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Short communication

On fine particulate matter and COVID-19 spread and severity: An *in vitro* toxicological plausible mechanism

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ABSTRACT

COVID-19 pandemic had a significant impact on global public health. The spread of the disease was related to the high transmissibility of SARS-CoV-2 virus but incidence and mortality rate suggested a possible relationship with environmental factors. Air pollution has been hypothesized to play a role in the transmission of the virus and the resulting severity of the disease. Here we report a plausible *in vitro* toxicological mode of action by which fine particulate matter ($PM_{2.5}$) could promote a higher infection rate of SARS-CoV-2 and severity of COVID-19 disease. $PM_{2.5}$ promotes a 1.5 fold over-expression of the angiotensin 2 converting enzyme (ACE2) which is exploited by viral particles to enter human lung alveolar cells (1.5 fold increase in RAB5 protein) and increases their inflammatory state (IL-8 and NF-kB protein expression). Our results provide a basis for further exploring the possible synergy between biological threats and air pollutants and ask for a deeper understanding of how air quality could influence new pandemics in the future.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has been a global significant public health emergency, yielding millions of deaths (Msemburi et al., 2023). The pathogenic agent involved is the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a highly virulent and aggressive enveloped RNA beta coronavirus that was first found in Wuhan (China) in December 2019 and subsequently spread throughout the world (Wang et al., 2020). In addition to respiratory manifestations, some patients experienced serious complications resulting in severe acute respiratory syndrome, organ failure and septic shock. Older people with pre-existing comorbidities, such as respiratory, cardiovascular, endocrine and digestive diseases were particularly at risk of a severe course of the disease (Callender et al., 2020; Huang et al., 2020). Although COVID-19 has had global impacts, significant differences in incidence, severity and mortality have been observed across countries. However, these differences cannot be explained by dissimilarities in health care systems alone.

It has been suggested that environmental factors such as air pollution, may have played a role in facilitating the spread and transmission of SARS-CoV-2, with a number of mechanisms suggested (Comunian et al., 2020; Conticini et al., 2020).

Exposure to fine particulate matter ($PM_{2.5}$) may possibly be related to increased severity and lethality of COVID-19 through its impact on chronic disorders, such as pulmonary and cardiovascular diseases and diabetes (Kim et al., 2018; Copat et al., 2020). Chronic exposure to air pollutants is known to lead to a general reduction in the immune response and an increase in hyper-inflammatory biomarkers, therefore, the ability of the human body to respond to infections is impaired. As a consequence, the pre-existing conditions could possibly facilitate viral penetration and replication (Kim et al., 2018; Roychoudhury et al., 2020). Ecological studies have explored, to different degrees, the potential associations by determining the attributable cases of COVID-19 cases or associated deaths related to poor air quality (Karimi et al., 2022; Tello-Leal and Macías-Hernández, 2021; Veronesi et al., 2022). The results from local epidemiological studies of the association

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between long-term, global exposure of the population to anthropogenic $PM_{2.5}$ pollution and COVID-19 death cases have been also extended to global scale thanks to numerical models (Pozzer et al., 2020). Recently, a regional study (De Angelis et al., 2021), over the Lombardy region in Italy, further supported the importance of air pollution during the first wave of COVID-19 cases and related mortality. Despite the results from ecological and epidemiological studies, these do not allow drawing any causal association linking airborne $PM_{2.5}$ to COVID-19 related fatalities (Taylor et al., 2022).

A number of systematic reviews have been conducted to assess the epidemiological evidence on the influence of air pollution on COVID-19. Recently, Carballo et al. (2022) (Hernandez Carballo et al., 2022) reviewed the literature up to mid-2021, finding that approximately half of the studies found associations between incidence, severity and mortality, with higher associations due to long-term exposure. They commented that course PM_{10} (particulate matter<10 μm in aerodynamic diameter), fine $PM_{2.5}$ (particles<2.5 μm in aerodynamic diameter), ozone (O_3), nitrogen dioxide (NO_2), and carbon monoxide (CO) were most strongly positively associated with COVID-19 incidence, and $PM_{2.5}$ and NO_2 with COVID-19 deaths (Hernandez Carballo et al., 2022). Epidemiological studies of COVID-19 have been criticized particularly with respect to confounder control and bias in exposure outcomes (Hansell and Villeneuve, 2021; Villeneuve and Goldberg, 2022).

