# University of Naples Federico II

## Department of Neuroscience, Reproductive Sciences and Dentistry

**Division of Pharmacology, School of Medicine**


# Neurotoxic effect of ultrafine carbonaceus components of air pollution in the central nervous system: UFPs putative mechanisms in neurodegenerative diseases

**Thesis submitted for the degree of**
**Doctor of Philosophy**

XXXVII Doctoral cycle
Academic year: 2023-2024


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# Summary

Ninety-nine percent of the global population has been estimated to be exposed to polluted air and indeed, air pollution is believed to be by many researchers one of the greatest scourges of our era, for its inevitable impact on our world. Besides having an active role in affecting the environment and ecosystem, it affects our society and most importantly, our health and life-expectancy - as it represents the second leading risk factor for early death worldwide.

Among all air pollutants, particulate matter (PM) is a heterogeneous class consisting of particles of different sizes, which has the infamous highest burden of disease globally.

The small size of these particles makes them especially harmful and, in fact, smaller particles are associated to greater adverse health outcomes than larger ones. In particular, the smallest fraction of PM, ultrafine particulate matter (UFP), has been shown to be able to penetrate virtually all human barriers: the skin, the alveolar-capillary, placental and blood brain barrier. Not surprisingly, UFPs have been associated to different nervous system pathologies, such as amyotrophic lateral sclerosis (ALS); yet the mechanisms underlying are still mysterious and the knowledge on the effects of UFPs is lacking. Moreover, current world regulations do not include UFPs, as data are not sufficient to provide recommendations, but only good practice statements.

Despite this, UFPs represent the dominant fraction of aerosols dispersed into the air and human exposure to it will increase drastically in the next decades.

Therefore, the main purpose of the present doctoral study was to evaluate the toxicity driven by the finest fraction of particulate matter in *in vitro* and *in vivo* models.

More precisely, this doctoral study focused, *in vitro*, on finding the cellular mechanisms through which UFPs exert their detrimental effects on the nervous tissue, specifically on motor neurons. Secondly, it aimed at establishing such mechanisms as pathological pathways shared with the neurodegenerative disease ALS - a motor neuronal disease in which the role of environmental etiology has been extensively addressed.

Whilst i*n vivo*, the aim was to explore the toxicity of UFPs in a more complex organism.

To this scope, UFPs were produced in a lab-scale manner, to yield nanoparticles having high similarities in size and microscopic structures with particles emitted by diesel exhaust combustion ($PM_{0.1}$ < 100 nm and NP20 < 20 nm); thus providing a model for anthropogenic diesel exhaust emissions.

Taken together, results show that the ultrafine fraction of PM, namely $PM_{0.1}$ and NP20, exerts neurotoxicity through several mechanisms targeting different cellular components and compartments, such as the plasma membrane, mitochondria, lysosomes and ER: in all cases, UFPs were able to impair organelles functionality and eventually to have a detrimental effect on cellular homeostasis. Furthermore, the present study indicated that UFP-driven toxicity shares several pathomechanisms with neurodegenerative diseases, in particular with ALS. $PM_{0.1}$ and NP20 were also able to yield toxic effects on the reproduction, growth and lifespan of more complex organisms.

Lastly, this doctoral research provided evidence for smaller sub-fractions of PM (i.e. NP20) being more toxic than larger UFPs (i.e. $PM_{0.1}$).

# Acknowledgments

*I would like to thank my research group at the University of Naples; Dr. Barbara Apicella , Prof. Dr. Stefania Loffredo and their team and the Worm Lab at the University of Kent - especially to Dr. Jennifer Tullet for hosting me in her lab and having me supervised in the in vivo part of this doctoral project.*
*An extremely warm thank goes to my professor and supervisor Prof. Agnese Secondo. Her lively personality has always been my first source of motivation and inspiration throughout these three years. She is a real example of female power; joining empathy, cleverness, strong morality and energy and I hope that she will keep inspiring other people inside of the Academia with her incredible contagious passion and curiosity.*

*A special thank goes to my colleagues and dear friends Daniele and Michele.*

*Lastly, I am thankful to my family who have supported me along this journey and who have contributed economically to my education, in this sad historical period of economic crisis - they are my everything, together with my friends, and I am so delighted to celebrate this accomplishment with them!*

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## Abbreviations

**AD** Alzheimer's disease

**ALS/PDC** ALS-Parkinsonism-dementia complex

**ALS/sALS** amyotrophic lateral sclerosis/sporadic form of amyotrophic lateral sclerosis

**AMPK** AMP-activated protein kinase

**AQGs** Air Quality Guidelines

**BBB** blood brain barrier

**BMAA/*L*-BMAA** β-N-methylamino-L-alanine/isomer L of BMAA

**CNS** central nervous system

**CO** Carbon monoxide

**CPM** condensable PM

**DOCs** diesel oxidation catalyst

**DPFs** diesel particulate filters

**DPM** PM emitted by diesel engines

**DMSO** Dimethyl sulfoxide

**EC** elemental carbon

**ER** endoplasmic reticulum

**FPM** filterable PM

**GDI** gasoline direct injection engine

**GSH/GSH-Px** glutathione/glutathione peroxidase

**HC** Hydrocarbons

**IAQ** indoor air quality

$I_{CRAC}$ $Ca^{2+}$ release-activated current

**IHME** Institute for Health Metrics and Evaluation

**LSDs** lysosomal storage disorders

$NH_4^+$ ammonium ion

$NO_2$ Nitrogen dioxide

$NO_3^-$ nitrate ion

**NOx** nitrogen oxides

**NPF** New particle formation

**NP20**

**NSC-34** Mouse motor neuron-like cells

**OC** organic carbon

$O_3$ Ozone

**PAHs** polycyclic aromatic hydrocarbons

**Pb** Lead

**PD** Parkinson's disease

**PM** particulate matter

$PM_{10}$

$PM_{2.5}$

$PM_{0.1}$

**ROS** reactive oxygen species

**SOA** secondary organic aerosol

**SOCE** Stored-operated Calcium Entry response

**SOD** superoxide dismutase

**SOF** soluble organic fraction

$SO_2$ Sulphur dioxide

$SO_4^{2-}$ sulfate ion

**SVOCs** Semi-volatile organic compounds

**TG** thapsigargin

**UFP** ultrafine particulate matter (or $PM_{0.1}$)

**UPS** ubiquitine-proteasome system

**VOCs** Volatile organic compounds

**WHO** World Health Organization

*Part of the data and methodology here presented have already been published in the following papers as first author manuscripts.*

*Sapienza, S., Tedeschi, V., Apicella, B., Palestra, F., Russo, C., Piccialli, I., Pannaccione, A., Loffredo, S., & Secondo, A. (2022). Size-Based Effects of Anthropogenic Ultrafine Particles on Lysosomal TRPML1 Channel and Autophagy in Motoneuron-like Cells. International journal of molecular sciences, 23(21), 13041. https://doi.org/10.3390/ijms232113041*

*Sapienza, S., Tedeschi, V., Apicella, B., Pannaccione, A., Russo, C., Sisalli, M. J., Magliocca, G., Loffredo, S., & Secondo, A. (2024). Ultrafine particulate matter pollution and dysfunction of endoplasmic reticulum $Ca^{2+}$ store: A pathomechanism shared with amyotrophic lateral sclerosis motor neurons?. Ecotoxicology and environmental safety, 273, 116104. https://doi.org/10.1016/j.ecoenv.2024.116104*

# 1. Introduction

## 1. Air Pollution

### 1.1. Components of air pollution

The term air pollution refers to a condition of the environment, in which a chemical, physical or biological agent modifies the natural characteristics of the atmosphere (World Health Organization (WHO), 2024). These contaminants are referred to as air pollutants and have an enormous and ubiquitous impact on the ecosystem at global scales, thus having tremendous health, social and economical implications on our society.

Air pollutants can be liquid, solid or gaseous. A first broad distinction is made whether the contaminant is emitted directly from a source (primary pollutant) or if it is formed reacting in the atmosphere (secondary pollutant). For instance, ozone is a secondary pollutant that it is formed when hydrocarbons (HC) and nitrogen oxides ($NO_x$) react with sun light, as well as $NO_2$: in this case, $NO_2$ is the combination of NO with oxygen.

Among all air pollutants, we can list the most important components from a health-related perspective:

1. **Airborne particulate matter** (PM), which will be extensively and thoroughly discussed in the following paragraphs.

2. **Nitrogen dioxide** ($NO_2$). A reddish-brown gas soluble in water, precursor of ozone. Even though $NO_2$ has different sources, the predominant one is represented by the combustion of fossil fuel and vehicle emission (Atkinson et al., 2018). It is a strong oxidant. A recent meta-analysis and systemic review (Huang et al., 2021) has provided robust evidence that long-term exposure to $NO_2$ is associated with a higher risk of all-cause, respiratory and cardiovascular mortality. A parallel analysis (Wang et al., 2021a) has found the same association following a short-term exposure.

3. **Carbon monoxide** (CO). An odorless, tasteless and colourless gas produced by the incomplete combustion of carbonaceous fuels (petrol, coal, wood, natural gas ..), although the main source of emission is represented by motor vehicles. With its high affinity to hemoglobin (Hb), tissue hypoxia is well recognized as its main pathophysiological effect. Specifically, CO binds to numerous heme-containing proteins, however, Hb has a 250-fold greater affinity to CO than for oxygen. Thus, CO competes with the latter, resulting in the displacement of oxygen to form carboxyhemoglobin and therefore, reducing the capacity of carrying oxygen (Hall J., 2010). Exposure to CO may cause difficulties breathing, exhaustion, dizziness, flu-like symptoms, headache, nausea, tachycardia (WHO, 2024; Chiew & Buckley, 2014). CO binds to several other elements, such as mitochondrial cytochrome oxidase, cytochrome c oxidase, platelets and leading to oxidative stress and inflammatory effects (Chiew & Buckley, 2014). CO poisoning is a common cause of emergency department admission and it is still related to a high level of morbidity and mortality worldwide (Rose et al., 2017); indeed, in some

countries death resulting from CO poisoning is comparable to death caused by diabetes mellitus (Hosseininejad et al., 2018; Braubach et al., 2013). CO exposure can ultimately be fatal, besides potentially leading to delayed neurological effects, including Parkinsonism, memory and concentration impairment, cardio-toxicity and reduced life-expectancy. (Chiew & Buckley, 2014).

4. **Sulphur dioxide** ($SO_2$). A primary pollutant mainly produced by the combustion of sulfur-containing fossil fuels such as oil and coal by power plants and industry, metal extraction, ships, vehicles and by volcanic activity (Khalaf et al., 2024). Moreover, its high water-solubility in raindrops and vapor, gives rise to secondary pollutants such as sulfuric acid, that damage object, buildings, plants and animal health. Khalaf et al., 2024, in his narrative review, has shown that $SO_2$ inhalation increases the incidence of allergic and respiratory diseases, cardiovascular diseases, as well as the risk of stroke, seizures, type-2 diabetes and the mortality from cardiovascular, respiratory diseases and type-2 diabetes. Additionally, whilst short-term effects have been associated with high concentrations of $SO_2$, long-term effects have been related to average concentrations (Goudarzi et al., 2016).

5. **Volatile organic compounds** (VOCs). VOCs are organic compounds whose chemical properties make them able to evaporate under normal indoor atmospheric conditions of temperature and pressure (United States Environmental protection agency (EPA), 2024). Although there are no universal health-based guidelines in Europe, they represent one of the most potentially toxic indoor air chemical class (Halios et al., 2022). They can be found both outdoors, as by-products of industrial and commercial activities, road traffic exhausts or indoors, generated by the use of everyday products. In residential environment sources are often

consumer products (detergents, cleaning and polishing products, air fresheners, personal care products, candle and incense burning), construction and building materials (paints, furnishing, solvents), space heating, electronic devices such as printers (Halios et al., 2022; Shrubsole et al., 2019). Halios et al., 2022, in his systematic review has presented the VOCs measured in Europe, with their health end-points (i.e. effects on the respiratory, nervous, cardiovascular system; allergenic effects and carcinogenity). It is worth mentioning the following VOCs, which are among the most health-relevant and commonly measured: acetaldehyde, acetone, benzene, ethylbenzene, formaldehyde, limonene, styrene, toluene, α– Pinene, naphthalene). Another important aspect of VOCs is volatility: depending on their relative volatility they can be found almost entirely as gases or in solids or liquids that contain them, or on surfaces, including dust and furnishing (EPA, 2024). Semi-volatile organic compounds (SVOCs) include polycyclic aromatic hydrocarbons (PAHs). PAHs are predominately petroleum hydrocarbons and consist of two or more fused benzene rings with different structural configurations generated by incomplete combustion of both organic matter (e.g.: cooking meat) and human anthropogenic activities (Ravindra et al., 2008). PAHs spread from one place to another through air and water and they can even be found in the soil, contaminating fruit, vegetables, grain and marine food and thus posing a sever threat to human health (Premnath et al., 2021). Adverse health effects include carcinogenity, genotoxicity, cytotoxicity, cardiotoxicity, hemotoxicity, neurotoxicity, hepatoxicity, immunotoxicity, teratogenicity, mutagenicity (Premnath et al., 2021).

6. **Lead** (Pb). This heavy metal and lead particulate compounds can be found indoors in contaminated dust from many products, namely

plumbing materials, batteries, gasoline, pipes, ceramics, paints, the arms industry, pigments, printing of books, cosmetics, medicine consumption and vehicle exhausts. Moreover, a major source of exposure is represented by drinking water (WHO, 2024; Sani & Amanabo, 2021; Raj & Das, 2023). Absorption can occur through four principal routes: inhalation, ingestion, transdermally and percutaneously (Sani & Amanabo, 2021). Its repercussions on human health are many and involve virtually all organs. They can be deleterious particularly to children and pregnant women. In children, lead can act as a neurotoxin disrupting neurotransmitter release and yielding symptoms such as behaviour and learning problems, lower IQ and hyperactivity, impaired development, hearing loss, anemia. Lead exposure in adults may cause cardiovascular effects, increased blood pressure, kidney failure and sterility issues, hallucination and respiratory and neurological disorders (WHO, 2024; Raj & Das, 2023).

