induced oxidative response. Namely, we argue if these PM induced oxidative responses follow the same consistent pattern because the exposure was dominated by combustion sources. In answering this question, we may understand if the $PM_{2.5}$ OP^{DCFH} can be used as one of the suitable proxies to predict the oxidative response to the combustion aerosol exposure (and perhaps to fresh traffic-related UFPs). In fact, it is still debated in the literature what OP assay is relevant for studies of health effects. In a recent review, Bates et al. (2019) conclude that overall the current studies have suggested that assays such as DTT and AA can be the suitable ones, though this could be due to the limited number of studies still carried out. Our study here provides an evidence that DCFH (which measures the OP in a significantly different way the DTT and AA do) also shows correlation with health markers. As such, this result highlights the complexity of assessing the OP and suggests that no single acellular assay should be exclusively used. Also, in answering this question, we may understand if the urinary excretion of the 8-Oxo-7,8-dihydroguanosine (8-OxoGuo) can be used as a molecular biomarker of the RNA oxidation induced by combustion aerosol exposure (and perhaps by fresh traffic-related UFPs). Although appealing, the latter point (about the biomarker) is clearly something that we cannot clarify at this time. Nor we can explain why the other metabolites determined in the urine, and in particular the 8-oxodGuo, a biomarker for DNA oxidation, did not show the same pattern (Tab.S1 of the supplementary material). We note here, however, that our results

on the 8-OxoGuo support very recent results in Gan et al. (2018) demonstrating that the urinary 8-oxoGuo is a potential biomarker of aging better than the 8-oxodGuo, and suggesting that RNA oxidation is a more sensitive parameter to follow for environmental pollution-related (and perhaps combustion pollution - related) oxidative damages. The fact that RNA is single stranded, while DNA is double stranded (the double strand sterically protects the molecule of the bases) is an argument to support the hypothesis that the guanine in RNA is more exposed to chemical modifications than in DNA. Our results are also supported by our concurrent analysis on the urinary biomarkers of oxidative damage to DNA and RNA in different groups of workers (approx. 200 workers, including CARE ones) compared to general population volunteers, showing that 8-oxoGuo is a potential biomarker of oxidative stress in humans in response to chemical agents, and is more sensitive than 8-OxodGuo and 8-OxoGua (Tranfo et al., 2019).

Finally, our findings show an association between $PM_{2.5}$ OP^{DCFH} and a biomarker of RNA oxidation (8-oxoGuo, Fig.6). These two values (OP and 8-oxoGuo) were measured through completely different and independent principles and methods and on different substrates ($PM_{2.5}$ and human urine samples, respectively), but in the same location and on the same days. Although independent of each other, these values match each other in response to the same stimulus (exposure to the same air), and match the in-vitro cellular-oxidative response. We can't provide clear evidences of the causal relation linking them. However, based on previous studies (in particular we refer the reader to Nel (2005); Møller et al. (2008); Valavanidis et al. (2013); Crobeddu et al. (2017); Gan et al. (2018); Bates et al. (2019)), we can speculate on it. The $PM_{2.5}$ OP^{DCFH} is one of the assays used for the detection of ROS. ROS can damage biomolecules such as DNA/ RNA. Cells defend against this damage using their key antioxidants, whose depletion can create a state of cellular stress (oxidative stress). This may be linked to an increased antioxidant enzymes production, under the control of a specific transcription factor, Nrf2. When this defence is insufficient to avoid cell damage, an inflammatory response may be triggered.

Oxidized DNA/RNA base lesions (8-oxoGua, 8-oxodGuo, and 8-oxoGuo) may be biomarkers of such oxidative stress in humans, in particularly in surrogate tissues such as the urine. Therefore, our values of the $PM_{2.5}$ OP^{DCFH}, possibly indicating PM-induced ROS generation, could be linked to an increased risk of oxidative damage to DNA/RNA, and our urinary excretion of 8-oxoGuo could be a biomarker of response evaluating the pro-oxidant effects on RNA. Their association presented here could imply the possibility that one or more components of $PM_{2.5}$ or some leaked constituents are either translocated systemically or that there are indirect effects caused by inflammatory mediators. Again, future studies should shed light on this relation.

Yet this is not a substantial basis for drawing any cause-effect relationship, in particular between traffic-related combustion aerosol and the oxidative response. We cannot conclusively infer this causality, but argue that our findings support (for the first time) previous literature studies (Delfino et al., 2013; Verma et al., 2014; Bates et al., 2015; Fang et al., 2016; Chen et al., 2017; Weber et al., 2018; Gan et al., 2018) with a strong consistency of results across completely independent measures of the aerosol induced oxidative response. This is a piece of information that merits an assessment of the importance of the combustion aerosol source in oxidative response related diseases. Clearly, on the basis of the available information, several issues cannot be ruled out, and these are only hints for future studies. Our work here intends to inform future research directions regarding these, and highlight some important questions on what should be considered to explain it.

Conflicts of interest. The authors declare no conflict of interest.

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HIGHLIGHTS

- Causal link between ambient $PM_{2.5}$ and adverse health effects not clear
- Aerosol properties compared to in-vitro and in-vivo oxidative responses
- Combustion-dependent association of in-vitro, in-vivo, and $PM_{2.5}$ oxidative responses
- Importance of the combustion aerosol in oxidative response related diseases
- Toxicological role of aerosol particle size, chemistry, solubility, physical state