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Review article

Toxicity of airborne nanoparticles: Facts and challenges

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ABSTRACT

Air pollution is one of the most severe environmental health hazards, and airborne nanoparticles (diameter <100 nm) are considered particularly hazardous to human health. They are produced by various sources such as internal combustion engines, wood and biomass burning, and fuel and natural gas combustion, and their origin, among other parameters, determines their intrinsic toxicity for reasons that are not yet fully understood. Many constituents of the nanoparticles are considered toxic or at least hazardous, including polycyclic aromatic hydrocarbons (PAHs) and heavy metal compounds, in addition to gaseous pollutants present in the aerosol fraction, such as NOx, SO_2, and ozone. All these compounds can cause oxidative stress, mitochondrial damage, inflammation in the lungs and other tissues, and cellular organelles. Epidemiological investigations concluded that airborne pollution may affect the respiratory, cardiovascular, and nervous systems. Moreover, particulate matter has been linked to an increased risk of lung cancer, a carcinogenic effect not related to DNA damage, but to the cellular inflammatory response to the pollutants, in which the release of cytokines promotes the proliferation of pre-existing mutated cancer cells. The mechanisms behind toxicity can be investigated experimentally using cell cultures or animal models. Methods for gathering particulate matter have been explored, but standardized protocols are needed to ensure that the samples accurately represent chemical mixtures in the environment. Toxic constituents of nanoparticles can be studied in animal and cellular models, but designing realistic exposure settings is challenging. The air–liquid interface (ALI) system directly exposes cells, mimicking particle inhalation into the lungs. Continuous research and monitoring of nanoparticles and other airborne pollutants is essential for understanding their effects and developing active strategies to mitigate their risks to human and environmental health.

1. Introduction

Air pollution is considered one of the greatest threats to human health (Kumar et al., 2014b; Kwon et al., 2020; Mannucci et al., 2015; Pope et al., 2009; Vouitsis et al., 2023). It is constituted by a complex mixture of particulate matter, volatile organic molecules, and gaseous compounds such as nitrogen dioxide (NO_2), carbon monoxide (CO), sulfur dioxide (SO_2), and ozone (O_3). Major sources of this pollution, and particularly airborne particulate matter are industrial settings, open biomass burning, and different modes of transport. Road traffic is responsible for more than 60 % of all airborne emissions in cities worldwide (Flood-Garibay et al., 2023; Kumar et al., 2017; Rönkkö and Timonen, 2019). Combustion of fossil fuels releases primary pollutants into the atmosphere, such as soot particles, nitrogen, and sulfur oxides originating from the combustion of fossil fuels, whereas friction processes of brakes, clutches, and tires also contribute to the release of airborne particulate matter (Amato et al., 2014; Flood-Garibay et al., 2023; Kumar et al., 2017; Rönkkö and Timonen, 2019).

According to the World Health Organization (WHO), air pollution is one of the most severe environmental health dangers (WHO, 2021). Globally, 99 % of the world's population in 2019 lived in regions where air quality standards were unmet (Mannucci et al., 2015). Respiratory infections, lung cancers, ischemic heart disease, cerebrovascular disease, and chronic obstructive pulmonary disease have been related to air pollution (Adamkiewicz et al., 2015). Long-term exposure to ambient PM2.5 was estimated to cause 4.2 million premature deaths in 2015, and

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exposure to ozone was related to 254,000 additional premature deaths (Cohen et al., 2017; Mannucci et al., 2015). This accounts for an estimated 7.6 % of global mortality in 2015, making exposure to air pollution the fifth most important global mortality risk factor (Cohen et al., 2017). Many studies document the link between outdoor air pollution and all-cause mortality. Every year, seven million people die from diseases attributable to air pollution, 59 % of which are found in East and South Asia (Cohen et al., 2017), and references therein.

Nanoparticles (aerodynamic diameter < 100 nm) have been identified as one of the most harmful components of atmospheric pollution (Kwon et al., 2020). Here, we discuss the current understanding of nanoparticles (NPs) that are emitted from sources such as internal combustion engines, wood, and fuel combustion, and how NPs can have severe effects on human health because of their systemic toxicity caused by substances such as polycyclic aromatic hydrocarbons (PAHs) and heavy metals.

In this review, we gathered literature data about the sources of NP and UFP. Primarily, we identified 20–25 research articles relevant to the topic, most published in the last 5 years. We did not extensively search the literature through scientific databases and search engines. Instead, we focused on summarizing relevant findings and discussing their implications for the future of environmental toxicology. The interest in measuring nanoparticles was emphasized because of their unique characteristics, particularly their small size, which is associated with various toxicological outcomes, as well as the challenging difficulties of different experimental approaches to test the toxicity of NPs, both *in vivo* and *in vitro*.

2. Why is it important to monitor nanoparticles?

Particulate matter (PM) has been identified as a key marker used to assess pollution and, therefore, it has been the subject of numerous toxicological investigations (Kelly and Fussell, 2020; Kumar et al., 2014b; Kwon et al., 2020; Leikauf et al., 2020; Vallabani et al., 2023). Airborne particles are often measured as PM2.5 or PM10 (particles of less than 2.5 or 10 μm in diameter, respectively), although it is known that the smaller size fraction, under 100 nm, dominates particle number concentrations in urban areas (Kumar et al., 2013). The terms NP (nanoparticles) and UFP (ultrafine particles) are used in original papers to refer to particles smaller than 100 nm. However, there is some preferential use of these names in toxicological and epidemiological fields. It is necessary to standardize the terminology for describing particle size in nanotechnology, health, and environmental sciences (Buzea et al., 2007). For the sake of convenience, here we will refer to them as NPs.

Nanoparticles (NPs) or ultrafine particles (UFPs) are not regularly monitored in outdoor air, despite being a significant part of environmental air pollution. Therefore, it is essential to bridge the knowledge gap between nanoparticle concentrations, their toxicity, and their impact on human health.

PM toxicity is determined by particle content and concentration, dose, size, and permanency (Stone et al., 2017). There is ample consensus that NP may represent a major hazardous component of the environmental particulate matter (Kwon et al., 2020; Newby et al., 2015; Vallabani et al., 2023). Nanoparticles may exhibit distinct properties compared to the corresponding bulk material, due to their small size and larger specific surface area (which means they have a much larger surface area per unit volume). This increased surface area can enhance the chemical reactivity of reactions by a factor of 1000 (Buzea et al., 2007). Moreover, the chemical reactivity may increase as the particle size decreases, depending on the chemistry of the nanoparticle (Buzea et al., 2007). Additionally, coatings and other surface modifications could further modulate the properties of NP and increase their toxic effects. There is a common consensus that these features also affect their toxic potential, with the result that NPs may be significantly more toxic than larger particles of the same material. For example, microparticles have similar sizes as cells and organelles, whereas NPs are similar in size to viruses, DNA, and proteins. This could make it easier for NPs to penetrate living cells and potentially harm the functioning of vital organs (Buzea et al., 2007; Malakar et al., 2021; Mannucci et al., 2015).

Inhaled environmental particles can affect human health by disrupting the regular activity of internal body systems (Fig. 1), and developing cardiovascular and lung disorders (Buzea et al., 2007; Daiber et al., 2020). The nasopharyngeal region captures many particles smaller than 10 nm, which means that the lungs are mainly exposed to particles larger than 10 nm (Oberdörster, 2001). The deposited fraction of inhaled particles (Fig. 2), as well as the amount of time that particles remain in the lungs before they are cleared, may determine how harmful they are (Malakar et al., 2021; Oberdörster, 2001). The large surface area of NPs can result in a dose-dependent increase in oxidation and DNA damage (Buzea et al., 2007). Despite the multiple potential health hazards linked to NPs (Buzea et al., 2007; Kumar et al., 2013; Mannucci et al., 2015; Münzel et al., 2021), their actual contribution to the toxicity of airborne pollution is largely unknown, mainly due to the lack of continuous monitoring data on nanoparticles. Current air quality legislation, like the European Air Quality Directives, only regulates PM10 and PM2.5, although the World Health Organization advises incorporating NP monitoring, measured as the total number of particles, into air quality monitoring stations (Kwon et al., 2020; WHO, 2021). To better understand the dangers of environmental nanoparticles and their impact on human population health, it is important to link NP monitoring with experimental identification of the fundamental toxicity mechanisms in living organisms. This can be, for example, achieved using animal models and/or cell cultures.

Heavy metals and organic compounds in combustion emissions contribute to NP toxicity, inducing oxidative stress, mitochondrial damage, inflammation, and activation of apoptosis (Buzea et al., 2007; Daiber et al., 2020; Vallabani et al., 2023). In addition to damaging lung tissue, NPs harm the cardiovascular and nervous systems (Flood-Garibay et al., 2023). Moreover, the organic fraction of the NPs has been related to genotoxicity in many cell types and tissues (Daiber et al., 2020; Kelly and Fussell, 2020; Kulmala et al., 2004; Olofsson et al., 2023; Seigneur, 2009; Vouitsis et al., 2023).