A number of studies have shown that elevated levels of air pollution could be associated with an increased incidence of respiratory viral diseases, including influenza and severe acute respiratory syndrome (SARS) in exposed individuals (Cao et al., 2014; Groulx et al., 2018; Hsiao et al., 2022; Chen et al., 2020; Su et al., 2019; Nor et al., 2021). However, it is still unclear how long the airborne microorganisms under different ambient atmospheric conditions remain infectious and whether the particulate matter (PM)-related viral load is sufficient to induce infection. Therefore, the impact of bioaerosols on human health is still poorly understood and the role of $PM_{2.5}$ as a vector or carrier for SARS-CoV-2 transmission continues to be controversial (Ishmatov, 2022).

Some authors have proposed that $PM_{2.5}$ could have a direct and/or indirect systemic impact on the human body by increasing inflammation and oxidative stress, ultimately leading to respiratory, cardiovascular and immune system dysfunction, thereby reducing resistance to infection and making people more susceptible (Pope et al., 2016; Santurtún et al., 2022). Furthermore, some studies have demonstrated the ability of $PM_{2.5}$ to induce an inflammatory response in the respiratory tract resulting in the induction of angiotensin 2 converting enzyme (ACE2), present in type-2 pneumocytes. ACE2 is highly expressed in the lung, the heart, the vasculature, the gastrointestinal mucosa and the brain, possibly explaining the heterogeneity of COVID-19 symptomatology. As for the lung, the membrane receptor is mainly expressed in the alveolar regions, explaining the deleterious viral effects at the respiratory level (Chen et al., 2021; Davies et al., 2021; Buzhdygan et al., 2020; Farahani et al., 2022). ACE2 acts as a negative regulator of the renin-angiotensin system (RAS) by reducing pulmonary inflammation, thereby protecting the lung from injury (Zhu et al., 2022; Monteil et al., 2020; Lin et al., 2018; Sagawa et al., 2021). Noteworthy, the cell surface receptor ACE2 has also been proposed as a viral entry into respiratory host cells due to the high affinity of SARS-CoV-2 for this membrane receptor (Borro et al., 2020; Zhu et al., 2021; Zhang et al., 2020). In fact, ACE2 is recognized by the virus spike (S) glycoprotein and exploited to enter host cells and spread.

Despite the number of mechanisms proposed, to date there is a lack of toxicological evidence on the role of $PM_{2.5}$ in increasing the SARS-CoV-2 infectiousness, as suggested by some epidemiological studies. From this perspective, it is important to identify the molecular pathways specifically triggered by environmental exposure to airborne particles and to identify a plausible mechanism of action by which air pollution may act as a predisposing factor for the incidence of the COVID-19 and consequent spread of the pandemic.

Activation of a strong inflammatory response, dysregulation of the immune system, and increased expression of the membrane receptor ACE2 have been suggested as probable links between air pollution and COVID-19 (Santurtún et al., 2022; Borro et al., 2020).

In the present paper, we investigate the hypothesis that $PM_{2.5}$ might facilitate SARS-CoV-2 infection, and lead to a more severe manifestation of the disease, through an increase in the basal state of lung inflammation and a consequent increase in the expression of ACE2 receptor. We hypothesize that pathophysiological conditions requiring ACE2 activation could represent a “Trojan horse” for viral infection, increasing one of the entry sites available to the virus, and thereby augmenting the susceptibility to infection. $PM_{2.5}$-induced ACE-2 over-expression may be therefore exploited by the virus to infect target cells. We also hypothesize that $PM_{2.5}$-related pathophysiological conditions, associated with an increased inflammatory status may ultimately exacerbate disease progression (Frontera et al., 2020; Kim et al., 2020). Specifically, we investigated *in vitro* the interplay of SARS-CoV-2 and fine urban aerosol particles ($PM_{2.5}$ collected in Milan during winter 2021) in a human alveolar epithelial cell line (A549), extensively used for inhalation toxicology purposes (Marchetti et al., 2019), to explore if $PM_{2.5}$ primes lung cells to viral infection by modulating ACE2 receptor expression; if the increased expression of this receptor determines an increased infectability of the cells; and whether $PM_{2.5}$ may aggravate the potency of the inflammatory response to viral infection. To sustain the proposed toxicological evidence, the results have been discussed in parallel to the modelling of global exposure of the population to $PM_{2.5}$ pollution and the attributable hazards and the recent epidemiological findings.