7. **Ozone** ($O_3$). Ozone can be found in different layers of the atmosphere: depending on which, it can be beneficial or detrimental to our species. In fact, in the stratosphere ozone filters the ultraviolet rays coming from the sun. However, in the troposphere, high concentrations of ozone are formed through photochemical reactions with other pollutants like VOCs, CO and nitrogen oxides, thus exerting its toxicity (WHO, 2024; Donzelli & Suarez-Varela, 2024). Ground-level ozone effects range from transient symptoms to chronic and life-threatening conditions. The respiratory tract is mainly affected; although $O_3$ is also able to cause other systemic disorders like cardiovascular pathologies, with an increased risk of stroke and heart attack. Moreover, it contributes to neurodegenerative disease, such as Alzheimer's disease (AD) and disorders like the attention-deficit/ hyperactivity disorder (ADHD). It also positively correlates with

mortality due to Parkinson's, dementia, multiple sclerosis and has been associated with an increased incidence of anxiety and depression. Moreover, inflammation driven by $O_3$ exposure is also able to impair the immune system (Donzelli & Suarez-Varela, 2024) .

## 1.2. **Indoor and Outdoor pollution**

Air pollution is often categorized into two microenvironment, depending on where the pollution is occurring: these are ambient (outdoor) air pollution and household (indoor) air pollution. Both of them are associated with a high incidence of premature death. However, it is important to stress that they are a product of conceptual simplifications and that in actual life, they are strictly associated, actively affecting each other quality. Pollutants are transferring with different mechanisms between the two environments, thus making the understanding of their interaction crucial to counteract air pollution with an effective intervention strategy. The latter includes the transformation of indoor space usage and cost-effective plant approaches, the so-called phytoremediation, which promotes the adoption of potted plants, green walls and active botanical filters for indoor air purification (Montaluisa-Mantilla et al., 2023; Kumar et al., 2023a; Dennis, 2015).

The three main determinants playing a role in transmissions are, according to Mohammadi and Calautit (2022): natural ventilation (intentional openings across the building fabric, e.g: window opening), mechanical ventilation (use of systems to force airflow) and infiltration (air leakage/entry via opening and gaps). Conditions outside and inside buildings further affect the transmission of air pollutants, such as weather conditions (i.e. temperature differences, wind, humidity, solar radiation), urban geometry and context

(urban “canyons”, trees and traffic), indoor activities and deposition. Conventionally, fixed air quality monitoring stations have been employed to monitor outdoor pollution levels. However, the exposure pattern is quite different, as people spend most of their time indoors. Therefore, a good indoor air quality (IAQ) is one of the major indexes monitoring people health and well-being and it is paramount to improve it (Mohammadi & Calautit, 2022; Dennis, 2015; Kumar et al., 2023b).

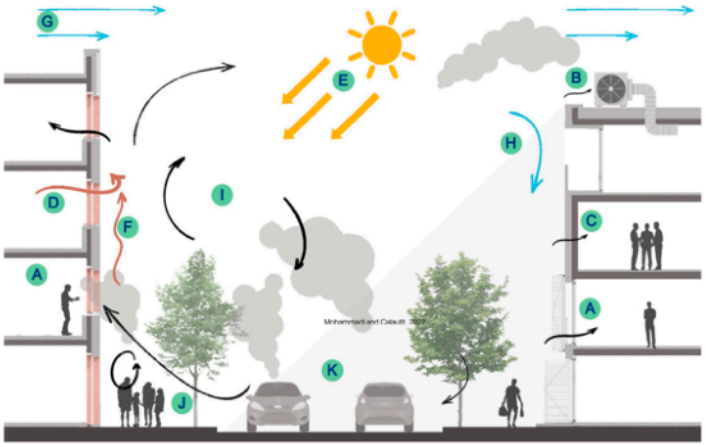



**Figure 1**. Transmission interactions between indoor and outdoor environments. (A) natural ventilation. (B) mechanical ventilation intake. (C) infiltration. (D) buoyancy-driven ventilation and cross-contamination. (E) solar radiation. (F) thermal plume due to surface heating. (G) prevailing wind. (H) flow into canyon. (I) recirculation in urban canyon. (J) local amplification due to street elements. (K) local sources.
Figure taken and adapted from Mohammadi and Calautit, 2022.

### *1.2.1.* ***Indoor pollution***

Not only people spend around 90% of their time indoors, where the concentrations of pollutants are often 2 to 5 times higher than outdoors; but indoor pollution levels can also quickly rise to 100 times the outside pollution level (EPA, Indoor air quality, 2020; IQAir, 2018).

Indoor air pollution levels have increased in the last decades due to an increase of use of synthetic building materials, furnishings, personal care

products, pesticides, household cleaners and of energy-efficient building constructions that lack sufficient mechanical ventilation (EPA, Indoor air quality, 2021). Not surprisingly, the urban population is highly exposed to air pollutants and the situation is worsened by phenomena like overpopulation and the presence of industries near cities (Liang & Gong, 2020).

However, inhabitants of rural places or developing countries are exposed to significant levels of indoor air pollution as a result of a lack of contemporary energy options available. For instance, kerosene combustion for cooking and heating (Kumar et al., 2023b).

Sources of indoor pollution generally include cooking, cleaning, smoking, but even the movement of the household occupants is able to cause the resuspension of particles (Dennis, 2015). Biological activities are another important source of contaminants - pets, plants and humans may bring on with them biological contaminants (e.g.: bacteria, spores, fungi, yeasts, viruses, microbial toxins and secondary metabolites - such as bacterial endotoxin and pollen, insects and plants allergens), (Moldoveanu, 2015). Common combustion sources such as oil, coal, tobacco and wood products, chimneys, furnaces, cookers and candle-burning, produce several gaseous and VOCs pollutants; as well as household cleaning products, furnishings (e.g.: carpets, heating and cooling systems - stoves, ovens, heaters and water heaters), humidification systems and the use of pesticides outdoors (Godish, 2016; Jarvis at al., 2010; Kumar et al., 2023b).

Radon is another common indoor pollutant. The main radon sources are represented by soil gas, tap water and building material that can emit radon, like stone, concrete and bricks (Kumar et al., 2023b). Indoor ozone is primarily produced by the use of electrical equipment or transmitted from

the external environment and has many harmful by-products when reacting with other indoor pollutants (Huang et al., 2019).

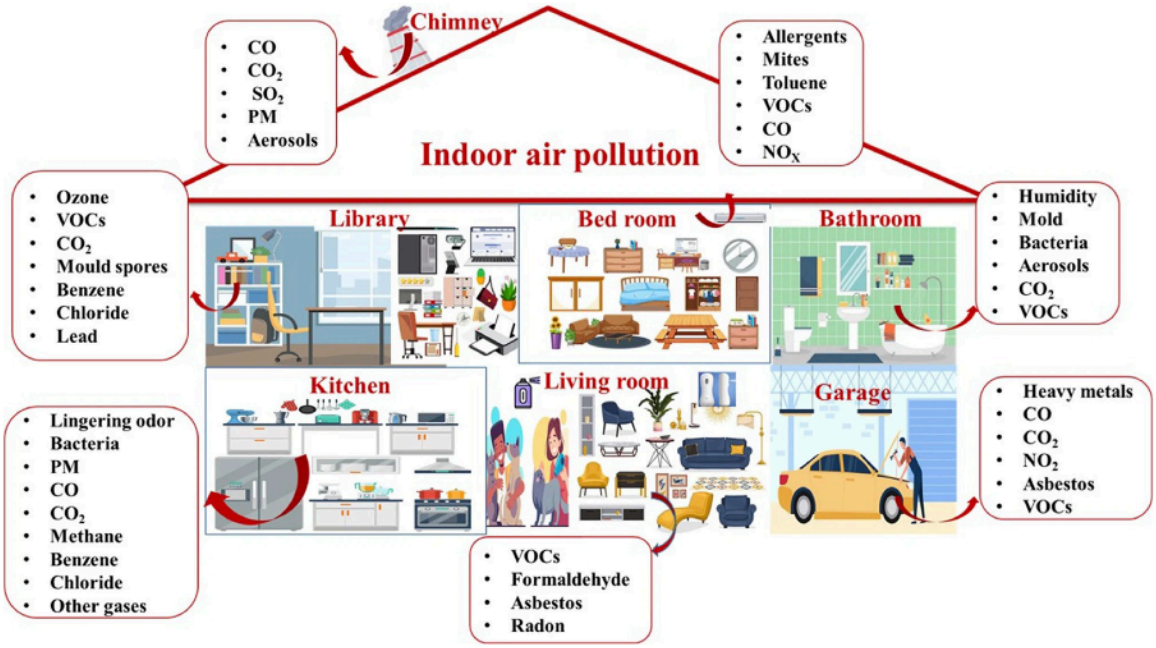



**Figure 2**. Indoor sources of air pollution. Figure taken and adapted from Kumar et al., 2023a.

### *1.2.2. **Outdoor pollution***

Outdoor air quality is not only important from a health perspective - as it contributes largely to the global burden of disease when the air is polluted, but also, it contributes to the phenomenon of acid rains; it can impair visibility and damages crops, as well as monuments and buildings. It is able to decrease the protective ozone layer and has an impact on indoor air quality (EPA, 2024).

Sources are numerous and can be both of natural and anthropogenic origins. Natural sources are mainly attributable to windblown dust and desert dust, emissions from vegetation (pollen and mould spores), volcanic activity,

spontaneous wildfires, sea spray but also natural disasters like flooding and hurricanes.

Anthropogenic sources are, however, the principal ones. Contaminants are emitted into the environment during combustion processes due to fuel burning - cars, planes, ships, trains; industrial and manufacturing activities, power plants burning coal, gas oil and biomass; heating and cooling and powering homes and last but not least - indoor related activities like using self-care products or detergents.

## 1.3. **The toll of air pollution: burden of disease**

Air pollution is thought to be, by many researchers, one of the greatest scourges of our century and even era, for its impact on our life and society (Manisalidis et al., 2020). Besides having an active role in affecting the environment and ecosystem, it affects our society and most of all, our health and life-expectancy.

On the one hand, 99% of the global population is estimated to be exposed to air that contains high levels of pollutants (WHO, 2024); on the other hand, air pollution exposure is associated with high levels of morbidity and mortality. Currently, it is the second leading risk factor for early death worldwide, surpassed only by high blood pressure and outranking tobacco as a leading cause of death and disability (Health Effects Institute, State of global air, 2024). Moreover, 34% of preterm births in 2021 were attributed to exposure to air pollution (Institute for Health Metrics and Evaluation (IHME), 2024). In the study of Lelieveld et al. (2019), the authors has estimated that the mean life-expectancy decreased per affected individual is about 26.5 years, whilst the global mean loss of life-expectancy is 2.9 years.

The two most widely-cited estimates of the toll of air pollution come from the WHO and the IHME. The estimated death toll is relatively similar. IHME, in collaboration with the Health Effects Institute, has very recently released the State of Global air 2024, a comprehensive report of risk assessment of exposure to fine particulate matter ($PM_{2.5}$), $NO_2$ and $O_3$, - the latest, worldwide.

The State of Global Air 2024 captures, with striking statistics and numbers, the magnitude of air pollution and the estimated death toll.

Deaths attributed to air pollution exposure were 8.1 million in 2021 alone - more than 1 in 8 deaths worldwide; of these, 90% are associated to noncommunicable diseases, that is those diseases that are not spread from person to person and are often chronic (e.g: cardiovascular diseases, cancer, diabetes, chronic respiratory diseases etc.).

Elders, children and pregnant women are the most vulnerable in the population - more than 700 000 deaths in children under five were due to household and outdoor air pollution-related causes.

And yet, the State of Global Air 2024 does not fully capture the scale of the phenomenon. Indeed, it lacks estimates of numbers of people affected and suffering from poor health as a result to air pollutants exposure, estimates of other pollutants - other air pollutants than PM, which are rarely considered in global studies or, as it is stated in the report, it lacks: “ The true human toll of each life that is lost too early, the human suffering that accumulates over the course of a chronic illness, or the burdens borne by families and communities”.

Roser in (2021) has recently put together and analysed data coming from the principal studies that have estimated the global impact of air pollution on the population. It is quite difficult to pin point the actual contributions of

indoor air pollution or outdoor pollution, in terms of deaths. Therefore, numbers of deaths span a wide range, although all authors agree on the toll being in the millions. As Roser (2021) points out, this indicates that research on the global impact of air pollution is still in its early stage. However, recent studies tend to report, generally, a higher death toll compared to older studies. This is probably not due to a worsening of air pollution at a global scale, but to increased knowledge, which suggests that the health impact is larger than expected (Roser, 2021). Therefore, in previous studies the impact of air pollution on human health was severely underestimated. For instance, Vohra et al. (2021) and Heft-Neal et al. (2018) with their data seem to suggest a higher toll than the one that has been described so far. In conclusion, numbers are certainly very high and more studies are needed to effectively established the health-price of air pollution. Indeed, other than reducing exposure to natural air pollution, exposure to anthropogenic air pollution can be reduced changing the technologies and products that are currently used in everyday life. Thus, addressing the problem of air pollution means bringing our society to the development and the advent of new technologies.