3. Sources and toxicity of airborne nanoparticles

Transport represents the main source of NPs in metropolitan environments (Olofsson et al., 2023; Stone et al., 2017). Diesel and gasoline exhaust are the primary sources of airborne nanoparticles in urban areas (Kumar et al., 2013; Vouitsis et al., 2023). The size of exhaust particles from vehicles ranges from 20 to 130 nm for diesel engines and from 20 to 60 nm for gasoline engines (Sioutas et al., 2005). High pollution episodes or proximity to high-traffic roads can significantly increase the number and concentration of nanoparticles in the air (Buzea et al., 2007). All the particles produced can also undergo alterations due to their stay in the atmosphere. Depending on the source and atmospheric processing, airborne particles differ in chemical composition, reactivity, mass, size, solubility, shape, and surface area (Kumar et al., 2014b; Rönkkö and Timonen, 2019; Vouitsis et al., 2023).

Air pollution and nanoparticle formation are commonly identified with human activities, such as road transport, industry, and coal burning (Buzea et al., 2007; Kumar et al., 2014b). In addition, natural processes that include photochemical reactions, volcano eruptions, and forest fires can produce huge amounts of nanoparticles, which profoundly impact air quality (Buzea et al., 2007). Transport-related NPs may arise from traffic exhaust such as airplane and ship emissions, and non-exhaust sources (e.g., brake wear and railways) (Bendtsen et al., 2019; Petzold et al., 2008; Vouitsis et al., 2023). Both exhaust and non-exhaust sources contribute to the concentration of NP in urban air. The relative impact of non-exhaust particulate matter on human health is likely to increase in the future (Amato et al., 2014). Technological progress has managed to reduce PM emissions from vehicle exhaust gases, but little attention has been devoted to minimizing non-exhaust pollutants.

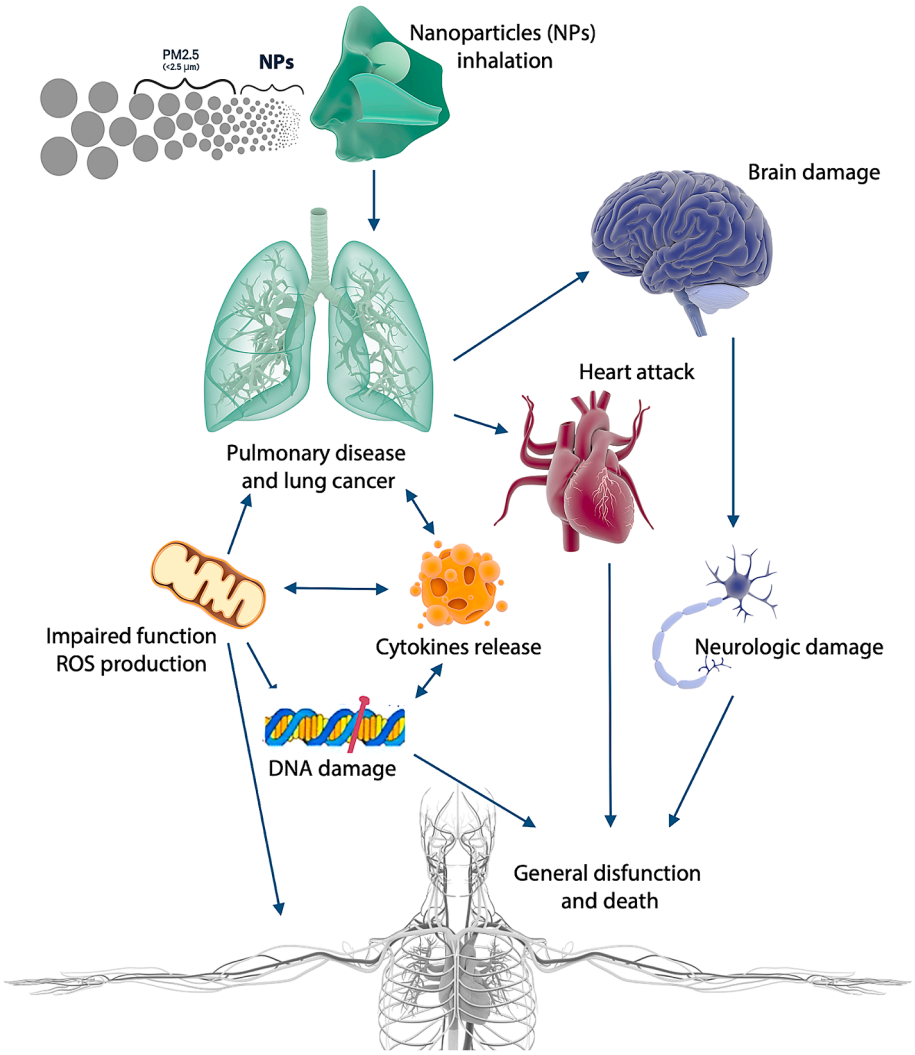

Fig. 1. Summary of the mechanisms of uptake of airborne nanoparticles and their fate in the human body. Main active routes upon exposure to nanoparticles and cellular components and damaged organs are indicated. The figure links different disease pathways associated with nanoparticle toxicity (based on *in vitro* and *in vivo* studies described in the main text).

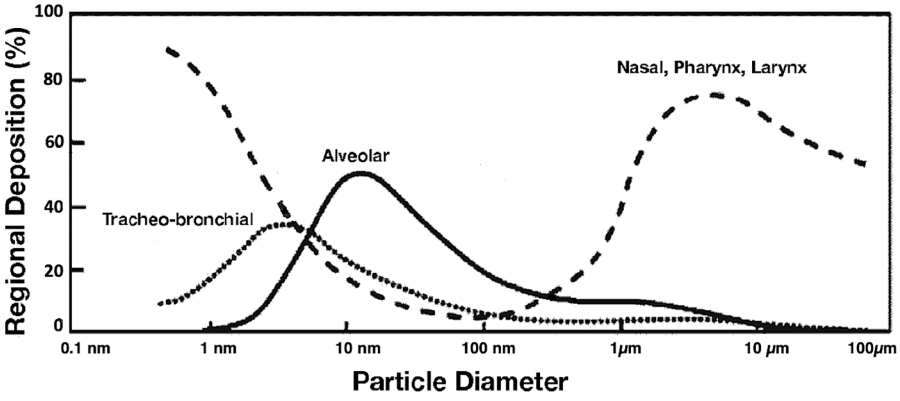

Fig. 2. Deposition of inhaled particles in the human respiratory tract versus the particle diameter. Particle size is a critical factor in determining deposition location within the respiratory system. The nasopharyngeal region captures particles smaller than 10 nm, while the lungs receive those between 10 and 20 nm. .
Adapted from (Oberdörster, 2001)

NPs may contain different types of chemicals that can lead to toxic effects, for example, extractable organic compounds and water-soluble transition metals (Kwon et al., 2020; Mesquita et al., 2014), which can both cause oxidative stress through overlapping mechanisms. High molecular weight PAHs are mainly found in gasoline vehicle emissions, while low molecular weight PAHs are usually found in emissions from

diesel vehicles, ships, and heavy oil exhaust emissions. Their emissions are also traditionally linked to sources of some gas-phase pollutants, like NO_2 and other nitrogen oxidation products. Among these products, which are strong irritants and contribute to respiratory diseases, nitrogen oxides may react with PAHs in NPs to produce nitroarenes through either direct nitration during combustion processes or by heterogeneous gas-particle interaction. These secondary components of NPs are considered more mutagenic and carcinogenic than the parental PAHs (Kielhorn et al., 2003; Mannucci et al., 2015). The fact that exhaust NPs consist of various potentially harmful inorganic and organic components, makes it difficult to identify their mechanism of action in toxicological studies.

Metals in airborne nanoparticles can be a health hazard owing to their role in both carcinogenic and non-carcinogenic processes, and they have a pronounced effect on lung responses (Baldwin et al., 2015; Lam et al., 2016; Ohlwein et al., 2019). Airborne lead-containing (Pb) nanoparticles pose a significant health risk to people and animals in contaminated areas, as they reduce inflammatory response to pathogens and cause persistent pathological changes. A reduced inflammatory response is observed in tissues after inhalation of particles containing Pb $(NO_3)_2$, which is associated with the negative effects of lead on tissues and persistent pathological changes in target organs (Dumková et al., 2017), including induced cerebellar damage (Zhang et al., 2024a). After inhalation of soluble Pb compounds, the total number of macrophages can be significantly reduced, leading to reduced immunological response (Smutná et al., 2022). Chronic exposure to metals poses a significant risk to public health due to their non-biodegradable nature (Buzea et al., 2007; Mitra et al., 2022). Also, both crystalline and amorphous silica particles have been reported to exhibit pro-inflammatory and pro-cytotoxic effects, with the crystalline variant causing persistent and gradual reactions leading to lung fibrosis (Calas et al., 2018),

Airborne Fe-containing NPs can be rather hazardous to humans as they are highly bioactive and toxic, as evidenced by indicators of oxidative stress found in the condensate of exhaled breath of workers exposed to Fe-containing NPs during pigment manufacturing (Pelclova et al., 2016). Magnetite (Fe_3O_4) causes acute cytotoxicity and generation of ROS in human A549 alveolar epithelial cells (Könczöl et al., 2013). A study of the effects of particulate matter from a subway station in Stockholm found that NPs contained magnetite as the predominant form of iron oxide (Karlsson et al., 2008).