2. Results and discussion

Time course and concentration–response experiments (data not shown) were preliminarily performed on A549 cells to select the optimal conditions for the study, especially aiming at establishing sub-acute exposure conditions at relatively low $PM_{2.5}$ concentrations for prolonged exposure time, as well as the concentration of viral particles. Based on the results obtained from viability (Alamar Blue) and cytokines release (IL-6 and IL-8) assays, a 72 h treatment at 2.5 $\mu g/cm^2$ was selected for $PM_{2.5}$ exposure. With regards to SARS-CoV-2, cells were treated with 2.4×10^3 genome copies.

2.1. *Increased infectability of lung cells by airborne $PM_{2.5}$*

The primary question we seek to answer is if there is a possible causal relation that links air pollution, airborne $PM_{2.5}$ in particular, to increased infectiousness of SARS-CoV-2. We explored, as a plausible link, the $PM_{2.5}$ ability to induce a state of alveolar cell inflammation that leads to an increased expression of ACE2. We show here that a 72 h exposure of human lung alveolar cells to airborne $PM_{2.5}$ is able to significantly increase the expression of the membrane ACE2 receptor. Compared to the control, in fact, cells exposed to $PM_{2.5}$, also in presence of SARS-CoV-2, showed a statistically significant increased expression of ACE2 that was not activated after exposure to solely SARS-CoV-2 particles (Fig. 1). $PM_{2.5}$ is able therefore to increase the receptor used by SARS-CoV-2 to infect the host cell even at relatively low *in vitro* exposure concentrations.

These results are consistent with recent studies demonstrating that particulate matter is able to induce lung injuries and increase the expression of ACE2 receptors in murine models (Chen et al., 2020; Ishmatov, 2022; Gunaratne and Marchant, 2022) and human epithelial cells (Lin et al., 2018). In line with these studies, Botto and colleagues (2023) (Botto et al., 2023) also reported that sub-acute exposure to $PM_{2.5}$ induces alterations in the ACE/ACE2 pathway in mice, which could predispose to increased susceptibility to SARS-CoV-2 infection.

The increased expression of ACE2 on the apical membrane of lung cells, therefore, represents the first step of a possible increased infectability. The capability of SARS-CoV-2 to bind to ACE2 and enter the host