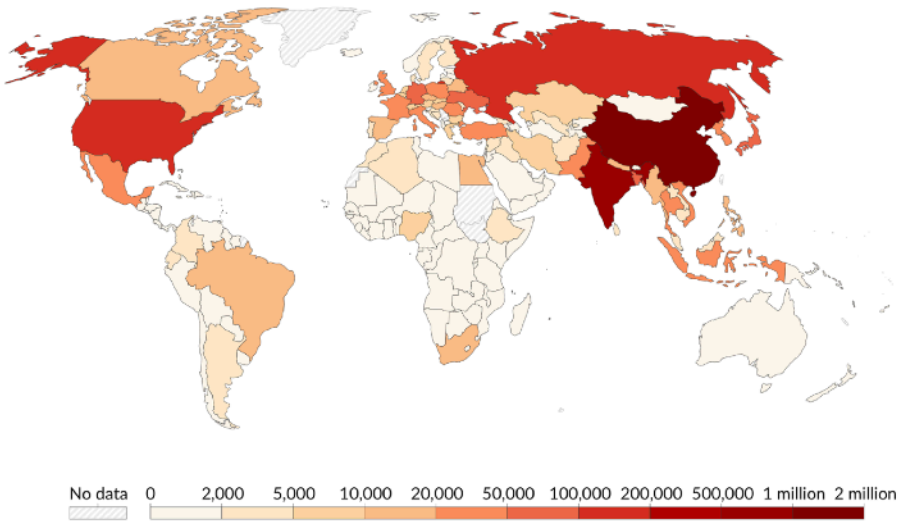



**Figure 3**. Air pollution deaths from fossil fuels in 2015. Figure taken and adapted from Lelieveld et al., (2019).

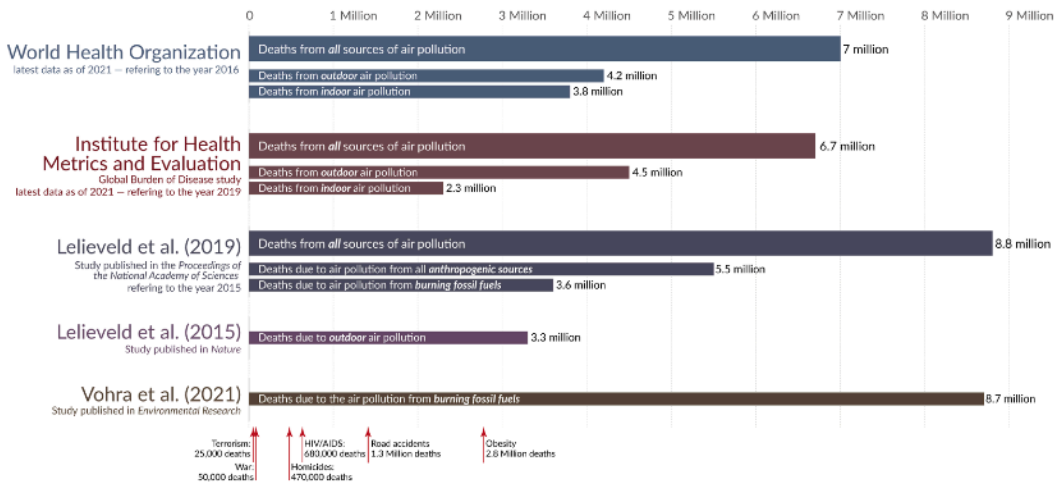



**Figure 4.** Estimates of the global death toll from air pollution published in major recent studies as to 2021. Figure taken and adapted from Roser, (2021).

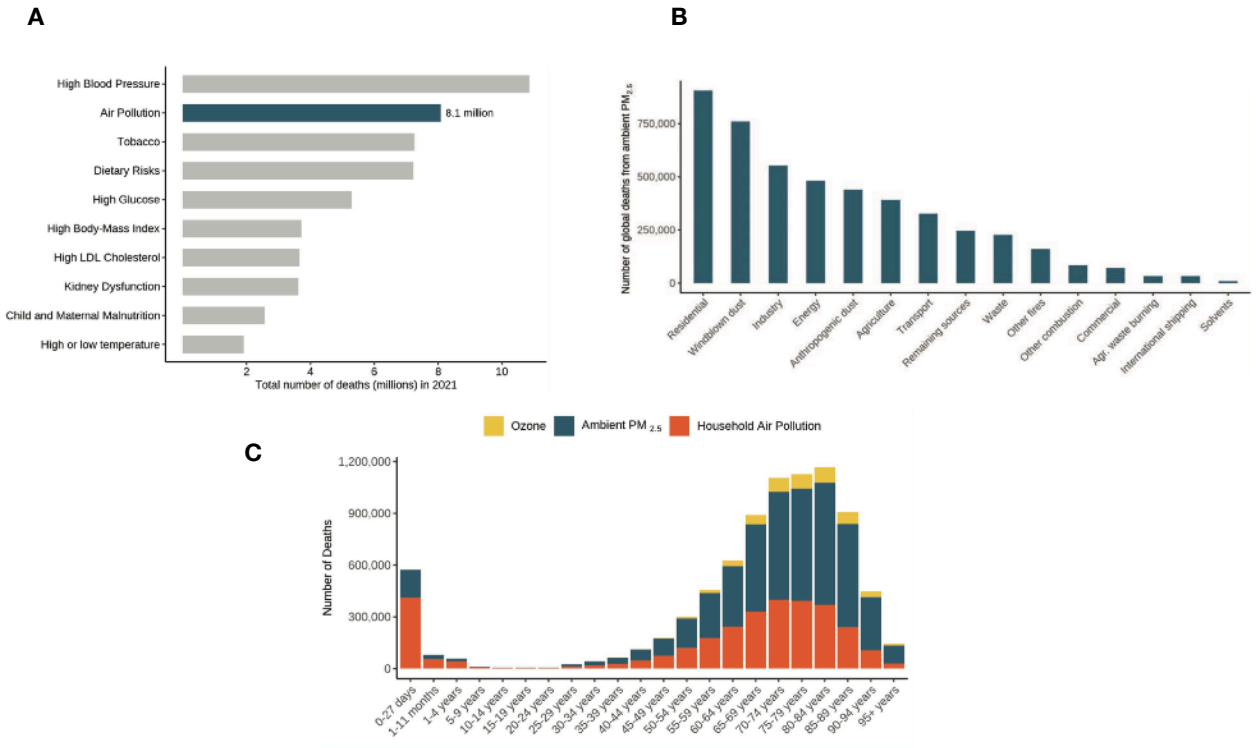



**Figure 5.** Global deaths data reported in the State of Global Air, 2024. (A) Global ranking of risk factors by total number of deaths in 2021. (B) Contribution of key sources of $PM_{2.5}$ to total number of global deaths in 2020. (C) Distribution of global deaths in 2021 attributable to ambient $PM_{2.5}$, ozone and household air pollution by age.

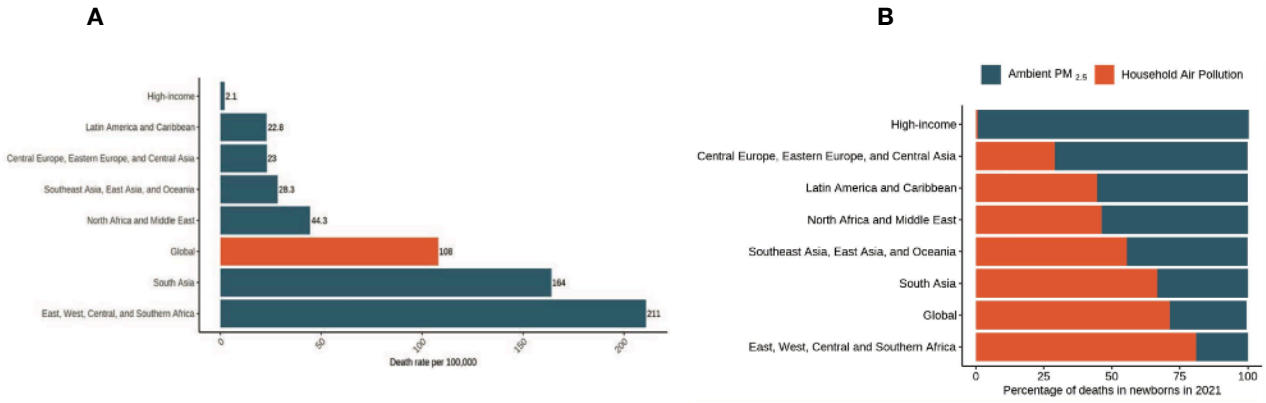



**Figure 6.** Global infant deaths data reported in the State of Global Air, 2024. (A) Death rate (number of deaths per 100000) in children under 5 linked to air pollution across GBD Super Regions in 2021. (B) Relative (percentage) contribution of ambient and household air pollution to deaths in newborns in 2021.

## 1.4. **Air pollution regulation policies**

Air pollution has been recognized as the single biggest environmental threat to human health and well-being (WHO, Air Quality Guidelines (AQGs), 2021). In 2021, the WHO revised the AQGs in response to a request by governments at the 68th World Health Assembly held in May 2015 in Geneva (Health Effects Institute, State of global air, 2024). In this report - the latest AQG, the WHO lists air quality guidelines and interim targets with the ultimate aim to improve air quality ad protect people's health across the globe. Such guidelines are expected to remain valid for up to 10 years.

The air quality guidelines provide evidence-informed, quantitative-based and non-binding recommendations; whilst interim targets are concentrations of a given pollutant associated with a specific decrease of health risk. Therefore, they are not end-points but steps towards a progressive reduction aimed at meeting the air quality guideline levels. It is important to note that epidemiological studies were unable to support any " Threshold of no

effect" and for this reason, adverse health effects may occur even at levels below the ones suggested in the guidelines (Kutlar Joss et al., 2017). Moreover, not all air pollutants have recommendations. Indeed, for some air pollutants such as ultrafine particulate matter (UFPs), elemental carbon (EC) and black carbon, data are not sufficient to provide recommendations, but only good practice statements.

For this reason, regulatory authorities adopt a given level (e.g: concentration, deposition level) of air pollutants as enforceable and thus, the process of developing legally binding regulations is responsibility of national policy makers and air quality policies. In the European Union, guidelines are referenced in the Ambient Air Quality Directive. The European Directive identifies geographical areas at risk of exposure to be monitored and assessed, so to record the emission sources and levels of air pollution (Manisalidis et al., 2020); the European Environment Agency (EEA) publishes every year a comprehensive report on air quality.

Therefore, WHO air quality guidelines have been used as a reference tool to support decision-makers across the world in setting standards and goals for air quality management (WHO, AQGs, 2021). However, the extent to which these are followed by countries is still not sufficient.

In their study, Kutlar Joss et al., (2017) gathered information on ambient air quality standards across the globe and compared them in relation to the previous AQGs (WHO, AQGs, 2005). Results show that, of 170 countries - including 57 countries which did not set any air quality standards; levels of standards varied greatly depending on the country and pollutant. Standards for 24-hour averaging time for $PM_{2.5}$ and $PM_{10}$ (respectively the 21% and the 46% of countries), met the air quality guidelines, while only 7 countries (2%) adopted the WHO annual mean air quality guidelines for these two pollutants. Thus, air quality standards for $PM_{2.5}$, $PM_{10}$, but also $SO_2$, poorly

alined with the WHO recommended guidelines.The agreement was higher for CO, $SO_2$ (10-min averaging time) and $NO_2$ (Kutlar Joss et al., 2017).

| Pollutant | Averaging Time | AQG | IT-4 | IT-3 | IT-2 | IT-1 | Change Compared to 2005 AQG |
|---|---|---|---|---|---|---|---|
| $PM_{2.5}$ (µg/m³) | Annual | 5 | 10 | 15 | 25 | 35 | Tightened |
| | 24-hour** | 15 | 25 | 37.5 | 50 | 75 | Tightened |
| $PM_{10}$ (µg/m³) | Annual | 15 | 20 | 30 | 50 | 70 | Tightened |
| | 24-hour** | 45 | 50 | 75 | 100 | 150 | Tightened |
| Ozone (µg/m³) | Peak season* | 60 | | | 70 | 100 | New |
| | 8-hour | 100 | | | 120 | 160 | Unchanged |
| $NO_2$ (µg/m³) | Annual | 10 | | 20 | 30 | 40 | Tightened |
| | 24-hour** | 25 | | | 50 | 120 | New |
| $SO_2$ (µg/m³) | 24-hour** | 40 | | | 50 | 125 | Loosened |
| CO (mg/m³) | 24-hour** | 4 | | | | 7 | New |

AQG = air quality guideline; IT-4 ITI = specific interim targets.
*Average of daily maximum 8-hour mean ozone concentration in 6 consecutive months with highest 6-month running average ozone concentration.
**Not to be exceeded more than 4 days/year.

**Figure 7**. WHO, Air Quality Guidelines, 2021.

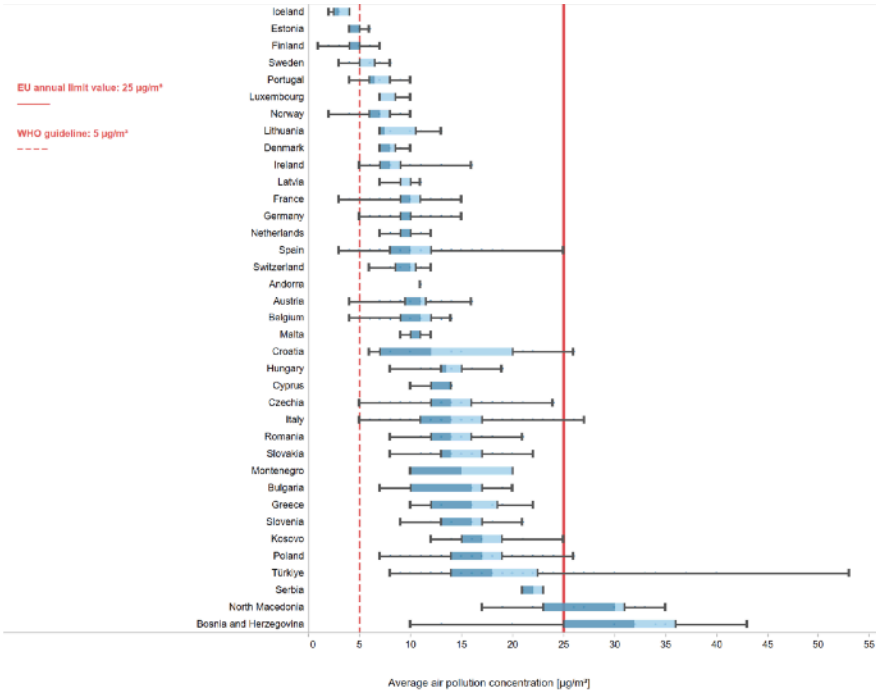



**Figure 8.** $PM_{2.5}$ concentration in Europe. Data from the European Environment Agency.

## 1.5. **The non-health effects of air pollution: environment, climate change, social welfare, global economy**

Before having an impact on human health, air pollution has clearly a huge impact on the environment. The major evident environmental phenomena associable with air pollutants are the decrease of stratospheric ozone and thus thinning of the protecting ozone layer, acid rain (wet or dry precipitations containing nitric and sulfuric acids which acidify water and soil, damage trees, crops and the human cultural heritage) and lastly, haze - caused by fine particles dispersed in the air, which reduces the transparency of the atmosphere (Manisalidis, 2020). Notably, these three phenomena cannot occur without the driving force of air pollutants (Feng et al., 2024).