Heavy metals are the main, yet not the unique, contributors to oxidative stress, as they can induce reactive oxygen species (ROS) (Horie and Tabei, 2021). Overproduction of ROS triggers the transcription of antioxidant enzymes, which stimulates compensatory mechanisms controlled by the pleiotropic Nrf2 transcription factor (Pardo et al.,

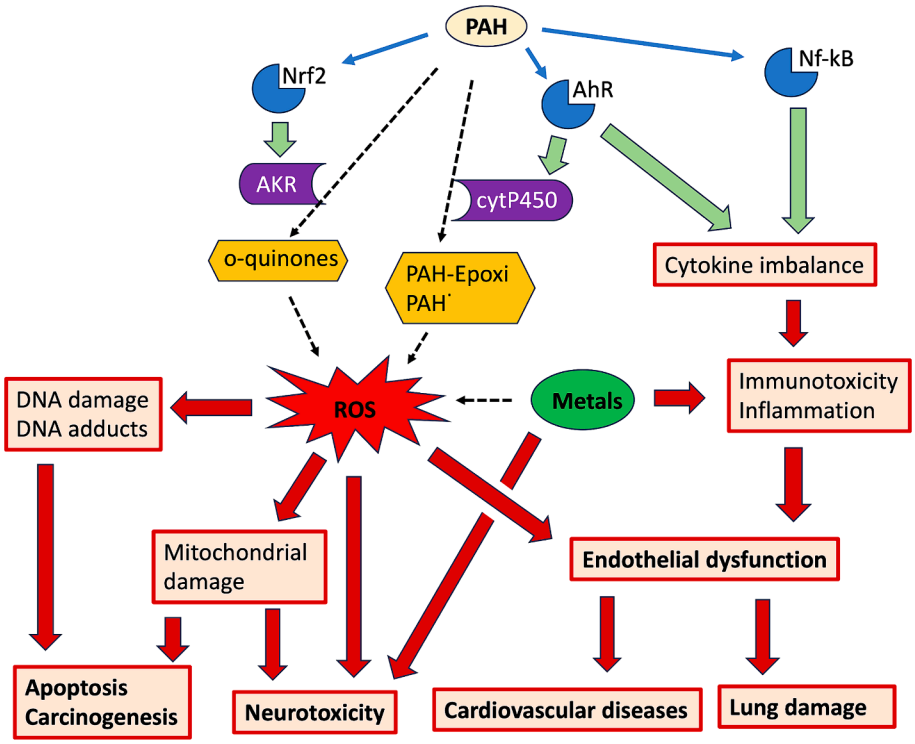

Fig. 3. Toxic effects of PAH and heavy metal constituents of nanoparticles on a characteristic vertebrate cell. Different PAH congeners can induce the ectopic activation of at least three families of receptors: the aryl hydrocarbon receptor (AhR), the nuclear erythroid 2-related factor 2 (Nrf2), and the kappa-light-chain-enhancer of activated B cells nuclear factor (Nf-kB) (blue arrows). AhR and Nrf2 regulate the expression (green arrows) of different enzymes implicated in redox metabolism and detoxification processes (magenta boxes), like members of the cytochrome P450 family (cytP450) or the alpha-keto-reductase multigenetic complex (AKR). In the presence of excess PAH and other xenobiotics, acting as ectopic substrates (black dotted arrows), these enzymatic activities generate highly active oxidated species, including radical species, epoxy derivatives, or oxidated quinolones (orange). These oxidizing compounds increase the levels of ROS (in red) in the cell, increasing lipid, protein, and nucleic acids modification by oxidation and/or adduct formation (negative outcomes marked as red boxes). This leads to apoptosis or/and carcinogenesis, depending on the cell type and the extent of the damage. On the other hand, ectopically activated AhR and Nf-kB receptors disrupt the normal expression of interleukins in macrophages and other cell types from the immunological system, leading to immunotoxicity and inflammation. Heavy metals (green oval) contribute to several of these negative outcomes since they generate ROS, which can participate in intracellular Fenton reactions (black dotted arrow), and some of them are recognized neurotoxicants and immunotoxicants (red arrows). Both ROS and immunotoxicity contribute to several endothelial dysfunctions, which can result in lung and cardiovascular damage (red arrows). Derived from references (Fröhlich and Salar-Behzadi, 2014; Mesquita et al., 2014; O'Driscoll et al., 2018; Sondermann et al., 2023; Vouitsis et al., 2023). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2020). In NP-exposed mice, Nrf2 is involved in angiotensin II-associated vascular damage (Gao et al., 2021). Coronary artery disease has a higher incidence and mortality rate in certain ethnic groups, which may be related to the impact of air pollution and some metabolic disorders (Sucato et al., 2023). It has been established that South Asian populations have a higher prevalence of premature myocardial infarction than other populations (Sucato et al., 2023).

During the so-called Phase I of the de-toxicant response, several PAHs can be over-activated in various tissues, enhancing the activity of multiple oxidizing enzymes, particularly those of the cytochrome P450 family, through the interaction with the endogenous Aryl Hydrocarbon Receptor (AhR), also known as "the dioxin receptor" (Fig. 3) (Billet et al., 2008; Machala et al., 2001; Mesquita et al., 2014; O'Driscoll and Mezrich, 2018). AhR also participates in other regulatory signaling pathways, such as T-cell regulation, and may induce cytokines independently of its ability to activate Phase I enzymes, leading to T-cell differentiation and inflammatory response, a mechanism of toxicity that can link oxidative stress with inflammatory responses (O'Driscoll and Mezrich, 2018).

Airborne particles can reach the lung and pass through alveolar cell membranes, leading to immune cell activation, cell transmigration, cytokine release, and systemic inflammation (Daiber et al., 2020; Leikauf et al., 2020). As mentioned above, air pollution can produce lung cancer by creating an inflamed environment that fosters the proliferation of mutated lung cells (Hill et al., 2023). Those particles can trigger lung tumors without causing any direct damage to DNA (Hill et al., 2023). In any case, PAHs contained in PM2.5 can directly act as genotoxins, causing mutations and promoting tumorigenesis (Lee et al., 2020). Furthermore, NPs increased hepatic lipid content in an experimental model of diabetic dyslipidemia, suggesting that nanoparticle-rich diesel exhaust disrupts some liver functions (Ito et al., 2016). NPs can also reach the central nervous system after translocation through the lungs and induce nervous disorders, reducing the number of neurons (Flood-Garibay et al., 2023) —see section 3.8—.

NPs can damage epithelial cells in the upper respiratory tract and affect macrophages, leading to inflammatory processes (Li et al., 2003). Particles not removed by alveolar macrophages, or cleared by mucus, can damage the lungs and other organs. Components like metals, black carbon, and PAHs can increase reactive oxygen species (ROS) formation, triggering inflammatory responses (Kumar et al., 2021; Kwon et al., 2020) and even cell death (Ruijter et al., 2023). NP-induced release of cytokines (IL-6, TNF-α), and chemokine MIP-2 has been assessed in rat lung alveolar macrophages, and alveolar epithelial type II cells (Sayes et al., 2007). Different cell types respond differently to the presence of NPs. For example, alveolar type II epithelial cells showed the highest increase in MIP-2 and IL-6 secretion, while TNFα release was elevated in macrophages. However, TNFα is not commonly produced in lung epithelial cells or macrophage/epithelial cocultures (Sayes et al., 2007).

Although it is difficult to associate the adverse effects of NP (and of atmospheric pollution in general) to a specific pollutant, the current view is that PAHs contribute to the overall toxicity in a very significant proportion. PAH and metals interact with multiple regulatory targets inside the cells, altering the redox metabolism and triggering stress responses at levels that overcome the normal metabolic and immunological balance, with negative outcomes ranging from inflammatory processes to cancer development or even cell death (Fröhlich and Salar-Behzadi, 2014; Mesquita et al., 2014; O'Driscoll et al., 2018; Sondermann et al., 2023). Fig. 3 summarizes the different adverse effects that can be derived from exposure to PAHs, heavy metals, or a combination of both and the main molecular mechanisms involved. In addition to carcinogenesis and neurotoxicity, the excess of ROS and immunotoxicity contribute to endothelial dysfunctions that may cause lung and cardiovascular damage, two known systemic effects of nanoparticle pollution (Marchini, 2023; Vouitsis et al., 2023).

3.1. Combustion particles

Combustion processes are one of the most important sources of airborne nanoparticles. NPs are formed at high temperatures often from gas-phase chemicals. Delayed primary particles are formed when gaseous chemicals nucleate through a cooling and dilution process. Finally, secondary nanoparticles are derived from gaseous precursors through atmospheric photochemistry (Kulmala et al., 2004).