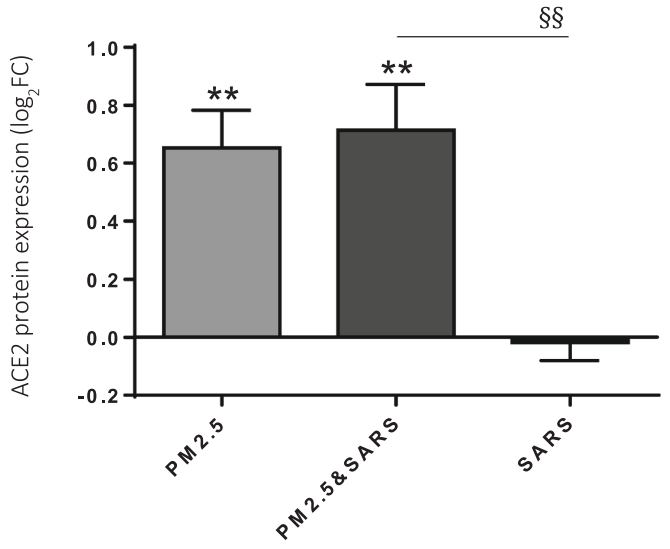

Fig. 1. Overexpression of the ACE2 receptor in PM and PM&SARS-CoV-2 exposed cells. ACE2 protein expression in A549 upon co-exposure to PM2.5 (2.5 μg/cm^2) and SARS-CoV-2 (2.4 × 10^3 genome copies) for 72 h. ACE2 protein was assessed by western blot in the whole cellular lysate. Bars represent the mean ± SEM of three independent experiments (n = 3). Control value (i.e., lung cells not exposed to PM nor to SARS-CoV-2) is equal to zero (0 ± 0.02) having considered the log$_2$ of the fold change. Statistical analysis was performed by One-Way ANOVA with Dunnett's multiple comparison test. $^{**}p < 0.01$ versus control cells, $^{§§}p < 0.01$ versus PM2.5&SARS exposed cells.

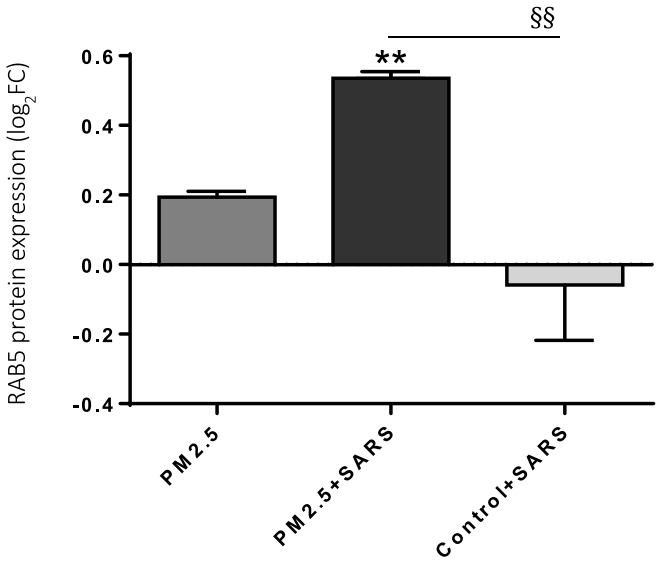

Fig. 2. Increased activation of the Rab5 protein expression. Rab5 protein expression. A549 cells were primed with PM2.5 (2.5 μg/cm^2) for 72 h, then SARS-CoV-2 (2.4 × 10^3 genome copies) was added for 2 h. Rab5 protein was assessed by western blot in the whole cellular lysate. Bars represent the mean ± SEM of three independent experiments (n = 3). Control value is equal to zero (0 ± 0.08) having considered the log$_2$ of the fold change. Statistical analysis was performed by One-Way ANOVA with Dunnett's multiple comparison test. $^{**}p < 0.01$ versus control cells, $^{§§}p < 0.01$ versus PM2.5 + SARS exposed cells.

cells is the second step to be assessed.

Many enveloped viruses use surface receptors as an entry site into the host cell. After binding to the receptor, endocytic uptake begins and they are transported through early endosomes (EE), late endosomes (LE) and late endosome-lysosome. At this point, they could escape the endolysosomal compartment by fusion between the viral envelope and endosomal membranes resulting in the release of viral nucleic acids into the cytoplasm. The final stages of the viral life cycle are the replication and assembly of the new virus particles and finally the cellular exit by budding through the plasma membrane of the host cell (Gunaratne and Marchant, 2022).

In order to gain more insight into the mechanism by which $PM_{2.5}$ favors SARS-CoV-2 internalization and hypothesizing that SARS-CoV-2 virions are internalized via an endocytic pathway, we checked the protein expression of the early endosome marker Rab5 in cells primed with $PM_{2.5}$ for 72 h and then exposed to viral particles for additional 2 h. Interestingly, in cells with high ACE2 expression (primed with PM) and then exposed to SARS-CoV-2, Rab5 expression was also found to be significantly increased (Fig. 2), suggesting an enhanced capability of viral internalization through endocytosis in $PM_{2.5}$ primed cells.