Wildlife and vegetation are exposed to toxic pollutants through the air, soil, water. This exposure impairs or boosts processes in animals and plants, ultimately changing the equilibrium of ecosystems. For instance, a phenomenon called eutrophication occurs when high concentrations of nutrients stimulate the blooming of algae, which in turn causes an alteration of the diversity of fish that is also able to lead to death (Manisalidis, 2020). Thus, indirect and direct effects on species are expected to be substantial. Crops yield and food productivity are another aspect of the impact of air pollution on the soil and water that has repercussions on our heath and economy. A review from Barton et al., (2023), found out that studies on the effects of air pollution on wild populations of birds, documented at least one species trait negatively correlated with pollution.

In general, the effects on our environment are difficult to evaluate and the ubiquity of our environment can easily mislead us on the real impact of air pollution (Aguilar-Gomez et al., 2022).

Global climate change is also affected and actively affecting air pollution: indeed, climate change and air pollution can worsen each other reciprocally, leading to several ecosystem-mediated effects and indirectly affecting human health (Alahmad et al., 2023).

Besides having an impact on our health and on the ecosystem at a global scale, air pollution yields a potent effect on our society. Exposure to pollutants is not equal across communities and this leads to social disparities. For instance, those facing a high burden of disease have a lower socioeconomic status or belong to historically marginalized groups (Wang et al., 2023).

More intuitively, air pollution is strictly linked with human capital and economic growth. Industry, from manufacturing to the use of products, plays a key role in the emission of pollutants. In addition to that, control strategies implemented to limit emissions may adversely affect the economy. Indeed, reductions in air pollution levels often coincide with short-term dips in economic growth (Chen et al., 2018; Feng et al., 2024). Secondly, control strategies may lead to unemployment, for instance in case of shutting down highly polluting businesses. And yet, on the other hand, the labor literature shows that air pollution decreases workers productivity and is able to decrease labor supply, among the many (Aguilar-Gomez et al., 2022).

Other “ Non-health” end points caused by air pollution are currently being the object of investigation within the field of economics, such as school performance, decision-making and crime. In fact, there are also subclinical effects stemming from the same physiological mechanisms causing health harm that may be imperceptible and, nonetheless, impact behaviour, performance and skills. Additionally, they may arise at considerably lower pollution levels (Aguilar-Gomez et al., 2022).

Collectively, implications after exposure, production and even control measures to limit the phenomenon, are various and include “ Non-health” endpoints that must be addressed to tackle and manage effectively air pollution.

# 1. Introduction

# 2. Particulate Matter

## 2.1. **Particulate matter: the air pollutant with the strongest impact**

The term particulate matter (PM), also known as, more appropriately, atmospheric aerosol, refers to an airborne mixture of liquid droplets and solid particles suspended in the air, continuously varying in size and chemical composition in space and time (WHO, 2024). Dimensions of such particles greatly vary, ranging from particles that can be seen with the naked eye - such as dirt, dust, soot, smoke, to others that can be detected only with an electron microscope (EPA, 2024). The small size of these particles makes them especially harmful to humans: in fact, epidemiological and toxicological studies have shown that smaller particles are associated to greater adverse health outcomes than larger ones (Schlesinger et al., 2006; Zhang et al., 2015) as they can be inhaled, reach the lower airways and enter the bloodstream, thus virtually making their way to all human body organs.

As previously discussed, not only are aerosols able to influence human health, but they are also able to influence the environment. For instance, they can alter the macro- and microphysical properties of clouds and regulate cloud lifetimes, as well as precipitation efficiency - by serving as cloud condensation nuclei or ice nuclei (Rosenfeld et al., 2008; Tao et al.,

2012; Wang et al., 2013; Koren et al., 2005; Fan et al., 2007; Yuan et al., 2008; Zhang et al., 2015). Moreover, aerosols are incorporated into atmospheric hydrometeors (i.e. fog, cloud droplets, rain drops, ice crystals) (Zhang et al., 2015). Urban precipitation is also affected: aerosols have been shown to increase the rainfall rate under clean conditions and, on the other hand, to decrease the rainfall rate under polluted conditions, suggesting a different effect on precipitation depending on the socioeconomic state of countries and region of the world (Li et al., 2008a; Zhang et al., 2015).

PM is also able to have a long-range effect due to transport. For instance, urban and regional PM from Asia have been implicated in climatically altered cyclones over the Pacific Ocean in different studies (Li et al., 2008b ; Zhang et al., 2007; Wang et al., 2014; Zhang et al., 2015).

Given its impact, PM has gained the attention of the science community at the beginning of the 80s and is currently a topic of extensive research, as it is the most pressing issue in air quality regulation worldwide (Fuzzi et al., 2015). As a matter of fact, recent data indicates PM, in particular its fine fraction ($PM_{2.5}$), as the main driver of air pollution's burden of disease worldwide, with ambient $PM_{2.5}$ accounting for 4.7 million deaths and household $PM_{2.5}$ accounting for 3.1 million; making up together 7.8 million deaths globally in 2021, according to the State of Global Air, 2024. Long-term exposure is associated with many illnesses and early deaths from, among the many: heart disease, lung cancer, stroke, type-2 diabetes, lower respiratory infections, adverse birth outcomes and chronic obstructive pulmonary disease (Health Effects Institute, state of global air, 2024). Moreover, ambient PM represented in 2021 the leading level 4 risk factor for the disability-adjusted life years (DALYs) index among environmental and occupational risks. This is an index of both the years of life lost from

premature deaths and years lived in poor health (IHME, Disease, injury, and risk factsheets, 2024).

### *2.1.1.* ***PM toxic effects on the central nervous system***

The ability of the fine and ultrafine fraction of PM to enter the brain through both the olfactory epithelium and blood brain barrier (BBB) (Ajmani et al., 2016; Oberdörster et al., 2004; Genc et al., 2012) makes PM a potential highly neurotoxic pollutant. The negative effect of air pollution exposure on the central nervous system (CNS) is indeed undeniable and many human epidemiological and animal studies have corroborated an association between PM exposure and CNS pathologies (Calderón-Garcidueñas et al., 2002; Block & Calderón-Garcidueñas, 2009; Genc et al., 2012; Block et al., 2012; Costa et al., 2014; Costa et al., 2017; Sram et al., 2017; Russ et al., 2019; Costa et al., 2020).

Increased markers of oxidative stress and neuroinflammation were found in post-mortem samples of individual exposed to high levels of urban PM (Calderón-Garcidueñas et al., 2008; Calderón-Garcidueñas et al., 2011; Calderón-Garcidueñas et al., 2012; Levesque et al., 2011; Costa et al., 2020) and these results are strengthen by findings in animal studies. For instance, dogs exposed to heavy polluted air in Mexico City presented chronic neuroinflammation and neurodegeneration occurring in different regions of the brain (Calderón-Garcidueñas et al., 2002, 2003; Costa et al., 2014, 2020); mice models exposed to traffic in a highway tunnel showed, similarly, neuroinflammation responses (Bos et al., 2012).

Therefore, it may be not surprising that higher functions seem to be affected: decreased cognitive function, olfactory impairments, auditory deficits, depressive symptoms and other neuropsychiatric disorders were

shown to be associated with PM exposure in humans (Costa et al., 2020; Calderón-Garcidueñas et al., 2011, 2010; Fonken et al., 2011; Freire et al., 2010; Guxens & Sunyer, 2012; Lee et al., 2019; Petkus et al., 2019; Ranft et al., 2009 ; Costa et al., 2020).

On top of that, human and animal studies indicate that air pollution may exert neurodevelopment toxicity and contribute to the etiology of neurodevelopment disorders, such as autism spectrum disorder (Costa et al., 2020; Shabani, 2021).

PM emitted by diesel exhaust, one of the major contributor to urban PM pollution, has been shown to induce EEG changes (Crüts et al., 2008) as well as to alter motor activity, spatial learning and memory, novel object recognition ability and emotional behaviour. In terms of mechanisms of toxicity, this type of urban PM has ben shown to lead to oxidative stress and inflammation in the brain and to an altered expression of genes related to inflammation and antioxidant responses (Mohankumar et al., 2008; Gerlofs-Nijland et al., 2010; Win-Shwe & Fujimaki, 2011; Levesque et al., 2011; Ehsanifar et al., 2019; Valand et al., 2018; Costa et al., 2020). In short, both short and long-term exposures to diesel exhaust were found to lead to neuroinflammation and oxidative stress - in the case of acute exposure, even to an impaired adult neurogenesis (Coburn et al., 2018; Cole et al., 2016; Costa et al., 2020).

## 2.2. **PM classification and formation**

Depending on the size of the aerodynamic diameter of its particles, particulate matter is often classified into coarse particles ($PM_{10}$) fine particles ($PM_{2.5}$) and ultrafine particles (UFP) (EPA, 2024; WHO, AQGs,

2024). However, only $PM_{2.5}$ and $PM_{10}$ are subject to the regulatory framework.

1. **$PM_{10}$**: coarse inhalable particles with an aerodynamic diameter generally of ≤ 10 µm. Compared to a human hair, which has an average diameter of approximately 70µm, $PM_{10}$ particles are 7 times smaller than a single hair (EPA, 2024).

2. **$PM_{2.5}$**: fine inhalable particles with an aerodynamic diameter of generally ≤ 2.5 µm. This means that a $PM_{2.5}$ particle is 30 times smaller than a single hair (EPA, 2024).

3. **UFPs**: ultrafine nano-sized inhalable particles having a diameter of generally ≤ 0.1 µm (100 nm), also referred to as $PM_{0.1}$.

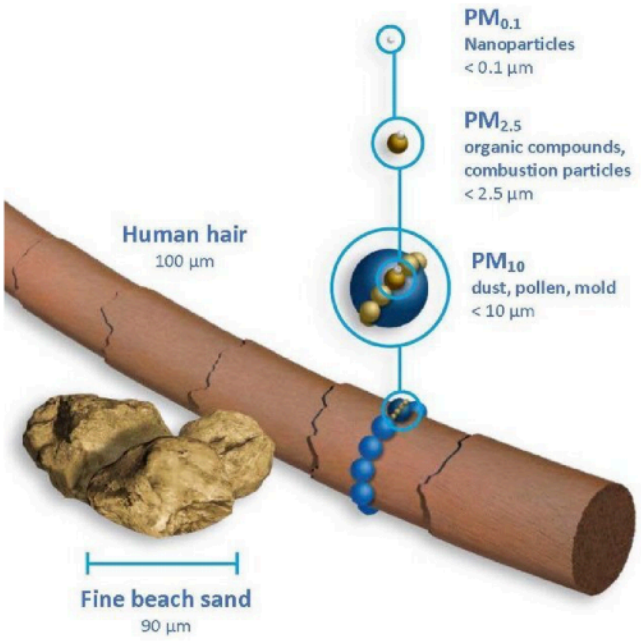



**Figure 9.** PM particles dimensions compared. Figure taken from "Ultrafine particle sensors for drastic improvement of air quality" by Eindhoven University of Technology. https://phys.org/news/2022-02-ultrafine-particle-sensors-drastic-air.html

Atmospheric aerosols can directly be emitted from anthropogenic or natural sources (primary PM) or can be formed through gas-to-particle conversions in chemical reactions involving other air pollutants, such as $SO_2$, NOx and other specific organic compounds (secondary PM) (Elmarakby, 2024; Daellenbach et al., 2020; Hopke et al., 2020; Schauer et al., 1996; Viana et al., 2008). Both of primary and secondary PM undergo chemical and physical transformations, transport, cloud processing and removal from the atmosphere (Zhang et al., 2015). Remarkably, urban primary PM is mainly emitted from anthropogenic sources (Zhang et al., 2015).

However, mechanisms leading to fine PM formation are still shrouded in uncertainty, especially in terms of processes related to PM origin and growth (Zhang et al., 2015). This is reflected in the variegated chemical composition of particles and the variegated sources of emissions, that span from primary to secondary; from natural to anthropogenic.

New particle formation (NPF), also known as aerosol nucleation, is one of the events that leads to the formation of secondary aerosol formation (Ling et al., 2019 ; Kulmala et al., 2004) and it is believed to be the largest contributor of sub-micron particles to global aerosol loading (Ling et al., 2019; Spracklen et al., 2006; Pierce & Adams, 2009; Seinfeld & Pandis, 2006). NFP occurs in two different steps of the making of a particle: during nucleation - to form a critical nucleus and during growth and enlargement of the freshly nucleated particle (Zhang et al., 2015). Nucleation seems to involve different species of stabilizing molecules, such as sulfuric acid, ammonia, water, ions, iodine species and organic compounds.

Besides primary emission and NPF, other processes that regulate the PM number concentrations are coagulation and cloud processing (Zhang et al., 2012; Seinfeld & Pandis, 2006 ; Zhang et al., 2015). While coagulation

decreases the number of particles, cloud processing can both decrease it or increase it. In addition to this, higher temperatures accelerate chemical reactions occurring in the atmosphere, whilst lower temperature make PM dissipate more slowly in the air (Bell et al., 2008; Ramli et al 2020). Weather condition is thus a major factor driving PM concentration (Hsu et al., 2017, Ramli et al., 2020).