Internal combustion engines, biomass and wood burning, fuel oil combustion, natural gas combustion, biogenic emissions, and cooking emissions are the primary anthropogenic sources of organic compounds in the air (Kumar et al., 2014b; Kumar et al., 2013; Rönkkö and Timonen, 2019; van Drooge et al., 2014), as well as fires in waste facilities (Elihn et al., 2023). In road and industrial areas, the complex mixing of particles from various sources is influenced by atmospheric processing, local co-pollutants, and climate conditions (Kumar et al., 2013; Vouitsis et al., 2023). Furthermore, indoor air can be more contaminated than outdoor air, and combustion particles can be produced, for example, during cooking and by poorly ventilated stoves utilizing wood and plant residues. Therefore, reducing emissions from combustion sources is extraordinarily important for controlling atmospheric pollution in cities and rural and semi-rural areas (Mesquita et al., 2015; S. R. Mesquita et al., 2014).

The presence of pollutants inside and outside buildings is a major environmental risk factor for human health and one of the main causes of premature death (Cohen et al., 2017). In rural areas during the cold season, air samples with a high proportion of emissions from biomass burning show very high levels of PAH, which are associated with dioxin-like activity and embryotoxicity (Mesquita et al., 2015; S. R. Mesquita et al., 2014). Using certain cooking fuels in residential buildings is the primary source of air pollution. Wood-burning household stoves contribute significantly to outdoor air pollution, especially in winter. The effects of wood smoke on air pollution and human health are most noticeable in developing countries where wood is the main source of energy for heating and cooking. Some compounds contained in wood are irritants, mutagens, and carcinogens (WHO). Still, it is unclear to what extent they contribute to the negative health effects in urban buildings (Dilger et al., 2023). Particulate matter and NP may exhibit dioxin-like activity, with peak activity observed during the cold period. The particle size distribution of urban atmospheric aerosols varies considerably depending on location and weather conditions, such as wind direction and speed, relative humidity, and temperature (Kumar et al., 2014a; Mesquita et al., 2015; Rönkkö and Timonen, 2019).

3.2. Road traffic exhaust emissions

Nanoparticles are not uniformly distributed in the environments affected by road transport, but they form an externally and internally mixed aerosol with varied particle characteristics (Rönkkö and Timonen, 2019). Diesel exhaust particles (DEPs) contribute significantly to the number of airborne nanoparticles, initiating several health impacts attributed to exposure to NPs. They may increase health problems, chiefly respiratory pathologies, heart disease, and even premature death. Since 2012, the International Agency for Research on Cancer (IARC), part of the World Health Organization, has classified diesel engine exhaust as a group 1 carcinogen, and gasoline engine exhaust as a possible carcinogen for humans (IARC/WHO, 2013). However, it has not been determined yet whether the effects of diesel exhaust NPs on human health are worse than those of PM2.5 because NP concentrations have been scarcely monitored in epidemiological studies (Boland et al., 2001, 1999; Olofsson et al., 2023).

PAHs (polycyclic aromatic hydrocarbons) are an important component of exhaust NPs. PAHs are toxic, carcinogenic, and mutagenic substances with well-known toxic modes of action (Billet et al., 2008; Machala et al., 2001).

3.3. Naval transport exhaust emissions

The use of heavy fuel oil in naval transport is the main source of NP emissions. The resulting NPs consist of organic carbon, elemental carbon, PAHs, and transition metals, which can cause oxidative stress and cytotoxicity (Petzold et al., 2008; Sinha et al., 2003). Oddly enough, the generation of NPs from marine engines is mostly unknown, but the high content of transition metals can affect human health. Further studies are needed to assess the impact of the fuel and the modes of operation (Oeder et al., 2015; Vouitsis et al., 2023). A comparison between heavy fuel oil and diesel fuel shipping emissions showed that heavy fuel oil predominantly contained particles smaller than 50 nm and diesel fuel contained mainly larger particles (>200 nm) (Vallabani et al., 2023). Reactions to heavy fuel oil emissions are dominated by oxidative stress and inflammatory responses (Vallabani et al., 2023; Vouitsis et al., 2023).

3.4. Aircraft transport exhaust emissions

Several reports point out the toxicity and similar health consequences of air pollution caused by airports and transportation (Bendtsen et al., 2021, 2019). Aircraft jet engines emit substantial amounts of volatile organic compounds with physicochemical properties like diesel exhaust gases (Bendtsen et al., 2021). Exposure to aircraft exhaust nanoparticles induces several toxic effects *in vitro* and in animals, such as inflammation, ROS production, and DNA damage. Interestingly, those exhausts appear to exhibit lung toxicity like diesel exhaust particles (Bendtsen et al., 2019; Vallabani et al., 2023). Both aircraft jet engine exhaust and diesel exhaust particles can be considered equally harmful to the lungs (Bendtsen et al., 2019; Vallabani et al., 2023). Bloodstream may transport those particles, reaching various organs (Ferry et al., 2011; Mannucci et al., 2015; Sharma et al., 2021). Particles emitted by aircraft mainly consist of soot. The lighter PAHs occur mainly in the gaseous phase, while heavier related compounds are mostly in the particulate phase and are generally the most carcinogenic and mutagenic. The organic fraction decreases as engine power increases, but then it slightly increases again due to the growing contribution from lubrication oil emissions (Vouitsis et al., 2023). It has been suggested that the use of paraffin-rich biofuels, which contain a blend composed of hydro-processed esters of fatty acids, could be an effective measure to reduce the exposure of airport workers to NPs bearing genotoxic PAHs (Heeb et al., 2023).

3.5. Railway and subway transport emissions

Studies on the health effects of airborne pollution have paid more attention to subway (underground) railways than surface railways. This could be because subway environments are indoor spaces with potentially higher concentrations of pollutants and worse health effects. It has been shown that the particulate matter (PM) found in the subway railway environment does not cause higher levels of oxidative stress or cytotoxicity than PM found in outdoor air (Spagnolo et al., 2015). However, another study on airborne particles indicates that subway PM can induce higher oxidative stress and DNA damage, seemingly because they can form intercellular ROS (Karlsson et al., 2005). Emissions from diesel locomotives show the presence of metal components (Na^{+} and Ca^{2+} as main ions) and they are dominated by the particle mass (Abad López et al., 2023; Kim et al., 2021).

3.6. Non-exhaust sources

In urban air, there are several sources of NPs, such as transport exhaust emissions described above. Among the non-exhaust particle emissions are brake wear, tire wear, road surface wear, and resuspended road dust (Vouitsis et al., 2023). Car brakes contribute considerably to traffic-related particulate mass, but less to the total number of nanoparticles. When braking, wear particles are emitted from the friction surfaces of the pads and discs, and some particles are released into the environment. Non-exhaust particle emissions mainly comprise heavy metals and various organic compounds (Vouitsis et al., 2023).

The main contributor to road dust emissions depends on environmental conditions, with road dust emissions having a clear seasonal effect, being higher in summer, possibly due to lower pavement moisture (Padoan et al., 2018). In countries that use studded tires during winter, there is a clear increase of wear particles in spring due to the combination of dry roads and resuspension (Hussein et al., 2008). Road powder dust appears to come primarily from street material rather than tires. The composition of primary solid particles from streetwear can change via chemical reactions in the air, called particle aging processes, and potentially become more toxic (Diaz et al., 2013; Lei et al., 2023). Finally, windblown dust and pollen are often the main sources of coarse particles in the air (Daiber et al., 2020). UV light-induced photochemical reactions on the surface of the particles could form highly reactive products, and the particles may also contain bacterial/fungal endotoxins, pyrogens, or urban heavy and transition metals, resulting in additional biological toxicity (Daiber et al., 2020).

Primary sources of airborne metals are mining, smelting, and metal processing (Pelclova et al., 2016), and metal-containing particles are also common in the subway. Brake wear dust and subway emissions are the principal sources of airborne Fe-bearing NPs. Metals can enter the body through inhalation of metal or metal-containing particles, causing adverse health effects. The NP fraction causes more severe DNA damage than larger particles (Könczöl et al., 2013, 2011).

3.7. Nanoplastics

Microplastics (plastic particles up to 5 μm) and nanoplastics (sizes between 1 nm and 1000 nm, although an upper limit of 100 nm has been suggested) are pollutant particles that raise increased environmental concerns. A hot topic in research is how plastic particles affect human health risks associated with airborne particles, as airborne nanoplastics and microplastics can be found almost anywhere. There is evidence of airborne plastics in crowded metropolitan areas, resulting in public health concerns (Kelly and Fussell, 2020).

Fibers are the most common type of microplastics found in various atmospheric environments. These particles are easily transported due to the low density of plastic particles. The original chemical composition of plastics can consist of a mixture of about 40,000 substances (Abad López et al., 2023). Polymers are the main component of this mixture and contain various additives such as plasticizers, stabilizers, and flame retardants (Abad López et al., 2023).