These data are consistent with a previous work, which indicates that enveloped viruses often enter cells via endocytosis. Influenza virus indeed requires both early and late endosomes for entry and subsequent infection of host cells, whereas Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV) only transit through early endosomes (Sieczkarski and Whittaker). Macovei et al. (Macovei et al., 2013) reported that human hepatitis B virus (HBV) infection also strongly depends on the expression of Rab5 and Rab7 in differentiated and permissive HepaRG cells (Macovei et al., 2013). Another group found that the rate of adenovirus infection is also related to an increase in early endosomes, as evidenced by over-expression of Rab5 (Rauma et al., 1999).

These findings strengthen the hypothesis that individuals (sub) chronically exposed to $PM_{2.5}$ are potentially more susceptible to the infection of SARS-CoV-2, mediated by an increased endocytosis of ACE-2-bound viral particles at alveolar level.

The capability of $PM_{2.5}$ to induce an increase of ACE2 and the capability of SARS-CoV-2 to exploit it support ecological studies, during the first wave of COVID-19, reporting positive correlations between the daily COVID-19 incidence and $PM_{2.5}$ concentrations, both in China (Zhu et al., 2020) and northern Italy (Fattorini and Regoli, 2020), two strongly polluted regions. The northern regions of Italy have been more strongly affected by the SARS-CoV-2 pandemic than other areas with similar socio–economic backgrounds (Conticini et al., 2020; Borro et al., 2020; Fattorini and Regoli, 2020). These regions represent one of the most severely polluted area in Europe. According to a recent study (Borro et al., 2020), high levels of $PM_{2.5}$ (up to 60–70 μg/m^3) were observed in most of the Po Valley in January and February 2020, before the spread of the virus. Subsequently, the greatest effects in terms of infections were observed in the same area. Findings by Borro and colleagues (Borro et al., 2020) show that increased $PM_{2.5}$ concentrations were closely associated with increased infection rates. Fattorini and Regoli also demonstrated a significant correlation of a number of pollutants (NO_2, O_3, $PM_{2.5}$ and PM_{10}) with the incidence of COVID-19 cases in 71 Italian provinces (Fattorini and Regoli, 2020).

2.2. *Increased responsiveness of lung cells exposed to airborne PM*

SARS-CoV-2 infection is known to trigger an exaggerated immune response characterized by increased levels of pro-inflammatory chemokines and interleukins such as IL-1β, IL-2, IL-6, IL-8 (CXCL8), and IL-10, resulting in worsening the severity of the disease (Hsu et al., 2022).

To further elucidate whether increased SARS-CoV-2 internalization also corresponds to increased inflammatory response, we investigated NF-κB activity and IL-8 release on cells primed with PM for 72 h and subsequently exposed to the virus for additional 24 h (Fig. 3A and B). Indeed, it has been reported that the increased activation of NF-κB leads to an elevated production of pro-inflammatory mediators, including IL-8. Furthermore, upregulation of the pro-inflammatory transcription factor NF-κB has been reported to be involved in the development of SARS-CoV-2 infection resulting in an uncontrolled systemic inflammatory response, increasing COVID-19 severity (Gudowska-Sawczuk and Mroczko, 2022; Su et al., 2021). Forsyth and colleagues reported that S1