Urban fine PM typically consists of a large part of secondary constituents, including sulfate, nitrate, ammonium and organic matter (Guo et al., 2014; Huang et al., 2014; Zhang et al., 2015). All these particle-phases, except for the ammonium, are attributable to emissions of other pollutants like SO, NOx, and VOCs (Guo et al., 2014; Zhang et al., 2015) . VOCs, represent a heterogeneous class of molecules which is able to engage in gas-to-particle conversions and contribute to the enlargement and growth of the particle, leading to the generation of secondary organic aerosol (SOA) (Seinfeld & Pandis et al., 2006; Odum et al., 1997; Donahue et al., 2006; Zhang et al., 2015). VOCs precursors are oxidized in air by $0_3$, OH, $NO_3$, Cl forming highly oxygenated, low volatility products, which condensation and coagulation play a pivotal role in NPF (Ling et al., 2019; Metzger et al., 2010; Finlayson-Pitts et al., 2020).

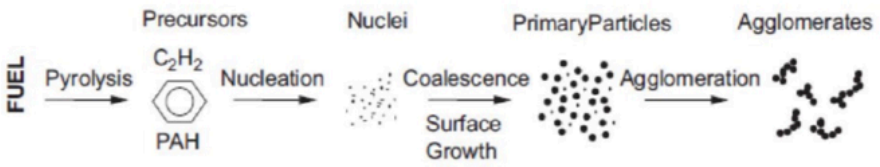



**Figure 1.** Steps involved in the formation of particulate matter (PM). From [10].

**Figure 10.** Formation of PM. Figure taken from Bans et al., 2013.

In addition to conventional processes of formation, airborne micro-plastics may contribute to particulate matter formation and pollution, although there are no data available at the moment on the influence of micro-plastics on air quality (Sridharan et al., 2021).

## 2.3. **PM composition**

As previously mentioned, the chemical composition of particles is such assorted, to the point that a complete molecular identification of all components has not yet been achieved - suffice it to think the exceptional variety of the organic component (Finlayson-Pitts et al., 2020). Moreover, there is currently no technique able to give a comprehensive analysis of all inorganic and organic species, limiting to analyses of subsets of the components.

### *2.3.1.* ***Inorganic component***

In terms of elements, C, H, O, N, S are common constituents, as well as trace metals (Samara & Voutsa, 2005; Ramli et al., 2020; Finlayson-Pitts et al., 2020).

Cadmium (Cd), arsenic (As), chromium (Cr), copper (Cu), cobalt (Co), iron (Fe), nickel (Ni), Pb , manganese (Mn), strontium (Sr), titanium (Ti), zinc (Zn) and vanadium (V) were found to be common elements in $PM_{2.5}$ (Niu et al., 2010; Wang et al., 2017a; Ramli et al., 2020); furthermore, such trace metal components are believed to be important in determining the toxic effects associated with aerosols (Wang et al., 2017a; Ramli et al., 2020). Traffic and industrial emissions, mining and non-ferrous melting, combustion of oil and flight emissions, burning of biomass, resuspension of

soil and soil crust are among the main sources of trace metals (Dubey et al., 2012; Das et al., 2015; Ramli et al., 2020).

Other inorganic compounds found in aerosols include water-soluble ionic species, such as sulfate ($SO_4^{2-}$), nitrate ($NO_3^-$), ammonium ($NH_4^+$) ions, which are virtually present in all particles (Jimenez et al., 2009; Finlayson-Pitts et al., 2020). Ammonium, calcium ($Ca^{2+}$), chloride ($Cl^-$), magnesium (Mg), nitrate, potassium (K), sodium (Na), sulphate, part of organic carbon, elemental carbon, are all water-soluble ions usually found in $PM_{2.5}$ (Ali-Mohamed and Jaffar, 2000; Tsai et al. 2012; Salam et al. 2015; Ramli et al., 2020). Importantly, the toxicity of PM is mainly ascribed to its insoluble fractions, such as EC, part of OC and minerals (Zhang et al., 2024).

### *2.3.1.* ***Organic component***

Another major component of aerosols is represented by carbonaceous species, which include organic, elemental carbon and carbonate (Yang et al. ; Ramli et al., 2020). In particular, organic carbon constitutes up to 70% of fine PM mass (Jacobson et al. 2000; Sharma et al. 2018; Ramli et al., 2020). According to Li et al. (2012) carbonaceous species can be divided into two groups: OC and EC (Turpin & Huntzicker 1995; Li et al. 2012). EC is a substance made only of carbon not bound to other elements (such as diamond and graphite). It is also improperly referred to as black carbon (BC), which is described as carbon with ideally light-absorbing quality, usually formed during incomplete combustion of fossil fuels and biomass (Fuzzi et al., 2015; Turpin & Huntzicker 1995; Kwon et al., 2020). The two are therefore not synonymous, although BC is mostly composed of EC.

OC is a big group comprising thousands of different species, such as PAHs. PAHs heavier species (usually five-ring chemical structure, atomic weight > 228) are found mostly in the molecular phase and thus in fine PM, whilst lighter species (two- and three-ring structures) are found predominantly in the vapor phase (Lu et al., 2008; Ramli et al., 2020). According to Evagelopoulos et al. (2010), fine PM- bound PAHs concentrations detected were times higher than coarse $PM_{10}$- bound PAHs. Together with other evidences, this shows that the level of airborne PAHs are majorly dependent on fine PM (Duan et al., 2005; Ramli et al., 2020).

As for the chemical properties of the organic component, in primary aerosols it is usually less oxidized (e.g.: polycyclic aromatic hydrocarbons in soot); whilst in secondary organic aerosols (SOAs) is more complex: it includes different oxygenated species (e.g.: acids, alcohols, aldehydes, ketones, esters, peroxides, aldols, hemiacetals, acetals etc.) and nitrogen- and sulfur- containing organic molecules (e.g.: alkyl- heterocyclic amines, organonitrates, organosulfate esters). In smaller particles, highly oxidized and high molecular weight oligomeric compounds with larger O:C ratios are relatively more predominant. On the contrary, particles growth involves less oxidized and lower molecular weight species (Akimoto & Hirokawa, 2020 ; Finlayson-Pitts et al., 2020). SOAs complexity is due to the high number of potential organic precursors found in the air, as well as further chemical reactions occurring in the condensed phase, which can form new products after the particle has formed (McNeill, 2015; Finlayson-Pitts et al., 2020). In addition to that, the chemical composition of SOAs depends on environmental conditions and their physical properties , such as relativity humidity, temperature, gas phase composition, viscosity and so on. (Finlayson-Pitts et al., 2020).

Aerosols are not only constituted of chemical components: bacteria, fungi, pollen and endotoxins are airborne biological agents, forming the so-called “Bio-aerosols”. This kind of aerosol is emitted from the biosphere and includes living and dead organisms (e.g.: bacteria, archaea, algae, viruses), as well as part of them, products of them and dispersal units (e.g.: fungal spores, pollen and other allergens, endotoxins, biopolymers, metabolites (Fröhlich-Nowoisky et al. 2016; Ramli et al., 2020). However, much is still unknown about bio-aerosols; although they have emerged as important players in atmospheric processes (Urbano et al. 2011; Ramli et al., 2020).

The size of aerodynamic diameters of bio-aerosols varies from typically 20nm to 100µm, depending on the bio-characteristics of the particle. For instance, pollen size spans from 5 to 100µm, the bacterial one from 1 to 30 µm and the size of viruses from 20nm to 10 µm (Hargreaves et al., 2003 ; Ramli et al., 2020). In urban areas, bacteria are generally associated with bigger particles (Haas et al., 2013 ; Ramli et al., 2020). However, it has been also shown a positive correlation between the respirable fractions of bacteria and $PM_{2.5}$, with proteobacteria as the predominant bacterial component identified in $PM_{2.5}$ (Ramli et al., 2020). The respirable fractions of fungi is mainly associated with $PM_{10}$ (Liu et al., 2016; Ramli et al., 2020), while fungal spores are believed to be the dominant biological component of PM (Zhang era al., 2010 ; Ramli et al., 2020). According to Fröhlich-Nowoisky et al. (2009), the capacity of spores to live under extreme conditions makes them able to be ever-present in airborne aerosols. In this case, the increase of fine PM is positively associated with the increase of spore concentrations of Penicillium and Aspergillus (Haas et al. 2013; Yan et al. 2016; Ramli et al., 2020). However, the concentration of fungal spores is higher in $PM_{10}$ than in $PM_{2.5}$ (Kalisa et al., 2019; Ramli et al., 2020).

Another relevant component of bio-aerosols are endotoxins, especially in terms of health-outcomes. A typical endotoxin is the lipopolysaccharide component of the cell wall of Gram-negative bacteria. Such endotoxin is ubiquitous in nature and highly toxic. It can be found in aerosols in the environment, given its stability under extreme conditions. As a matter of fact, many studies have shown that airborne endotoxins are correlated with $PM_{2.5}$ and $PM_{10}$ (Sandle, 2016; Ramli et al., 2020).

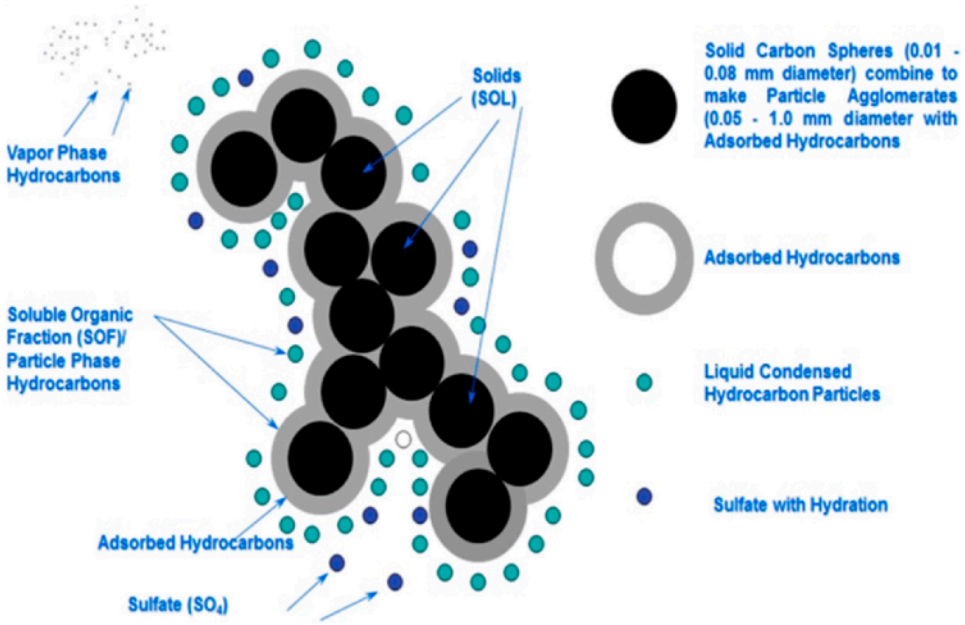



**Figure 11.** PM composition. Figure taken from Mohankumar and Senthilkumar, 2017.

Among the sources of bio-aerosols, there are natural and anthropogenic sources. The latter includes composting crops, wastewater for treating plants, feedlots, wetlands and wastes; although bio-aerosols can be generated and carried also by unexpected sources, such as pets and animals (Bowers et al., 2011; Gangamma, 2014; Jacobson, 2012; Kalisa et al. 2019; Ramli et al., 2020).

## 2.4. **Ultrafine particulate matter**

### *2.4.1.* ***Chemical and physical properties***

Aerosols with an aerodynamic diameter of 0.1 μm or less (UFPs), despite their minor mass and size, represent the dominant fraction of aerosols dispersed into the air, resulting in being 80-90% of all particles (Hofman et al., 2016). If $PM_{10}$ and $PM_{2.5}$ are typically described in terms of mass of distribution, UFPs are described, by contrast, in terms of number of concentration, due to their small size (Kwon et al., 2020; Hofman et al., 2016).

Particular concern for human health has risen, as UFPs small size make them able to reach the most distal lung regions - the alveoli, where they can penetrate the alveolar-capillary barrier and distribute throughout the body through the blood stream. In fact, harmful systemic effects of $PM_{10}$ and $PM_{2.5}$ are often attributable to UFPs, especially extrapulmonary effects related to PM exposure (Kwon et al., 2020; Li et al., 2016; Terzano et al., 2010). Moreover, in virtue of their physical characteristics, UFPs have a high specific surface area (total exposed surface area per unit of mass) which is highly reactive; thus UFPs result in being capable of adsorb several hazardous compounds.

As anticipated with PM in general, beyond having a direct impact on health, UFP emissions from fossil fuel combustion affect the globe indirectly; for instance through precipitation. Evidence have shown that UFPs do not necessarily alter the total precipitation amount, but do alter the spatial and intensity distribution of precipitation (Junkermann et al., 2011; Kwon et al., 2020). For instance, UFPs are associated to less steady rain or more extended drought periods in some areas, and to heavy torrential rain and flooding in other areas thousands of kilometers away (Junkermann et

al., 2011; Fan et al., 2018; Rosenfeld et al., 2011; Kwon et al., 2020). For all the above reasons, it appears that UFPs are potentially way more threatening to human health than bigger particles.

Other physical features include UFPs fast evolution, particularly of their smallest fraction (< 20 nm). This means that UFPs have short atmospheric lifetimes (a few hours) and their concentration sharply decay from the emission source (Zhang et al., 2001; Kwon et al., 2020). UFPs indeed follow the Brownian motion with movements based on diffusion due to concentration gradients. Therefore, near emission sources where there is high number of concentration, UFPs tend to promptly collide with adjacent particles in a process called coagulation, forming larger particles or deposit onto surfaces, or evaporate if condensable (Seigneur, 2019; Wang et al., 2011; Kwon et al., 2020). Coagulation, besides having a role in particle growth, can thus be considered as an important process for removing UFPs from the ambient, although it has little effect on PM mass concentrations (McMurry et al., 2004; Zhao et al., 2015; Kwon et al., 2020).