Numerous studies have shown that nanoplastics, especially in combination with other pollutants, have adverse effects on the environment and living organisms (Buzea et al., 2007). These effects include bioaccumulation oxidative stress, inflammation, metabolic disorders, genotoxicity, and cytotoxicity. Nanoplastics can damage important organs like the lungs, liver, kidneys, and brain via the bloodstream. The interaction of nanoplastics with various components of the bloodstream can harm human health. Nanoplastics can bind to plasma proteins changing their behavior in biological systems (Rajendran and Chandrasekaran, 2023).

Nanoplastics can penetrate deep into the respiratory tracts, infiltrating cells and eliciting multiple health problems (Choudhury et al., 2023). For example, intratracheal instillation of small nylon fibers can cause a rapid inflammatory reaction in the lungs of rats (Daiber et al., 2020). In addition, airborne microplastics are likely carriers of chemical and biological pollutants, so they may represent a crucial, but still unquantified, source of exposure to humans. Recent reports confirmed their presence in human blood (Leslie et al., 2022), placenta (Ragusa et al., 2021), breastmilk (Ragusa et al., 2022), and their possible interaction with diverse cardiovascular events (Marfella et al., 2024),

suggesting that they may represent a much more severe hazard than anticipated. For example, it is not well understood whether microplastics carry biohazards, such as antibiotic-resistant genes (Maguire and Gardner, 2023).

3.8. On the toxicity of nanoparticles

Although exposure to nanoparticles has been associated with toxic effects, it is difficult to establish a direct correlation between exposure to NPs and specific health disorders, such as induced carcinogenesis (IARC/WHO, 2013; Vallabani et al., 2023). Exposure to airborne NPs, whether acute or long-term, can increase the risk of developing respiratory and cardiovascular diseases, and cerebrovascular events (Kelly and Fussell, 2020; Mannucci et al., 2015; Münzel et al., 2021; Ohlwein et al., 2019; Vallabani et al., 2023), and neurological disorders (Flood-Garibay et al., 2023) (Fig. 1). Furthermore, there is a growing interest in the effects of NP at the reproductive level. This includes concerns about the presence of endocrine disruptors and their impact on reproductive organs (Park et al., 2024; Zhang et al., 2024b), as well as potential adverse effects of NP on pregnant women that can pass from the bloodstream to the fetus, thereby inhibiting fetal development (Drury et al., 2023).

Modern diesel engines are equipped with technologies to filtrate and reduce particle emissions. However, some of these new technologies, such as diesel oxidation catalysts or diesel particulate filters, contribute to increasing the overall number of particles and the NO_2 content of the exhaust by reducing the medium size of emitted particulates, reaching diameters below 30 nm even as small as 8 nm (Karthikeyan et al., 2013; Ko et al., 2016). Total NOx emissions are not affected, but the NO_2/NO_x ratio is significantly reduced by Diesel particulate filter (DPF) regeneration due to the reduction of NO oxidation by the diesel oxidation catalyst and increased NO_2 reduction by the DPF. Lastly, DPF regenerations increase PM emission factors several times compared to a trip without DPF regeneration, resulting in a significant exceedance of the emission limit (Huang et al., 2022). The ensuing exhaust produces inflammation and lung injury in exposed rats, with the circumstance that vascular oxidative stress and endothelial alterations correlate with the number of NPs rather than with the mass of inhaled particles or NO_2 concentration (Karthikeyan et al., 2013).

The harmful effects of NP exhaust emissions of gasoline, especially from gasoline direct injection (GDI) engines, are not fully understood (Yang et al., 2019). Transition metals present in particles can generate ROS that can develop detrimental respiratory symptoms (Daiber et al., 2020; Diaz et al., 2012). Many stress signals begin in the lungs and induce the release of cytokines which can, thereafter, influence the whole organism (Holme et al., 2019) (Fig. 1). Furthermore, systemic inflammation can lead to additive effects since it can induce damage to the cardiovascular and nervous systems (Holme et al., 2019; Karoui et al., 2019; Lawal, 2017).

Airborne nanoparticles can cause lung cancer by inducing the activation of pro-inflammatory pathways that foster the proliferation of mutated cells in the lungs (Hill et al., 2023). Those particles can induce lung tumors without directly harming DNA (Hill et al., 2023). Furthermore, in diabetic animals with dyslipidemia NPs have been reported to increase lipid deposition and affect some liver functions (Ito et al., 2016). NPs can reach the central nervous system after translocation through the lungs (Calderón-Garcidueñas et al., 2021; Flood-Garibay et al., 2023), mainly through the bloodstream, and induce nervous degeneration by reducing the number of neurons (Ito et al., 2016). Also, they can reach the brain directly through the olfactory bulb (Oberdörster, 2001; Schraufnagel, 2020).

Inhaled nanoparticles are generally considered to have little impact on wildlife populations, but that may be because some of the toxic effects associated with them, such as the generation of oxidative stress, inflammation, and DNA damage, have been linked to other environmental stressors. Air pollutants can harm wildlife through organ damage, increased susceptibility to stress and disease, reduced reproductive success, and even death. Besides, metal contamination, which in urban environments can be linked to air pollution (and NP in particular), has been shown to alter the morphological traits of the tree sparrow *Passer montanus* (Li et al., 2021).

Neurological disorders are recognized as one of the main public concerns, and there is growing evidence that their increase may be related to air pollution (Flood-Garibay et al., 2023; MohanKumar et al., 2008). In animal models inhaled NPs can reach the brain after deposition in the nasal or alveolar epithelia (Figs. 1 and 2) and trespass the blood–brain barrier (Calderón-Garcidueñas et al., 2020). Systemic inflammation, followed by the massive release of cytokines and related factors, can damage the cardiovascular and nervous systems, inducing atherosclerosis and related pathologies (Holme et al., 2019; Karoui et al., 2019; Lawal, 2017), brain inflammation, and neurodegenerative diseases (Flood-Garibay et al., 2023; O'Driscoll and Mezrich, 2018; Zhu et al., 2020). Some studies using animal models have investigated the effects of NP on brain development (Flood-Garibay et al., 2023), while others have documented that NPs alter emotional behavior, learning capability, and neurotransmission (Flood-Garibay et al., 2023; Schraufnagel, 2020). In addition, inflammation of endothelial cells and/or the lungs is considered a key link between PM exposure and cardiovascular disease (Donaldson et al., 2001). Early systemic oxidative stress would exacerbate endothelium-dependent vasodilatation (Holme et al., 2019).

Inhaling nanomaterials has been associated with the development of cancer (Fig. 1). This association has usually been linked to NP genotoxic properties (Leikauf et al., 2020), but recent reports have proven that inflammatory response to airborne pollution can promote lung cancer (Kelly and Fussell, 2020; Vallabani et al., 2023). Chronic inflammation, which can be promoted by nanoparticles and triggered by interleukin IL-1β, is essential for tumor growth in cells carrying mutations that cause cancer (Hill et al., 2023).

While technological improvements have reduced particulate emissions from engine exhaust (Durga et al., 2014; Tzamkiozis et al., 2010; Vouitsis et al., 2023; Yang et al., 2019), little attention has been devoted to reducing emissions from brake and tire wear. NPs generated by tires and road wear contain metals, are considered genotoxic, generate ROS, and cause damage to epithelial cells (Gualtieri et al., 2005; Lindbom et al., 2007, 2006; Wik and Dave, 2009). NPs from tires and road surfaces pose a potential hazard to living organisms by generating ROS formation and damaging cells, causing genotoxicity (Gualtieri et al., 2005; Lindbom et al., 2007; Vallabani et al., 2023).

4. Monitoring the health effects of nanoparticles

Methods for assessing the health effects and toxicity of airborne NPs embrace epidemiological and clinical studies, animal models, and cell models (Kumar et al., 2013; Ruijter et al., 2023; Sayes et al., 2007; Vallabani et al., 2023). Although in silico toxicity evaluation is currently in its early phases, it is foreseen as a constructive approach in conjunction with *in vitro* and *in vivo* analyses (Chou et al., 2017; Portugal et al., 2022). Understanding the mechanism behind the toxicity of NPs will help establish the actual danger posed by them (Table 1).

4.1. Epidemiology

Epidemiological and experimental evidence indicate that NPs can lead to several pathological conditions, including poor birth outcomes and slower cognitive development in children, and affect adults with cognitive impairment (Buzea et al., 2007; Muoth et al., 2016). Epidemiologic analyses have associated high levels of NPs (and other combustion products, such as gaseous NOx and O_3) with poor health (Kumar et al., 2013; Li et al., 2003; Münzel et al., 2021). Moreover, oxidative stress and the triggering of apoptosis appear to be the two main mechanisms of embryonic toxicity induced by NPs (Chen et al., 2021).

Table 1
Comparison of different methods for assessing NP toxicity.