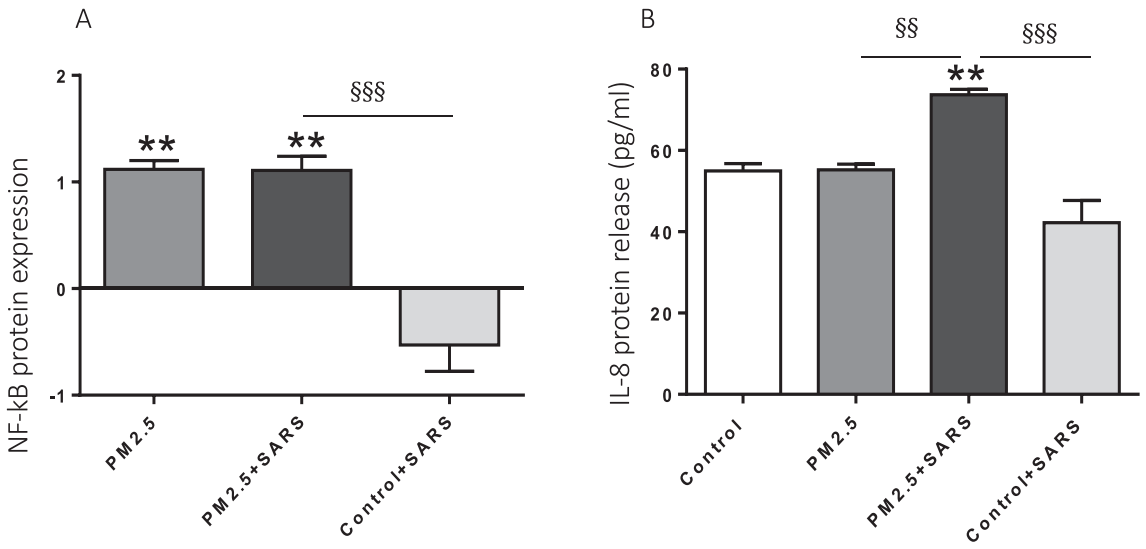

Fig. 3. Increased pro-inflammatory pathway activation. A. NF-κB protein expression. A549 cells were exposed only to PM2.5 (2.5 μg/cm^2) for 72 h, then SARS-CoV-2 (2.4×10^3 genome copies) was added for additional 24 h. NF-κB protein was assessed by western blot in the whole cellular lysate. Bars represent the mean ± SEM of three independent experiments (n = 3). Control value (0 ± 0.05) is equal to zero having considered the $\log_2$ of the fold change. Statistical analysis was performed by One-Way ANOVA with Dunnett's multiple comparison test. $^{**}p < 0.01$ versus control cells, $^{§§§}p < 0.001$ versus PM2.5 + SARS exposed cells. B. IL-8 release. A549 cells were primed with PM2.5 (2.5 μg/cm^2) for 72 h, and then SARS-CoV-2 was added for additional 24 h. Bars represent the mean ± SEM of three independent experiments (n = 3). Statistical analysis was performed by One-Way ANOVA with Dunnett's multiple comparison test. $^{**}p < 0.01$ versus control cells, $^{§§}p < 0.01$ PM2.5 exposed cells and $^{§§§}p < 0.001$ versus PM2.5 + SARS exposed cells.

Spike protein promotes the activation of the inflammatory signaling through NF-κB expression and cytokines production on both human lung (A549) and intestinal epithelial cells (Caco-2) after 24 h of exposure, regardless of the virus SARS-CoV-2 (Forsyth et al., 2022). A study on mice and human cells infected with SARS-CoV-2 also showed upregulation of several inflammatory chemokines, many of which are regulated by NF-κB (Lacasse et al., 2023).

In agreement with these studies, our results demonstrated that $PM_{2.5}$-primed cells respond to SARS-CoV-2 infection within 24 h with activation of NF-κB signalling (Fig. 3A) and a significant release of IL-8 (Fig. 3B), suggesting a role of airborne pollutants in sustaining the activation of the inflammatory response pathway that can worsen the progression of COVID-19 disease.

Our results, therefore, suggest that the *in vitro* exposure to fine PM, besides determining an increased infectability, may also lead to an increased inflammatory response in alveolar epithelial cells, likely contributing to the promotion or worsening of the cytokine storm related to poor prognosis in the worst COVID-19 cases. The data provided show the absence of variations on the inflammatory biomarkers after SARS-CoV-2 alone exposure. However, this could be related to the testing conditions used and longer exposure times may provide more insight in the SARS-CoV-2-related responses.