### *2.4.2.* ***Monitoring, detecting and collecting UFPs emitted***

The total aerosol emitted from PM sources includes two type of PM: filterable PM (FPM) and condensable PM (CPM). PM that is solid or nonvolatile, can be captured on a filter upon emission; whilst CPM are particles initially in the vapor phase after combustion (forming initially as UFPs), which quickly condense upon cooling in the air forming solid salts or liquid droplets (Feng et al., 2018; Kwon et al., 2020). Recent studies indicate that organic compounds make the major contribution to CPM (Qi et al., 2017; Kwon et al., 2020).

Fixed monitoring stations, which operate at ambient air temperature, measure both FPM and CPM. However, measurements in heated air (which is typical within specific emitting sectors), often include only filterable aerosols, leaving out condensable UFPs - a critical and conspicuous part of UFPs. This means that the total PM registered is often underestimated, especially during cold seasons (Aas et al., 2012; Tsukada et al., 2008; Kwon et al., 2020), as well as not being routinely monitored.

To add up to the difficulties of detecting in the first place the total PM emitted, given the physical features of UFPs, it is evident that fixed monitoring air quality stations cannot properly capture individual exposure to UFPs, which should be assessed where individuals reside or pass through (Kwon et al., 2020). Moreover, the time resolution of particle measurements should be on the time and space scale of their evolution (Manigrasso et al., 2013; Kwon et al., 2020) - as their concentration steeply declines from the source of emission and they tend to grow in size from nucleation via coagulation and condensation (Wang et al., 2011; Kwon et al., 2020). Although there is a condensation particle counter, a device invented more than a hundred years ago, able to reach into the sub-10 nm range and detect UFPs with its subsequent developments (Aitken, 1888; Pollak & Metnieks, 1959; Kwon et al., 2020), there is no consensus, currently, for a standardized method for measuring ambient UFPs; as well as guidelines on UFP emissions level, as previously discussed. This is in part due to a historical under-appreciation of UFPs, both in literature and in public awareness.

Collecting UFPs is also possible; however, the analyses that come after are quite limited due to UFPs chemical and physical features.

### *2.4.3. **UFP sources of emission***

In the European Union, the total UFP emissions was estimated to be 272 kilotons, in 2008 (Grebot et al., 2011; Kwon et al., 2020): a number which was with all likelihood highly underestimated.

Major sources of indoor UFPs in residential environments are either a product of combustion (e.g.: use of incense, mosquito coil, candle burning, cooking) or noncombustion (e.g.: heat-not-burn tobacco, domestic appliances driven by brushed electric motors, hot flat irons, spray air fresheners (Liu et al., 2014; Löfroth et al., 1991; Manigrasso et al., 2017, 2018; Protano et al., 2017; Kwon et al., 2020), with higher emissions occurring from combustion sources rather than non -combustion ones. In office environments, lasers printers are one of the main UFPs contributors. Another source of UFPs which should be addressed to more investigations, given its nowadays popularity, is the use of e-cigarettes. Studies have in fact shown that mainstream e-cigarettes contain a significant amount of UFPs - beyond all the VOCs, heavy metals and carbonyl compounds found (Fromme & Schober, 2015; Zhao et al., 2016a; Mikheev et al., 2016; Williams et al., 2013; Kwon et al., 2020). A study detecting UFPs from e-cigarette users, observed particles of 15 nm and 85 nm (Zhao et al., 2017; Kwon et al., 2020); one study on vape shops detected particles with a diameter of 60 nm and 250 nm (Nguyen et al., 2017; Kwon et al., 2020).

In the ambient, not negligible UFPs long-term emissions are associated with wildfires, in particular with the smoldering phase of wood burning (Urbanski et al., 2008; Kwon et al., 2020). UFPs resulting from wildfire smoke are thus a heterogeneous mixture of chemical species, depending on wet or dry vegetation burning, hardwood or softwood, or on the different stages of combustion (e.g.: smoldering or open-flame) (Zhang et al., 2013; Kwon et al., 2020). Another important UFPs source is represented by solid

fuel combustion, which emissions chemically differ from the ones emitted from combustion of gasoline and diesel. UFPs generated by coal combustion, in particular, may be highly toxic for the greater amount of VOCs and Fe-oxides adsorbed. Moreover, compared to larger fractions, UFPs are constituted to a greater extend of unburned carbon - presumably soot, thus having a high carbonaceous content, which is associated to a greater toxicity (Linak et al., 2007; Kwon et al., 2020; Zhang et al., 2024).

It is worthy mention that clean coal technologies have been modified to limit fine particles and NOx emissions according to air quality guidelines; however, they have, at the same time, led to a dramatic increase of UFPs particles. Thus, coal combustion keeps being one of the major contributor of anthropogenic PM emissions. It has also been found that the most persistent UFPs sources in the low atmosphere are modern technology fossil fuel-burning power stations, refineries and smelters (Junkermann & Hacker, 2018; Kwon et al., 2020).

However, in urban ambients, the leading source of UFPs emission is certainly due to vehicles exhaust, followed by traffic-related activities (Harrison et al., 2011; Kwon et al., 2020). A recent study conducted in the US has highlighted the role of gasoline and diesel vehicles emissions, contributing with a 14% to regional UFPs emissions, which may be underestimated due to technical limitations (Venecek et al., 2019; Kwon et al., 2020). As road vehicles are the primary emitting source of UFPs in urban ambients, UFP emissions from diesel exhausts will be further discussed.

#### *2.4.3.1.* ***Diesel particulate matter***

PM emitted by diesel engines (DPM) is produced through a series of physical and chemical reactions occurring in the exhaust pipe and the post-treatment catalyst at the exhaust stroke. Such reactions involve gaseous, liquid and solid substances and therefore, the resulting PM emitted is a complex mixture (Long et al., 2013).

Other components emitted at diesel exhausts are the soluble organic fraction (SOF), which represents the majority of PM emitted. SOF includes, among the many, unburned organic compounds, oxygenated organic compounds and hydrocarbons. Sulfates are generated due to the presence of sulfur in diesel fuel (Dong et al., 2022). Soot, which by definition is an undesired byproduct of a high-temperature incomplete combustion of fuel oil and biomass, in diesel engines is produced during the diesel cycle operation, when a high-pressure fuel is injected into a charge of high-temperature recompressed air. Soot represents the main component of the solid phase of DPM (Meloni & Palma, 2020; Long et al., 2013; Watson & Valberg, 2001; Dong et al., 2022) and soot particles span from UFPs to coarser particles. DPM having an aerodynamic diameter size of < 800 nm are made of a carbonaceous core of EC, arranged in a graphitic-like "spherule" structures and coated with a wide variety of organic compounds such as PAHs, aldehydes, VOCs, SVOCs and, gas phase like CO and $CO_2$, sulfates and metals in traces etc. (McLaughlin et al., 2020; Kwon et al., 2020; Meloni & Palma, 2020). Because of the grade of complexity of diesel combustion, studies on soot formation have been made in much simpler environments, such as one-dimensional premixed flames and diffusion flames. These two models provide soot particles having high similarities in size and microscopic structure, as well as the effects of temperature,

pressure and reactants ratio (Mahamulkar et al., 2016; Meloni & Palma, 2020).

Theoretically, in a gasoline engine the combustion environment is, on the contrary, much more homogenous and thus the resulting soot formation lower (Meloni & Palma, 2020). However, PM mass and number emissions of gasoline direct injection (GDI) vehicles may exceed those of diesel vehicles equipped with modern diesel oxidation catalyst (DOCs) and diesel particulate filters (DPFs). GDI has become one of the predominant types of engine, to improve fuel economy. Contrarily to conventional port fuel injection engines (PFI), the GDI leads, nonetheless, to an incomplete air-fuel mixture and thus combustion of the fuel - due to the short time, resulting in a higher output of UFPs (Piock et al., 2011 ; Kwon et al., 2020).

At the same time, strategies to reduce diesel emissions are either focusing on changing the properties of fossil fuels/substituting them with synthetic fuels (Rojas-Valencia et al., 2017; Meloni & Palma, 2020), or on the engine, to alter the combustion, or to simply abate soot emissions before they leave the tailpipe through modern technologies such as DOCs, DPFs and SCR catalysts (Palma et al., 2011; Meloni & Palma, 2020; Kwon et al., 2020).

To maximize PM abatement both DPF and DOC are present in diesel engines. DOCs are flow-through catalysts that contain metals able to initiate the oxidation of hydrocarbons, CO, unburned fuel and oil. They significantly reduce the amount of semi volatile HC-based condensable UFPs, although potentially increasing the $NO_2$ emission while assisting soot oxidation in the DPF (Kittelson et al., 2006; Shrivastava et al., 2010; Kwon et al., 2020). This latter, represent the best device available to reduce, with high efficiency, PM and more specifically, UFPs. It consists of a ceramic filter system that captures any soot and other particles that the DOC is not

able to oxidize. Soot trapped onto the filter will deposit, generating a layer of soot which is surprisingly able to enhance the filtration efficiency for UFPs; however heavy depositions can compromise the engine efficiency and a proper level of deposition must be reached to keep the engine performance high. Therefore, the entrapped soot is burned periodically in a process called "regeneration" in which the excessive deposited particles are oxidized, yielding a great amount of SVOCs and smaller UFPs which are released into the ambient air (Meloni & Palma, 2020; Kwon et al., 2020). Such particles comprise organic compounds, carbonaceous soot, metallic ash, minerals and residue of additives and lubricants of the engine (Ko et al., 2016; Liati et al., 2018; Kwon et al., 2020). These temporary "spikes" of PN emissions occurring during the regeneration phase are not taken into account in the current emission standards, although their contribution for on-road vehicles can be significant (Giechaskiel et al., 2018; Kwon et al., 2020).

Therefore, the introduction of the DOCs and DPFs technologies has had the unexpected outcome of shifting the particle-size distribution of emitted PM to smaller diameters of 20-30 nm (Wilson et al., 1977; Kwon et al., 2020). This means that, while there is a reduction in particle mass, there is an increase of smaller sized particles, suggesting new nucleation mode particles associated with new technology during and after filter regeneration (Holmén & Ayala, 2002; Khalek et al., 2011; Kwon et al., 2020). Of note, the current European regulation of particle emission takes into consideration only solid particles > 23 nm, suggesting that this size cutoff should be lowered. On top of that, particles emitted by modern fossil fuel combustion systems are able to absorb more reactive oxygen species with their functional groups, potentially leading to an increased toxicity (Frank et al., 2013; Kwon et al., 2020).

### *2.4.4. **UFP-driven toxicity***

The small size of UFPs, combined with the high carbon content and oxygen-containing functional groups onto the surface, as well as a large surface area; make the UFPs, among all PM fractions, the potentially most toxic fraction. In addition to physical and chemical properties, toxic effects are strictly related to exposure route, time, frequency and dose and, last but not least, to their composition and on the hazardous/bio-hazardous substances absorbed (Zhang et al., 2024; Deng et al., 2019; Wang et al., 2021b; Kelly et al., 1998; Zhao et al., 2020).

Molecularly, there are many ways in which UFPs exert their toxicity, once entered in the human body. These can, essentially, be broken down to oxidative stress, inflammation and signaling related pathways. More specifically, oxidative stress caused by excessive reactive oxygen species (ROS) is able to lead to protein alteration, lipid peroxidation, mitochondrial membrane potential depolarization, DNA damage and genes regulation. Inflammatory factors are released once the particles have gained their access

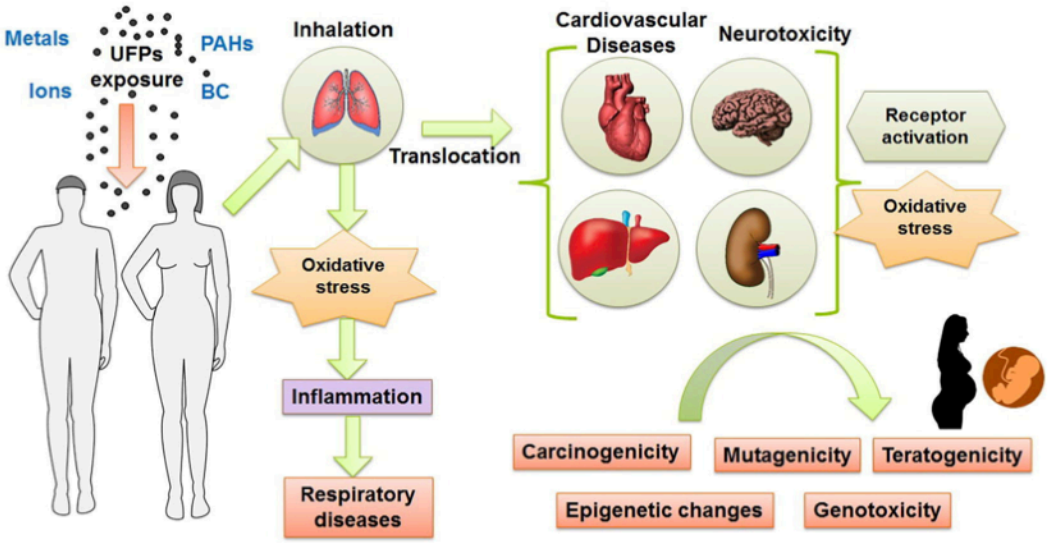



**Figure 12.** UFPs effects on human health. Figure taken from Moreno-Ríos et al., 2022.

to the organism, such as TNF-α and IL-10; the level of the superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) can decrease and the Th2/Th17 response can result in being inhibited (Xia et al., 2006; Lu et al., 2015; Zhang et al., 2024). Moreover, cascades of signaling pathways can lead to the expression of apoptotic proteins and genes, such as P53, Caspase-3, PI3K, p-AKT/AKT and p-FOXO3a/FOXO3a (Zhang et al., 2024).

#### *2.4.4.1.* ***Cardiovascular and respiratory effects***

In humans, exposure to UFPs occurs primarily through inhalation, but can also happen through the digestive tract or skin - for instance, through contamination of food ingested (Ta et al., 2018 ; Zhang et al., 2024). Once UFPs enter our organism, they can virtually reach and target all districts and organs, given their capacity of crossing all barriers, including the BBB and the placental barrier (Oberdörster et al., 2004 ; Bovè et al., 2019).