Method	Direct Animal Testing	Toxicological Laboratory Tests From Filter Extracts	Air-Liquid Interface (ALI)
Definition	Exposure of test animals (normally, rats) to air samples, either by natural breathing or by forced tracheal instillation	Toxicity analyses of air particles retained in specific filters by different NAMs (Non-animal Alternative Methods)	Direct exposure to air samples of cell cultures growing in the air-culture medium liquid interface.
Advantages	• Similar to actual human exposure • Integrative analysis of all toxic elements of the air sample	• No ethical or legislative issues • Possibility to explore different NAM methodologies (cultured cells, mini-organs, *in vitro* interaction, modified microorganisms, zebrafish embryos, etc.)	• NAM system • Intrinsically on-line • Integrative testing of toxic effects from both gas and particulate phases • Potentially portable, relatively compact (van scale)
	• Possibility to analyze whole-body, systemic endpoints	• Possibility to simultaneously analyze toxicity and chemical composition from sections of a same filter	• Cell exposure more similar to the lung situation than conventional cell culture systems
	• Analysis of reproductive and developmental toxic effects	• Capacity of accumulating particles for long periods of time and of using concentrated extracts	• Potential use of co-cultures imitating lung epithelium and many different types of cell lines.
		• Ability to test samples from distant, even remote locations	• Possibility to test toxicity of different particle sizes by using size selectors with different size cutoffs. • Compatible to air concentrators for testing low-particle environments.
Disadvantages	• Animal testing. Requires specific facilities and permits	• Requires capture systems with appropriate particle size cutoffs	• Needs an expert assessment to translate the results to whole body, physiological toxic effects (like all NAMs)
	• Very intrusive (particularly, for forced instillation methods)	• Unable to recover pollutants from the gas phase	
	• Ethical and legislative issues	• Limited by the capacity to extract the particles from the filters	
	• Limited mobility, in practice only to be used inside animal-housing facilities	• Bias towards organic or inorganic pollutants depending on the method of extraction • Risk of altering of particle size distribution during the process. • Needs an expert assessment to translate the results to whole body, physiological toxic effects (like all NAMs)	

Airborne NPs are commonly perceived as a "human problem" because they mostly impact urban human populations. In people suffering from metabolic syndrome, several cardiovascular-related effects linked to NPs have been identified (Devlin et al., 2014), but the global effects of NPs on human health remain essentially unknown (Rajagopalan et al., 2024). Average exposure to NPs in Asian cities is about four times greater than in European cities, but future research should also consider more comprehensive individualized monitoring of NPs to precisely estimate the overall daily exposure of people living in different urban microenvironments (Kumar et al., 2014b). On the other hand, there is insufficient epidemiological evidence regarding the effects of NP on morbidity and mortality (Ohlwein et al., 2019).

Since epidemiological data are necessarily descriptive and limited to the power of statistics, we need to identify sources and causes of toxicity. In vivo models and cell cultures are particularly important in toxicological analyses because they allow us to conduct in-depth studies that cannot be performed by an epidemiological or clinical approach, such as identifying induced activation of cell signaling pathways. Long-term monitoring of atmospheric nanoparticle concentrations and properties in metropolitan areas should be correlated with their impact through epidemiological approaches (Ohlwein et al., 2019; Rönkkö and Timonen, 2019; Sioutas et al., 2005).

4.2. *In vivo animal studies*

While epidemiological studies are fundamental to monitoring the actual impacts of airborne pollution on human population, the identification of pollutants causing these impacts requires different experimental approaches. Two main aspects are to be pondered when examining NP's effects. First, the variable toxicity of airborne nanoparticles, resulting from changes in composition due to global pressure to reduce combustion emissions, and, second, the increasing incidence of diseases linked to exposure to airborne particles (Schraufnagel, 2020). Exposing animals such as rats, mice, and other vertebrates helps us understand the biological effects of pollution. To understand the mechanisms of action and potential health risks associated with NPs, assays using whole organisms are essential. For instance, *in vivo,* assays using rodents are particularly useful for assessing pulmonary effects. Such studies have shown that NPs are more likely to cause pneumonia than larger particles since they can impair the immune system of the lung and induce inflammation, underscoring their potential toxicity (Buzea et al., 2007; Oberdörster, 2001). Also, rats and mice have been used in experiments to prove that NPs have true harmful effects on the brain. However, because of growing public awareness of animal welfare, regulatory restrictions have been applied to the use of animals in laboratory tests for toxicological evaluation.

Using animals in NP inhalation research is challenging due to the complexity of experimental conditions, which are more complex and intrusive than traditional methods of administering toxic substances through food or injection. Since the implementation of the Animal Welfare Guideline 86/609/ECC (The Council of the European Communities, 1986), European institutions committed themselves to encouraging the development and application of alternative methodologies, a concept usually referred to as the "three Rs principle" (reduction, refinement, replacement). This paves the way to develop methods requiring the use of the lowest possible number of animals and to extract as much information as possible from every experiment (as fewer experiments would render the same information), and, definitively, the replacement of sentient animals with body parts, which are obtained through appropriated euthanizing methods or using some species (Arthropods, Nematodes or Mollusks), or developmental stages in higher organisms (zygotes, embryos) for which is accepted a low or an absent level of pain and physical stress (Tannenbaum and Bennett, 2015; Vasbinder and Locke, 2017).

Another approach is to use zebrafish (*Danio rerio*), which is considered a rather informative model of human development, disease, and

toxic effects. It is worth noting that experiments conducted before the self-feeding larval stage conform with the principle of relative displacement of animal testing. Fish embryos and larvae (mainly from zebrafish *Danio rerio*) are increasingly used in toxicological studies, including testing of the toxicity of airborne NPs (Duan et al., 2017; Lee et al., 2008). Although fish do not have lungs, they are generally accepted as an excellent human model for assessing systemic and embryonic toxicity of air pollutants (Roper et al., 2018). Maintenance and spawning procedures follow the OECD test guideline for zebrafish embryos (Mesquita et al., 2015). Exposure to polluted samples is habitually maintained from 24 h post-fertilization (hpf) until 120 hpf, renewing growing media every 24 h. The development of embryos is observed under the microscope, and embryos can be frozen after exposure for posterior experiments.

4.3. *In vitro models*

In vitro models are often used to examine the toxicity of NPs. The use of animal-free studies has fueled the development of *in vitro* cell models that might preserve the features of genuine target cells and tissues of the body (Fröhlich and Salar-Behzadi, 2014; Rothen-Rutishauser et al., 2023). Several of these cell models include primary cells, in which cells are freshly isolated and cultured, and prevalently established cell lines, in which immortalized cells, often carcinoma ones, can be cultured almost indefinitely.

Cultured cells are helpful models for identifying the mechanism behind PM-induced changes in the lung and other tissues and organs (Durga et al., 2014; Jaén et al., 2021; Reyes-Zarate et al., 2016; Sayes et al., 2007). Nonetheless, extrapolating the impacts of a dose given to cells to the outcomes of inhaled levels reaching respiratory epithelial cells in animals or humans is frequently puzzling, and we should be cautious about extrapolating human health effects from *in vitro* studies (Sayes et al., 2007). The application of NPs to cells or tissue is expected to differ significantly from the short- or long-term exposure of animals or humans. To be as close to real exposure as possible, co-cultures of different cell types in layers like the tissue layers present in organisms and organ/tissue cultures have been proposed as proper exposure targets. When testing air pollutants using *in vitro* cellular models, it is necessary to consider some aspects such as cell models, cell exposure conditions, and toxicity endpoints. In addition, it is important to devise strategies to integrate *in vitro* findings into the context of *in vivo* toxicity, keeping in mind the diversity of pollutants that are present in the air (Zavala et al., 2020).

4.3.1. *Cell and tissue models*

In vitro human tissue models range from monolayer cell cultures to three-dimensional co-cultures. While *in vivo* studies allow us to identify the relationship between dose and occurrence of adverse effects, *in vitro* models can render important information on the mechanisms of action. The endpoints of interest determine the cellular model to choose. Many cell types have been used in modeling biological targets. Human bronchial cells have been used to compare the oxidative potential and harmful consequences of particulate matter (PM2.5) produced directly from an aviation turbine engine and a low-sulfur diesel fuel (EN 590) (Bendtsen et al., 2019). The pro-inflammatory effects of conventional diesel exhaust particles and first- and second-generation biodiesel on the gene expression of interleukins IL-6 and IL-8, as well as CYP1A1, and heme oxygenase (HO-1), have been compared using human bronchial epithelial BEAS-2B cells (Bendtsen et al., 2021; Skuland et al., 2017). *In vitro*, studies have also been performed to better understand the anti-inflammatory response by using human THP-1 monocytes differentiated into macrophages (treated with phorbol myristic acid) (Fukagawa et al., 2013). Macrophages, which represent one of the first lines of defense of the lungs after exposure to particles, make it possible to evaluate the increase in the level of various inflammatory mediators, including various interleukins and TNFα (Fukagawa et al., 2013).