The combined effect of $PM_{2.5}$ and SARS-CoV-2 to increase or sustain the inflammatory response, which has been related to unfavorable COVID-19 prognosis, supports the ecological results showing a positive correlation between excess mortality from COVID-19 to the long-term exposure to PM2.5 (Wu et al., 2020), in the USA, where air quality monitoring and the large Medicare cohort (performed in > 3,000 counties) data were combined. Coker and colleagues (Coker et al., 2020) found that the incremental 1 μg/m^3 $PM_{2.5}$ increase is associated with 9 % (95 % confidence interval: 6–12 %) additional COVID-19 - related mortality. More recent studies based on novel data indicate mortality increases of ~ 10.5 % ± 2.5 % per μg/m^3 for different locations in the world (e.g. Bozak et al. (Bozack et al., 2022) for New York, Hou et al. (Hou et al., 2021) for China and Renard et al. (Renard et al., 2022) for Western Europe). A recent review, however, pointed out that positive ecological associations for mortality were found in half of the studies analyzed and up to a 20% found negative or mixed associations, while stronger associations were found between long-term exposure to air pollution and COVID-19 incidence (Hernandez Carballo et al., 2022). Alternative explanations related to residual or uncontrolled confounding due to population density, population mixing, deprivation and ethnicity also need to be considered – there were null associations between air pollution and COVID-19 mortality in a large individual-level analysis in London after detailed adjustment for these confounders.

3. Conclusion

The present study provides a biologically plausible mechanism of action supporting the high rate of infection associated with ambient $PM_{2.5}$ exposure, and explaining, at least in part, why individuals living in highly polluted areas may be more susceptible to SARS-CoV-2 infection and are more likely to develop severe lung infections and injuries, ultimately leading to breathing difficulties and a negative prognosis. Together, our findings indicate that the air pollution-driven increase in the membrane receptor ACE2 represents an important determinant of the susceptibility of human alveolar epithelial cells to SARS-CoV-2 infection and stands at the base of a synergistic mechanism involving PM and viral particles to promote the pro-inflammatory response. These could be considered as key events of a more complex molecular mechanism to explain the link between air pollution and the spread of SARS-CoV-2, and for the first time demonstrate at cellular level the direct link between COVID-19 severity and exposure to PM pollution, which until now has been debated only basing on the environmental and epidemiological evidence.

A limitation of the results reported here is that our data are based on the use of a single cell type and relatively short exposure time. We are aware that biological responses in co-cultures systems of lung cells with other cell types (including endothelial cells and macrophages) and the use of air liquid interface exposure protocol could be useful to further explore the potential effects of air pollution and SARS-CoV-2. We also acknowledge that the limitation of the *in vitro* models in terms of exposure time, also reduces the possibility to mimic chronic exposure (extending the treatment to several days, possibly also at lower exposure concentrations).

Despite the limitations, the present research provides new insights

into the molecular mechanisms by which PM appears to facilitate infectiousness of a relevant human pathogen, such as SARS-CoV-2. This research also opens up a relevant question aimed at understanding the role of particulate pollution in favouring the biological interaction with the lung epithelia of other relevant airborne-related pathogens (such as bacteria, pollen, fungi, endotoxins and other viruses) and associated diseases. This should be thoroughly investigated and, if further confirmed also by clinical studies, air pollution policies should be revised in view of possible future pandemics to reduce the morbidity and mortality burdens of air pollution.

4. Methods

4.1. $PM_{2.5}$ collection and preparation

$PM_{2.5}$ samples were collected during winter 2021 at the Torre Sarca sampling site (Milan, Italy), a representative urban monitoring location. Particles were collected on Teflon filters (47 mm Ø, 2 μm, Whatman, USA) using a low-volume gravimetric sampler (EU system 38.33 l min^{-1}, FAI Instruments, Italy). Samples and blank filters were pooled and extracted by four subsequent cycles of sonication (20 min each in an ultrasound bath Sonica Soltec, Italy) in sterile water (2 ml per cycle). Detached particles were then aliquoted in sterile tubes and dried in a desiccator. The resulting pellets were stored at − 20 °C until further use. For in vitro exposures, particles were suspended in sterile water at a final concentration of 2 μg/μl. Just before use, the resuspensions were briefly sonicated (30 s SONOPULS Bandelin, 0,105 kJ, Amplitude 10 %, 001.0 s pulses) to allow homogeneous distribution of the particles. More details on the extraction procedure are reported in Gualtieri et al., 2010 (Gualtieri et al., 2010).