When inhaled, UFPs deposit, the more the particle is small, throughout the whole lung and can eventually reach the most distal part of the respiratory system, the alveoli, where gas exchanges occur (Sharifi et al., 2012; Chalupa et al., 2004 ; Zhang et al., 2024). In the alveolar region, the air-blood barrier is at its thinnest and therefore, the permeability of UFPs stronger. PM water-soluble ions are diluted by surfactants and serous fluid, then absorbed by the blood and lymphatic circulation. Most of what remains in the lungs is the insoluble component of PM. This fraction can react to proteins and receptors. Moreover, UFPs can easily enter, from the alveoli, in the lymphatic system by phagocytosis, or in the blood circulation, thus having access to all organs. In addition to that, particles inhaled show a variety of shapes (spheres, fragments, needles, agglomerates, dendrimers) that influences the membrane warping process during phagocytosis. For example, spherical particles are phagocyted easily, compared to non-

spherical ones, which can impair macrophages function (Sharifi et al., 2012; Zhang et al., 2024). Moreover, non-spherical particles tend to pass through capillaries and to stick to the blood vessel wall (Ta et al., 2018 ; Zhang et al., 2024), interacting with atherosclerotic plaques. UFPs can therefore disrupt organs function; interfere with lung ventilation-perfusion ratio and reduce the angiogenic capacity. In fact, it has been shown that PM increases oxidative stress and inflammation states in the lung, leading to respiratory diseases such as lung cancer and asthma (Li et al., 2016; Zhang 2024; Pope & Dockery, 2006). Furthermore, exposure to diesel exhaust PM has been associated to a potential increase in lung tumors (Benbrahim-Tallaa et al., 2012). Molecularly, it has been observed by Guan et al., (2020), an impaired lysosomal function and authophagic flux block, with the alterations of markers like LC3-II/LC3-I, p62 and Beclin (Zhang et al., 2024).

Besides interacting with atherosclerotic plaques in the vascular endothelium or arterial wall, other vascular effects include the alteration of oxygen and nutrients transportation, the interaction with circulating coagulation factors (thus promoting thrombosis), the interference with the tricarboxylic acid cycle (TCA cycle) in the heart with a reduction of ATP production and ultimately promoting acute coronary syndrome, ischemia, myocardial infarction and hypoxia (Zhang et al., 2024). Of note, UFPs are able to trigger mitochondrial dysfunction and oxidative stress in the ventricle, driven by the up-regulation of PrPc and GRP78 (Maher et al., 2020).

#### *2.4.4.2.* ***Effects on the central nervous system***

As the respiratory system is the main route of entrance of airborne particles, it is not surprising that most literature has focused on the effect of

PM on the cardiorespiratory tract. However, emerging body of evidence has pointed out the potential highly toxic effect of urban ultrafine PM on the brain (Calderón-Garcidueñas et al., 2019; Cory-Slechta et al., 2023; Costa et al., 2020).

UFPs access to the brain occurs, more indirectly, through blood circulation and the crossing of the BBB. UFPs are indeed able to pass through the tight endothelial junctions of the BBB, which can also be more permeable in inflammation states or higher levels of peripheral oxidative stress (Lochhead et al., 2010). Due to their small size, UFPs can also directly have access to the brain through the olfactory bulb pathway, through the nasal mucosa. Thus, they can easily enter the CNS through the olfactory nerves and, subsequently, distribute in potentially all regions of the brain, such as brain cortex and cerebellum (Shang et al., 2021; Oberdörster et al., 2004; Oberdörster & Utell, 2002; Genc et al., 2012; Forman & Finch, 2018). As a matter of fact, a study by Maher et al. (2016) showed that UFPs were present in the brain of individuals from the urban area of Mexico City, even at an early age (Costa et al., 2020).

Not surprisingly, UFPs seem to play a role in the etiopathology of neurological and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Calderón-Garcidueñas et al., 2019; Costa et al., 2020) and having important consequences on brain development (Cory-Slechta et al., 2019). For instance, a study of Andersen et al., (2010) showed a positive association between short-term UFPs exposure and mild strokes, whilst a very recent study by Cory-Slechta et al. (2024) investigated, on animal models, the effects of UFPs on particular time-frames important for brain development. Results indicated a particular vulnerability to UFP exposure in adolescence male brains, with alterations in different neurotransmitters and

on corpo callosus astrocytes and myelination, suggesting an important role of gender in neurotoxic effect related to UFPs.

Diesel UFP exposure, in a study of Ehsanifar et al. (2021), was shown to cause neuroinflammation and oxidative stress in a mouse model. Of note, toxicity due to ROS production is more pronounced in the brain than other districts in the body, thanks to the high amount of unsaturated fatty acids that can be peroxidized (Zhang et al., 2024). Moreover, the authors suggested that long-term exposures may alter the expression of hippocampal NMDA receptor subunits and highlighted, again, a possible gender role in modulating susceptibility to UFPs neurotoxic effects (Ehsanifar et al., 2021).

Another study of Li et al. (2020) showed that indoor UFPs were able to abnormally promote both astrocyte proliferation and a decline in cell viability, as well as to increase oxidative stress via the Keap1-Nrf2-ARE pathway in a 3D human organotypic chip model of human BBB. Oxidative stress is able to lead to mitochondrial dysfunction, an organelle known to be the target of environmental toxicants (Nabi & Tabassum, 2022) and whose dysfunction is associated with neurodegenerative processes that damage brain structures and functions. For instance, lesions on the striatum have been shown to be associated with UFPs exposure (Liu et al., 2020; Zhang et al., 2024 ).

Although there is growing evidence for UFPs-driven neurotoxicity and associations with neurodevelopmental and neurodegenerative disorders, the mechanisms of actions are still largely unknown and will require extensive research in the future.

#### *2.4.4.3. **Effects on other organs***

UFPs are able to induce changes in the gut and oral microbiota (Li et al., 2021; Zhang et al., 2024) and to yield toxic effects on abdominal organs, such as the liver, spleen, kidneys, pancreas and gastrointestinal tract (Voss et al., 2021; Yu & Choi, 2021; Zhang et al., 2024). In particular, there is strong evidence showing an association between fine and ultrafine PM and the incidence and mortality from diabetes (Münzel et al., 2018; Zhang et al., 2024). Du et al. (2019) showed that diesel particles exposures damaged normal β-cell function by significantly reducing their cellular viability and decreasing insulin secretion, ATP and GSH production, in a rat model.

Besides mitochondria, other organelles seem to be affected by UFPs. Adeel et al. (2019) for instance, showed that exposure to UFPs led to a mitochondrial dysfunction, as well as an endoplasmic reticulum (ER) abnormality, which displayed an abnormal appearance, in the intestinal tract of earthworms.

Interesting findings have indicated a potential association of UFPs exposure and joint diseases, especially considering the material and nanotechnology used traditionally in joint replacement surgeries.

UFPs may also alter the functionality of the human reproductive system. In fact, they were shown to be able to reduce sperm motility, velocity and testosterone levels in rats and to significantly lower sperm count/quality and to reduce fertility; besides yielding oxidative stress, inflammation and apoptosis (Zhang et al., 2020a; Zhang et al., 2024).

Given the ability of UFPs to pass through the placenta barrier (Bovè et al., 2019; Fournier et al., 2020), ultrafine PM seems to have a potential early toxic effect on the fetus and progeny that can affect both the mother and fetus - not to mention the development of the latter. Studies on embryos are

still at early stages, however, there is evidence on physical alterations occurring in the offspring of exposed mothers; such as reduced body weight and length, together with inflammation and oxidative stress processes (Luo et al., 2020; Rychlik et al., 2019).

Moreover, black carbon particles were found in human breast milk and were associated with ambient air pollution (Cosemans et al., 2024).

Lastly, UFPs have been implicated in transporting viruses and worsening viruses lethality, namely the association between SARS-CoV-2 and UFPs exposure (Yao et al., 2020; Botto et al., 2024).

To conclude, more toxicological and epidemiological are required, especially given that, with the increasing large-scale use of nano-material products, exposure to UFPs will increase substantially (Goel et al., 2019; Ray et al., 2020; Zhang et al., 2024). Subchronic and chronic toxicity, genotoxicity, carcinogenesis and embryotoxicity require, as well, further attention (Zhang et al., 2024).

# 1. Introduction

# 3. Amyotrophic Lateral sclerosis and Neurodegeneration

## 3.1. The role of the environment in neurodegenerative diseases

The process of neurodegeneration describes the collapse and loss of neuronal structure and function. Therefore, the neurological consequences of a degeneration of the central and peripheral nervous system are able to lead to severe cognitive and physical impairment.

Neurodegenerative diseases make up a heterogenous group of neurological pathologies including complex multifactorial diseases, monogenic diseases or diseases whose different forms are known to exist (i.e. inherited, sporadic and transmissible): all of them are characterized by common hallmarks of neurodegenerative processes occurring in cells (Coppedè et al., 2006; Wilson et al., 2023).

Because of the multifaceted nature of most neurodegenerative diseases, etiologies are largely not understood (Cannon & Greenamyre, 2011). For instance, about 5% of Alzheimer's disease cases are caused by mutations in genes implicated in AD's onset, while the remaining 95% sporadic cases are not associated to known genetic variants and are probably due to complex

interactions between the genetic make-up of each individual and the environment (Suresh et al., 2023).

As a matter of fact, neurodegenerative diseases can be often ascribed to a continuous interplay between the human genome and the environment, pointing out to the crucial role of the latter.

Environmental exposures over the course of a lifetime are collectively referred to as the exposome of an individual. The exposome operates through different biological pathways - some of which are detrimental to cells and are shared with neurodegenerative processes (Erzin & Gülöksüz, 2021; Wild, 2005; Miller & Jones, 2014) and can be conceptually broken down to three overlapping categories: an external general one, comprising urbanity, social capital, stress; a specific external one - diet, exposure to pollutants and chemicals, infections, physical activity, sleep and so on; and lastly, to endogenous factors, such as gut microbiota, oxidative stress and metabolism (Rappaport & Smith, 2010; Wild, 2012; Erzin & Gülöksüz, 2021). This means that our lifestyle and our own body built-up can influence the risk of developing neurodegenerative pathologies.

The role of environmental contaminants - including air pollution and in particular of $PM_{2.5}$, has been shown to be important for understanding common high-burden diseases etiologies (Suresh et al., 2023) and environmental contributions have been demonstrated to play a part in the ethiopathology of several common neurodegenerative diseases, such as AD, Parkinson's disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Cannon & Greenamyre, 2011). Because acting on the environment offers easier ways to limit or counteract the progress, prognosis and outcome of neurological pathologies and disorders, rather than acting on genes (Coppedè et al., 2006); studies in this field have gained more and

more attention worldwide and their numbers are bound to increase exponentially.

### *3.1.1.* ***Environmental risk factors for Amyotrophic Lateral Sclerosis***

Amyotrophic lateral sclerosis is a lethal neurodegenerative disease characterized by the progressive loss of neurons in the brain and upper/ lower motor neurons in the spinal cord (Van Es et al., 2017). Degeneration of motor neurons is responsible of a rapid progressive failure of the neuromuscular system, leading to death for respiratory failure or cardiac arrest. Life-expectancy is quite short, with 50% percent of ALS patients dying within 3 yers of symptoms onset (Huisman et al., 2011). It is the most common type of motor neuron disease and the incidence has been estimated to be around 1-3/100.000 cases. This number has increased steeply in the last decades, with a current estimated lifetime risk of approximately 1 in 300 (Huisman et al., 2011; Seelen et al., 2017). As in the AD's case, the majority of ALS cases are sporadic (90-95%); by contrast only a 10% of cases are associated with known genetic mutations (Al-Chalabi & Hardiman, 2013). Such disease is also known as Lou Gehrig's disease - as the famous baseball player and curiously, a higher frequency of ALS has been reported among athletes, compared to the general population (Filippini et al., 2020), thus highlighting the crucial environmental component.

A solid body of evidence has indeed pointed out to the influential role of environmental exposures in sporadic ALS (sALS) (Martin et al., 2017; Andrew et al., 2021; Goutman et al., 2022). Exposure to lead and mercury, pesticides, solvents, cyanotoxins, as well as smoking, military service and electromagnetic fields are thought to be associated with an increased risk of

ALS and have been addressed in literature (Wang et al., 2017; Filippini et al., 2020; Fiore et al., 2020; Oskarsson et al., 2015).

A case-control by Andrew et al. (2021), reported an increase of risk for lifestyle, behavior and occupational factors. Among the many: head trauma, electrical burns, hobbies involving lead, holding a job in mechanics, painting and construction. Their finding showed a long interval between exposures and ALS diagnosis: some exposures of several years prior to diagnosis had the largest effects on ALS risk (Andrew et al., 2021).

Moreover, studies have indicated a higher rate of ALS among football players (Piazza et al., 2004; Chio et al., 2009), especially in midfielders, who head the ball back from goal kicks, suggesting a role for head trauma in ALS.

Air pollution exposure has also been addressed. Long-term exposures to traffic-related $PM_{2.5}$ concentrations (well below the existing European annual mean limits) were associated with increased risk for ALS in a population-based case-control study in the Netherlands, involving data of 917 ALS and 2.662 controls (Seleen et al., 2017). A further extension of their analysis, which included a total of 1.636 patients with ALS and 4.024 controls, confirmed this association and pointed to other significant associations, such as with $PM_{0.1}$ (Yu et al., 2021). In line with this finding, another very recent study of Peters et al., (2024) found an association between UFPs and ALS.

## 3.2. **Pathomechanisms of Amyotrophic Lateral Sclerosis**

Many molecular and cellular mechanisms are implicated in the pathophysiology of ALS. Such mechanisms are not mutually exclusive and

contribute all together to the progression, onset and outcome of ALS. These, include excitotoxicity, oxidative stress, organelles dysfunction, protein aggregates and cytoplasmic inclusions, dysfunction of transport mechanisms, immune/inflammatory processes, genetic modifications and altered calcium homeostasis.