Several "reporter cells" are available. These are cell lines that have been genetically modified to monitor a specific response or harmful effect, such as human lung model cells used to study airborne particles in the respiratory tract (Rothen-Rutishauser et al., 2023). *In vitro*, human cell models represent certain cell types due to their embryological origin or the tissue organs from which they originate (Alfaro-Moreno et al., 2008), but *in vitro,* results should be carefully verified. All these methods have advantages and setbacks, but a common limitation is the loss of systemic effects like the influence of the entire animal or human metabolism, the immune system, and the regulatory responses to the toxic effects. A recently developed way to tackle this problem is using "micro-organs" or "3D cultures", in which cells (either in single or composite cultures) are grown in sophisticated microstructures that simulate organs such as the liver, heart, or brain. There is also a rising interest in the development of "organ-on-a-chip" (Yang et al., 2020), which consists of integrated circuits (chips) that contain multi-channel 3-D cell cultures that could mimic the functions and physiological reactions of a whole organ. Artificial "mini-lungs" have been made utilizing variants of this method. The development of "organ-on-a-chip" provides a promising alternative to utilizing animal models in toxicological testing. In any case, compared to animal models or animal cell lines, results with human organoids are more likely to apply to human disease.

Genetically modified yeast strains can be used to detect ligands for various cellular receptors (Noguerol et al., 2006), and constitute an extremely cost-effective alternative to cell culture experiments. By using the well-developed genetic engineering tools in yeast, it is possible to generate yeast strains that replicate essentially any regulatory animal pathway, the so-called 'Recombinant yeast assay', or RYA, strategy (Noguerol et al., 2006). For example, the replication of the AhR regulatory pathway in genetically modified yeast cells (the AhR-RYA assay) provides an easy methodology to monitor the PAH-mediated toxicity of aerosols (Machala et al., 1996; Mesquita et al., 2014).

4.3.2. *Air-liquid interface (ALI) system*

Although cells growing in 2D cultures have a basic phenotype and respond to environmental disturbances, they do not react to spatial–temporal signals present in the extracellular matrix or local microenvironment that regulates the response of individual cells. Understanding the structure–function relationship between tissues is essential for the development of accurate *in vitro* models in toxicology. Therefore, it is necessary to reconstruct high-fidelity tissue models that mimic *in vivo* conditions. The use of air–liquid interface (ALI) cultures permits the study of the whole aerosol (gas phase and particles) in cell cultures (Mülhopt et al., 2016). Given that the physicochemical characteristics of the particles remain unchanged, they can be studied with the same properties as when individuals inhale them and aerosols are forced into lung cells.

Unlike traditional (submerged) cultures, some models of bronchoepithelial cells grown in an air–liquid interface (ALI) show variations in cell shape, biochemistry, and sensitivity to test a variety of compounds, including environmental pollutants (BéruBé et al., 2010) and airborne nanoparticles carrying some metals (Fromell et al., 2023a). Direct deposition of aerosol particles onto cell surfaces, which resemble *in vivo* exposure by inhalation, is made possible by ALI cultures (BéruBé et al., 2010; Latvala et al., 2016), while secretory products can be collected and analyzed. In simulating in situ exposure of human proximal lung tissues to airborne particles, the objective is to develop a portable ALI system that could be modified and optimized (Latvala et al., 2016; Olofsson et al., 2023). In addition, combining primary cells with this approach can result in the development of a differentiated lung model composed of various cell types.

Compared to submerged exposure models, where a suspension of particles is in contact with the cells, NPs exposure at the air–liquid interphase allows a more realistic assessment of inhalation toxicity of NPs since the aerosol is directly distributed over the cell layer and the properties of the particles are preserved. Furthermore, ALI exposure

systems can imitate airflow as it enters the respiratory system, posing a more realistic particle deposition and flow/shear stress imposed on cells. (Latvala et al., 2016; Mülhopt et al., 2016; Yang et al., 2020).

Several human cell lines, mostly from the respiratory tract, are often used in environmental research. These include, but are not limited to, BEAS-2B, an immortalized, non-tumorigenic human cell line established from normal human bronchial epithelium; A549, an immortalized alveolar basal epithelial cell line derived from a human adenocarcinoma isolated from the lungs of a patient; and Calu-3, a human lung cancer cell line. These cell lines have been cultured in air–liquid interphase (ALI) systems, allowing them to grow in conditions that closely mimic *in vivo* conditions (Silva et al., 2023). An objective of using ALI is to create an *in vitro* environment that accurately represents lung exposure (Fromell et al., 2023b; Latvala et al., 2016; Olofsson et al., 2023). It can monitor NP toxicity in a context more representative of physiological real-life situations (Latvala et al., 2016; Olofsson et al., 2023; Upadhyay and Palmberg, 2018). For example, Calu-3 lung epithelial cells grown at the ALI were used to compare airport and non-airport emissions. At the highest exposed dose (around 1.5 $\mu g/cm^2$) there was a release of LDH and of IL-6 and IL-8. Airport and road traffic NPs and NP samples from a turbine engine had similar toxic properties (He et al., 2020). Likewise, combining primary cells with ALI can develop a differentiated lung model composed of various cell types, such as basal cells, ciliated cells, and goblet cells (Bhowmick and Gappa-Fahlenkamp, 2016). It is worth noting that there are various models of ALI exposure systems for studying the toxicity of airborne particles (Fromell et al., 2023; Latvala et al., 2016; Pontes et al., 2024; Rossner et al., 2019)). Some systems, such as EAVES, ALICE, and ExpoCube, have been developed by research groups, while others are commercially available (e.g., Cultex LTC-C or Vitrocell). They differ in terms of particle deposition and airflow introduction methods, and some are specifically designed for studying nanoparticles and examining the gas phase separately (Fromell et al., 2023; Latvala et al., 2016).

We can conclude that the best approach to studying NPs is to use primary cells in an ALI exposure system, which is the most realistic way to simulate *in vivo* conditions. Nonetheless, using an ALI system is more technically demanding and requires more equipment than conventional cell culture.

4.3.3. Toxicogenomics

Systems toxicology (omics) is utilized to evaluate toxicity by combining high-throughput techniques, such as genomic sequence analysis, proteomics, and metabolomics, to identify harmful changes in genetic expression, protein expression, and metabolites (Portugal et al., 2022). Toxicogenomics refers to genomic technologies that seek to identify toxicological pathways with negative consequences (Martins et al., 2019). This is an important step towards strengthening epidemiological evidence linking air pollution to an increasing number of diseases. Hazard identification and early assessment of potentially toxic compounds can be facilitated by toxicogenomic approaches.

Environmental 'omics' have been used to define toxicity mechanisms and short- and long-term effects of environmental chemicals on human health outcomes, and to define the acceptable levels and potential impacts of environmental toxicants on sensitive target species and ecosystems (Ge et al., 2013). Omics technologies can address a variety of environmental and human health issues, such as the effects of climate change on different species. In an epigenomic study, differentiated levels of global DNA methylation between lungs and blood cells were observed in rats exposed to traffic-related air for up to seven days. Gene-specific increases and decreases in methylation occurred (Zhang et al., 2024b). Epigenomics is particularly relevant as a powerful tool that can be utilized to study the cellular response to endocrine-disrupting chemicals that can be transmitted to offspring across generations (It is understood that offspring are not directly exposed to the endocrine-disrupting substances) (Zhang et al., 2024b). Moreover, toxicogenomics might identify existing challenges for future research (Halappanavar et al., 2020; Moffat et al., 2015; Piña et al., 2018). Besides, environmental metabolomics has evolved into a technique for comprehensive metabolic monitoring, which can identify biological effects and toxicological pathways linked to NP exposure (Kelly and Fussell, 2020; Portugal et al., 2022).

5. Technical difficulties and challenges in nanoparticle toxicity testing

Collecting enough nanoparticles (NP) for toxicity testing is a challenging process. Moreover, the aggregation of NPs during collection may alter their physicochemical properties, making the results of any toxicity test unreliable. It is worth noting that NPs can change their properties when separated from the collection filters. Additionally, during sampling, some volatile and semi-volatile compounds can stick to the surface of the NPs and be lost. Water-soluble compounds are also at risk of being lost. These challenges must be considered when performing toxicity tests with a group of NPs. Additionally, for particulate collection, it is important to select samplers, such as cascade impactors (whose design contemplates multiple impactors to cover different particle sizes), to manage particulate collection time and efficiently extract particles without altering their physicochemical properties (Kumar et al., 2021).

The amount of NP mass collected for toxicity and physicochemical characterization depends on various factors, such as the flow rate of the collection equipment used, the size and mass of particles, and the atmospheric conditions at different locations (Kumar et al., 2013; Vouitsis et al., 2023). Collecting nanoparticles for chemical identification and toxicological studies can use cascade impactors with flow rates ranging from 9 to 30 Liters per minute (LPM). For instance, high-volume impactors used are the High-volume five stages plus back-up cascade impactor (HVFCI) and the high-volume impactor sampler (HVIS) with a flow rate of 400 LPM. Efficient measurements have to consider the target concentration level in the microenvironment, and the equipment capacity to collect nanoparticles within a practical timeframe. Although not detailed in this review, additional features related to sampling tools are available elsewhere (Kumar et al., 2013; Vouitsis et al., 2023), along with specific recommendations to enhance the likelihood of obtaining the required number of particles (Kumar et al., 2013; Vouitsis et al., 2023).