4.2. Cell culture maintenance and treatments

Human alveolar epithelial cells (A549 cell line, ATCC® CCL-185, American Type Culture Collection, USA) were maintained in Opti-MEM medium (Gibco, Life Technologies, Italy) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco) and Penicillin/Streptomycin (100 X, Euroclone, Italy). Cells were maintained at 37 °C in humidified atmosphere with 5 % CO_2. For experiments, cells were seeded 24 h before treatment at a density of 4×10^3 cells/cm^2 in 6-well plates. Exposures to $PM_{2.5}$ and SARS-CoV-2 were performed in Opti-MEM medium supplemented with 1% FBS and antibiotics. Preliminary experiments using blank filters extracts revealed the absence of viability and inflammatory effects.

To investigate the capability of air pollution to increase ACE2 protein expression cells were first treated for 72 h with PM (2.5 μg/cm^2); co-exposure with the Heat-inactivated SARS-CoV-2 (2.4×10^3 genome copies, ATCC® VR1986HK™) was also analyzed. Thereafter, cells were primed with PM for 72 h (ACE2 activated), and afterward, SARS-CoV-2 was added for additional 2 or 24 h to evaluate the endosomal pathway and the inflammatory response activation.

The $PM_{2.5}$ concentration selected for experiments could be considered representative of a daily lung particle deposition. In fact, modelled deposition of $PM_{2.5}$ at airborne concentration of 79 μg/m^3, corresponds to 2.3 and 0.05 μg/cm^2 of $PM_{2.5}$ deposited in the tracheobronchial and alveolar regions (Li et al., 2003).

4.3. Western blotting

Cells were scraped from 6-well plates and lysed on ice in RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0) and 0.1% of proteases inhibitor, added just before use. Protein concentration was assessed by the Bicinchoninic Acid Protein assay kit (Sigma Aldrich) according to the manufacturer's instructions. Equal amounts of proteins were loaded, separated by 10 % SDS-PAGE gels, and finally transferred to nitrocellulose membranes. The membranes were then incubated for 1 h with blocking buffer (TBS + 0.1 % Tween20 + 5 % (w/v) BSA). Afterwards, membranes were incubated O/N at 4 °C with specific primary antibodies diluted according to datasheets (ACE2, RAB5 and NF-κB p65). After incubation for 1 h RT with the specific HRP-linked secondary antibodies (anti-rabbit IgG, Cell Signalling Technology, USA), membranes were incubated with enhanced chemiluminescent (ECL, Euroclone). Digital images were taken by a luminescence reader (Biospectrum-UVP) and densitometry analysis was performed with dedicated software (VisionWorks LS). As loading control, anti-β-Actin antibody was used.

4.4. Analysis of secreted inflammatory biomarkers by ELISA

After exposure to the different treatments, cell culture supernatants were recovered and centrifuged to remove particles and debris (12,000 rpm, 6 min, 4 °C). The resulting supernatants were collected to determine IL-8 protein levels by sandwich ELISA, according to the manufacturer's guidelines (Invitrogen, Life Technologies, Italy). The absorbance of each sample was measured by a multiplate reader (Infinite 200 Pro, TECAN, Switzerland) at the wavelength of 450 nm. The amounts of proteins were calculated based on standard curves and data were shown as pg/ml.

4.5. Statistical analysis

Mean and standard error of mean (SEM) of three independent experiments are reported. Statistical analyses were performed using GraphPad Prism 6 software, One-way ANOVA with Dunnett's post hoc multiple comparisons tests. Values of $p < 0.05$ were considered statistically significant.

CRediT authorship contribution statement

S. Marchetti: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **M. Gualtieri:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **A. Pozzer:** Methodology, Writing – review & editing. **J. Lelieveld:** Methodology, Writing – review & editing. **F. Saliu:** Data curation, Writing – review & editing. **A.L. Hansell:** Methodology, Writing – review & editing. **A. Colombo:** Supervision. **P. Mantecca:** Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Recovery and Resilience Plan (NRRP) Mission 4 Component 2 Investment Line 1.5: Strengthening of research structures and creation of R&D "innovation ecosystems", set up of "territorial leaders in R&D"

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