Given that the relationship between NP in the environment and adverse health effects is well established, the main gap in understanding the toxicity of NP is which components of airborne NP, as well as their characteristics, are responsible for those effects. Researchers are faced with a dilemma. They must choose between using custom-made NPs with specific properties that do not accurately represent the particle variability of airborne particles, or, for example, using naturally produced NPs from emission, and trying to evaluate which component is responsible for the toxic effect. Closing gaps in our knowledge seems essential to advise on recommendations and laws to improve air quality. Moreover, it is worth creating appropriate mitigation strategies that would contribute to the purpose of protecting the environment and human health.

Epidemiological and toxicological research has shed light on several aspects of the cardiorespiratory effects of NPs (Münzel et al., 2021). However, deeper knowledge is required. There are two main points to consider. First, the variable toxicity of airborne nanoparticles is caused by the likely change in their composition due to global pressure to achieve lower combustion emissions, and, second, the increasing number of people suffering from diseases associated with exposure to airborne particles. Long-term monitoring of atmospheric nanoparticles and their impact on human health in urban areas should be linked through epidemiological approaches. The characteristics of the different experimental setups to assess NP toxicity are summarized in Table 1.

In general, the accumulation of nanomaterial (NP) mass for toxicity and physicochemical characterization depends on the flow rates of the

collection instrument and the size and mass of the particles. NPs aggregate during collection, storage, and/or analytical processes, making it difficult to investigate their toxicity (Malakar et al., 2021). Another factor limiting the assessment of the toxicity of NPs is their highly complex structure, which might include hundreds or thousands of potentially hazardous components with multiple additive or synergistic effects (Malakar et al., 2021; Vouitsis et al., 2023). As a result, the methodologies used to assess the toxicity of exhaust NPs consider a wide variety of factors, comprising sizes, state of aggregation, organic and inorganic composition, and modes of uptake.

Airborne nanoparticles are abundant, but their mass is tiny (Buzea et al., 2007). More information is still needed on their composition and origins, as well as on their nucleation and transformation in the atmosphere, along with how nanoparticles are dispersed in the human body upon inhalation, including the pathways used, how efficiently they are transported, and how long the nanoparticles remain in the human body (Kulmala et al., 2004). A challenge for future studies is to establish whether the particle number, mass, or surface concentration of nanoparticles is the best fundamental parameter for assessing health effects (Rönkkö and Timonen, 2019).

A standardized procedure has not yet been implemented to guarantee that the samples of NPs are representative of the chemical mixtures found in the environment. Methods for gathering particulate matter have been thoroughly explored (Demokritou et al., 2004; Kumar et al., 2021; Roper et al., 2019) and specific advice has been given about improving the probability of obtaining the required particles. However, standardized protocols are required to prevent any possible biases in chemical recovery resulting from the chosen protocol.

Transferring NPs from collected filters to organic solvents can change the physicochemical characteristics of NPs (Demokritou et al., 2004). Extraction into organic solvents by sonication can alter the properties of NPs. On the other hand, extraction into aqueous solvents can also dissolve part of the inorganic fraction due to ion redistribution (Choi et al., 2017). Moreover, particle extraction could contaminate filters. Depending on the separation method, the filter type used, and the properties of the collected compounds, the amounts of recovered NP can vary considerably. However, differences in extraction solvent and filter (Teflon or quartz) do not appear to alter significantly different parameters measuring the oxidative potential of samples (Yang et al., 2014). Besides, significant differences have been observed in the percentage of incidence and timing of mortality in zebrafish, which have been linked to the extraction method (Roper et al., 2019). A zebrafish assay used as a model to examine exposure to NPs may represent a unique opportunity to establish a rapid standardized filter extraction-hazard assessment protocol for NPs in an entire animal model (Ayres et al., 2008; Mesquita et al., 2015; Roper et al., 2019).

The air–liquid (ALI) exposure systems, described in section 4.3.2, can directly expose cells, mimicking the inhalation of particles into the lungs. It is a useful "surrogate system" that provides direct *in vitro* exposure that can, for example, mimic the inhalation of particles into the lung (Latvala et al., 2016; Olofsson et al., 2023). In addition, it has the unique advantage of integrating toxic effects from both gas and particulate phases, and minimizing the risk of altering the original particle size profile, as it does not require a particle extraction step.

6. Concluding remarks

Air pollution is considered a main environmental risk factor for human populations and, in some respects, it acquires truly global proportions, affecting large sectors of the biosphere. Among air pollutants, NPs are of increasing concern. Their main sources are the burning of fossil fuels and biomass, followed by industrial emissions (Kumar et al., 2013; Vouitsis et al., 2023) The toxicological properties of NPs are closely linked to their composition and size, which determines their presence in the air, regional deposition in the human lungs, and potential toxicity (Buzea et al., 2007; Kwon et al., 2020; Vallabani et al., 2023).

When living organisms are exposed to NPs, various effects can occur that are not caused by individual components. The uptake of airborne NP by living organisms can produce both direct and indirect effects on the respiratory tract, heart, and nervous system. Those effects may be significantly more severe than the addition of the toxicity of their constituents (Daiber et al., 2020; Holme et al., 2019; Kelly and Fussell, 2020; Vallabani et al., 2023).

In toxicological studies, sample collection depends on the instrument, site characteristics, and current atmospheric conditions. Various factors influence the mass of NP required for any toxicological tests. Typically, large sample sizes are needed for several experiments during toxicological evaluations to examine exposure effectively, which implicates the use of large collectors and appropriate filters. Whereas the traditional knowledge indicates that the filter type and solvent used for particle removal did not influence the relative oxidative potential reactivity, it is currently established that the removal method could alter the toxic profiles of the particles (Kumar et al., 2021).

Studies using *in vitro* cell culture methods or animal models are useful for understanding the basic mechanisms behind airborne NP toxicity. Many cell types can uptake NPs, which cause oxidative stress and inflammatory responses (Horie and Tabei, 2021). The metal fraction of NPs may also initiate oxidative stress. Nanoparticles can increase ROS generation and chromosomal damage, resulting in proinflammatory reactions. ROS formation and oxidative stress have been linked to responses to inflammation, release of cytokines, and altered cell structures (Daiber et al., 2020; Holme et al., 2019; Li et al., 2003). In addition, NPs organic fraction is assumed to be primarily responsible for the genotoxic activity associated with airborne pollution. The combination of all these toxic activities has severe systemic impacts, including cardiovascular and nervous system diseases (Daiber et al., 2020) in addition to damaging the respiratory and pulmonary tracts and inducing different lung disorders (Fig. 1). The negative effects of airborne NPs on cardiovascular health are mostly related to inflammation, oxidative stress, and elevated blood pressure, followed by myocardial infarction, and acute coronary syndromes (Daiber et al., 2020; Ohlwein et al., 2019).

There are grounds for considering that airborne particulate matter can lead to lung cancer (IARC/WHO, 2013), which seems to occur with an intensification of systemic inflammation rather than direct genotoxic damage. Tumor development does not appear to result from DNA damage. Instead, it is caused by a cellular inflammatory response to pollutants in which the release of cytokines stimulates the proliferation of already existing mutated cancer cells (Hill et al., 2023). Additionally, the genotoxicity and carcinogenicity of PAHs remain well-established mechanisms of toxicity (Holme et al., 2023).

Identifying the constituents and physical characteristics of NPs that cause toxic effects requires the development/selection of appropriate animal and cellular models. However, designing realistic exposure setups for inhalation in vertebrates is much more complicated than standard toxicological procedures that use oral, dermal, peritoneal, or intravenous substance administration. Moreover, the growing public awareness of animal welfare makes it more difficult to develop these models following current ethical and legal standards. *In vitro*, exposure systems, such as the air–liquid interface (ALI) system, provide valuable screening data on the relative toxicity of inhaled particle types, standardized, and validated by comparison with *in vivo* effects (Latvala et al., 2016; Mülhopt et al., 2016; Sayes et al., 2007). Observations based on *in vitro* methods or animal models need to be validated with epidemiological or clinical data to demonstrate their relevance in assessing the risks to human health posed by NP emissions. The correct application of risk assessment, public health, and multidisciplinary intervention strategies in all populations exposed to nanoparticles should be reinforced.

CRediT authorship contribution statement

José Portugal: Writing – review & editing, Writing – original draft.

Carmen Bedia: Conceptualization. **Fulvio Amato:** Conceptualization. **Ana T. Juárez-Facio:** Writing – review & editing. **Rodopi Stamatiou:** Conceptualization. **Antigone Lazou:** Conceptualization. **Chiara E. Campiglio:** Writing – review & editing. **Karine Elihn:** Writing – review & editing. **Benjamin Piña:** Writing – review & editing, Writing – original draft.

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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