The ones most relevant to the purpose of this research will be further discussed below.

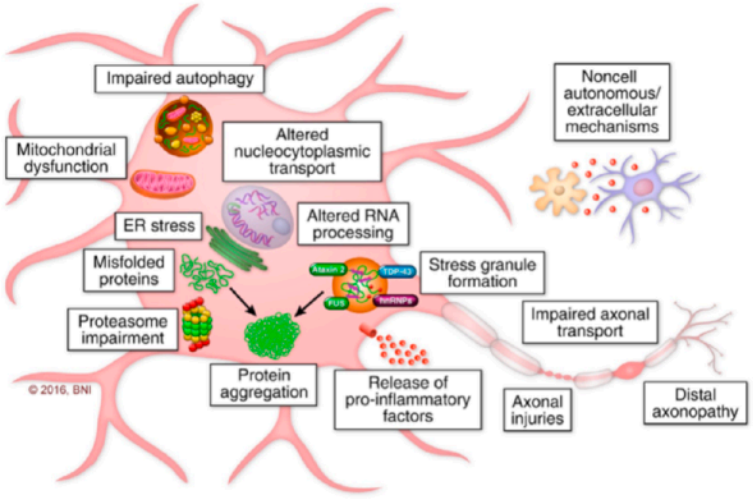



**Figure 13**. Molecular mechanisms of ALS. Figure taken from Moreno-Ríos et al., 2022.

### *3.2.1.* ***Calcium dyshomeostasis and organelles impairment***

In ALS, abnormal calcium handling and intracellular $Ca^{2+}$ levels have been reported (Patai et al., 2016). This global calcium dyshomeostasis is strictly linked with the general dysfunction of cellular organelles.

Moreover, many of the major toxic processes associated with ALS are calcium dependent, therefore making calcium a common denominator which, through a positive feedback loop, may unify these pathological mechanisms into a multifactorial escalating calcium-related mechanism leading to neurons death (Appel et al., 2001; Patai et al., 2016).

For instance, excitotoxicity driven by $Ca^{2+}$ is a major hallmark of ALS and other neurodegenerative diseases. Elevated intracellular levels of $Ca^{2+}$ in excitatory presynaptic terminals increase glutamate release, which in turn increases inward calcium currents through the postsynaptic permeable AMPA receptors (Williams et al., 1997). This postsynaptic increase of $Ca^{2+}$ inside the cytosol can determine an activation of endonucleases and proteases, resulting in DNA damage and cytoskeletal breakdown. Elevated levels of $Ca^{2+}$ are also able to induce disruptions of cytosolic proteins and misfolding, as well as to impair mitochondrial functions with a mitochondrial calcium overload: increasing ROS production and oxidative stress, ultimately leading to apoptosis or necrosis (Kawabata & Manfredi, 2010; Cozzolino & Carrì, 2012; Leal & Gomes, 2015; Patai et al., 2016; Mead et al., 2023).

The abnormal calcium handling and signaling of $Ca^{2+}$, among the many effects, can activate calcium-dependent apoptotic proteins (e.g.: calpains, caspases) and/or mitochondrial responses, that lead to apoptotic cell death (Mattson et al., 2008; Kruman & Mattson, 1999; Patai et al., 2016; Wang et al., 1999; Kim et al., 2002). Furthermore, elevated intracellular calcium levels may lead to the formation of free radicals and superoxide anions, which may perturb the plasma membrane (Dykens, 1994; Beckman et al., 1990; Lafon-Cazal et al., 1993). A depletion of $Ca^{2+}$ levels in the ER, which is believed to occur through a persistent shift of $Ca^{2+}$ from the ER to the mitochondrion, leads to impaired ER functions and ER stress; thus to protein folding dysfunctions, proteasome impairments and thus, to apoptosis (Prell et al., 2013; Tadic et al., 2014; Leal & Gomes, 2015; Leal et al., 2013).

In summary, $Ca^{2+}$ indirect and direct mechanisms are established factors contributing to ALS neurodegeneration.

#### *3.2.1.1. **Autophagic impairment***

Autophagy is a major catabolic pathway for the clearance of aggregated and defective proteins, as well as of damaged organelles. It can be promoted by stress stimuli, such as nutrient depletion, oxidative stress and protein aggregation - as it is a survival-related pathway (Menzies et al., 2017; Fleming et al., 2022).

Importantly, nutrient starvation represents a major regulatory mechanism, which acts through the inhibition of Target of rapamycin complex1 (mTORC1), a highly conserved repressor of autophagy (Saxton & Sabatini, 2017). Under nutrient-rich conditions, the latter phosphorylates ULK1 blocking ULK1 autophagy-promoting role. However, upon starvation, ULK1 dissociates from its repressor and can initiate autophagy (Hosokawa et al., 2009).

Autophagy can also be induced by low levels of cellular energy and glucose. These two parameter are detected by cells through the AMP-activated protein kinase (AMPK), which senses the AMP/ATP ratio: if the cell is glucose-deprived, AMPK is able to activate ULK1, which in turn promotes catabolic autophagy to produce new energy (Karabiyik et al., 2021).

Thus, given the pivotal role in degrading proteins, autophagy is involved in maintaining cellular homeostasis (Ravikumar et al., 2010; La Rovere et al., 2016). The process, briefly, leads to the formation of an initial double membrane formation called phagophore that stretches out, engulfing and englobing portions of the cytoplasm to be cleared, forming a vesicle called autophagosome. Cargo englobed can be either trapped in a bulk or a selective manner, through autophagic cargo receptors, such as p62. The autophagosome subsequently fuses with a lysosome forming an

autolysosome, in which lysosomal enzymes can degrade the substrate (Fleming et al., 2022).

In both sporadic and familial ALS, aggregates of proteins and cellular inclusions have been observed, suggesting that clearance mechanisms are defective - namely, the autohagy-lysosome pathway (Blokhuis et al., 2013). These processes are of particular relevance in neurons, which, unlike mitotic cells, cannot get rid of unwanted material through cellular division and have to rely on two specific pathways: the ubiquitine-proteasome system (UPS) and the autophagy-lysosome pathway (Fleming et al., 2022). Whilst, with the first one, cells mostly degrade short-lived and soluble proteins, with the latter they degrade mostly long-lived proteins and organelles (Rubinsztein, 2006; Rujano et al., 2006); the dysfunction of the two have been implicated in the pathogenesis of many neurodegenerative diseases, such as ALS (Rubinsztein, 2006; Cipolat et al., 2016; Matus et al., 2008). Not by chance indeed, many of the mutated genes implicated in ALS are involved in autophagic pathways at different stages, such as SOD1, p62, TDP-43, and optineurin (Lee et al., 2015a). Increasing evidence support the hypothesis that defects in the autophagic flux may exacerbate ALS motor neurons loss and therefore ALS progression (Cipolat et al., 2016; Song et al., 2012), along with the hypothesis that modulation of the autophagic flux represents a potential therapeutic target (Lee et al., 2015a).

#### *3.2.1.2.* ***Lysosomal impairment***

Although the lysosome plays an undeniable important role in molecules degradation, it has emerged and has established as a $Ca^{2+}$ signaling center of the cell: in fact, the lysosome controls several $Ca^{2+}$ dependent functions and is a actual store of $Ca^{2+}$; having an average free $Ca^{2+}$ concentration similar

to the one reached in the ER, being approximately 500 μM (Bygrave & Benedetti, 1996). Thus, the lysosome is undoubtably crucial for maintaining $Ca^{2+}$ homeostasis (Raffaello et al., 2016; Lloyd-Evans & Platt 2011; Patel & Docampo, 2010; Morgan et al., 2011).

For this reason, a solid body of evidence has indicated lysosome as a factor contributing to the pathology of many diseases. A handful of these are characterized by neurodegeneration, such as AD, PD, Hungtington's disease and lysosomal storage disorders (LSDs), which comprise the Niemann-Pick type C disease (Lee et al., 2015b; Croce & Yamamoto, 2019; Koh et al., 2019; Lloyd-Evans et al., 2008) - the first human disease associated with defects in lysosomal $Ca^{2+}$ functions (Lloyd-Evans & Platt 2011). Since then, defects in lysosomal $Ca^{2+}$ homeostasis have been observed in other models of neurodegenerative diseases, such as ALS (Tedeschi et al., 2019a).

Several lysosomal pumps and channels control the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and, to add up to this complexity, these are working in a fine regulated network of other $Ca^{2+}$ storing organelles. It is worth mentioning the close physical and functional interaction between lysosomes and the ER (Kilpatrick et al., 2013). For instance, the ER $Ca^{2+}$ sensor stomal-interacting molecule 1 (STIM1) has been shown to be a key regulator of the lysosome/ER coupling (Tedeschi et al., 2019b).

There are different lysosomal $Ca^{2+}$ release channels, such as voltage-gated $Ca^{2+}$ channels (VGCCs), two-pore channels (TPCs) and transient receptor potentials (TRPs). One member of the latter, the Transient receptor potential mucolipin subfamily 1 (TRPML1) has been widely studied as it contributes in the regulation of several $Ca^{2+}$-dependent functions and it is implicated in many diseases (LaPlante et al., 2002; Shen et al., 2012; Bae et al., 2014). Loss-of-function mutation in its gene causes a neurodegenerative LSD: the Mucolipidosis type IV (Bargal et al., 2000). This channel and its

activator, PI(3,5)P2 are of particular interest in ALS studies, as PI(3,5)P2 levels were found to be altered in ALS-related forms (Chow et al., 2009; Osmanovic et al., 2017).

Furthermore, TRPML1 seems to be a pivotal player in the lysosomal $Ca^{2+}$ dependent induction of autophagy, acting as a real "stress sensor": ROS have been, in fact, shown to activate TRPML1; so that the $Ca^{2+}$ released promote the translocation of TFEB into the nucleus leading to autophagy, in order to restore a balanced redox homeostasis (Settembre et al., 2013; Medina & Ballabio, 2015; Zhang et al., 2016; Tedeschi et al., 2019a). Therefore, a dysfunction of the lysosomal $Ca^{2+}$ handling system determines an impairment of autophagy - likely through an impaired TFEB regulation (Zhang et al., 2017; Cortes & La Spada, 2019; Tedeschi et al., 2019a).

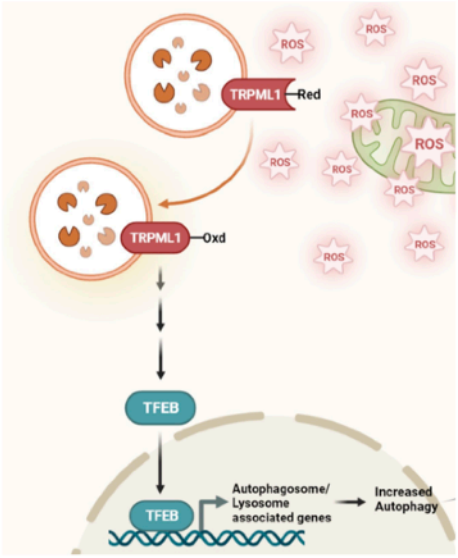



**Figure 14**. TRPML1 as a stress sensor promoting autophagy. Figure taken and adapted from Park et al., 2022.

### *3.2.1.3. **ER stress and Endoplasmic reticulum dysfunction***

The ER and the ER stress pathway are now considered as strongly implicated in the pathogenesis of ALS, as well as in many other

neurodegenerative disorders triggered by neurotoxicants (Cannon & Greenamyre, 2011; Jeon et al., 2023; Ilieva et al., 2007; Vijayalakshmi et al., 2011; Atkin et al., 2008; Atkin et al., 2017; Bugallo et al., 2020). The main functions of the ER include the folding and post-translational modifications of proteins, but also the important function of generating $Ca^{2+}$ signals upon receptor stimulations - as the ER represents one of the principal intracellular $Ca^{2+}$ stores. Therefore, the ER is paramount to sustain cellular responses and keep cellular homeostasis (Koch, 1990; Helenius et al., 1992; Berridge, 2002). However, organelles stress and overload can lead to the accumulation of unfolded/misfolded proteins within the ER, which activates several pathways to counteract and restore homeostasis. Further and persistent ER stress can lead to an increase of ROS production and, eventually, cause cell death and inflammation (Kwon et al., 2023).

ER stress protective mechanisms include the unfolded protein response (UPR) (Moon et al., 2018; Kadowaki and Nishitoh, 2019), the ER-associated degradation (ERAD), in which misfolded proteins are removed by 26S proteasome (Hampton, 2002; Lemus and Goder, 2014; Hwang and Qi, 2018), and the ER-phagy, which is the autophagic process able to remove damaged ER (Yang et al., 2021; Kwon et al., 2023).

The UPR signaling pathway can be initiated by three different ER membrane-associated sensors: inositol-requiring transmembrane kinase/ endoribonuclease 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription Factor 6 (ATF6). In normal conditions, such proteins are bound to a 78 kDa glucose-regulated protein/Binding immunoglobulin protein (GRP78/BiP), a ER resident chaperone that can dissociate from them, when ER stress occurs (Kopp et al., 2019). The free proteins can then initiate counteracting pathways (Kwon et al., 2023).

Even though the UPR is a protective mechanism, chronic stress leads to apoptotic cell death via the UPR. Therefore, prolonged ER stress and its consequent dysfunction are currently recognized as a common cause of neurodegeneration (Moreno et al., 2012; Ismael et al., 2021).

Furthermore, multiple studies link altered ER structure to neurodegenerative diseases like ALS and AD (Sree et al., 2021).

However, ER stress is also tightly connected to a dysfunction in the ER $Ca^{2+}$ handling system. Given the ER pivotal role in cell signaling and ionic homeostasis, a complex and fine machinery is required to serve several cellular functions. This is particularly true for neurons: the ER maintains, indeed, ionic gradients which modulate the mobilization of synaptic vesicles in the axon, ensuring neurons functionality (Brini et al., 2014).

Because ER reactivity to signals depends on resting $Ca^{2+}$ levels, it is fundamental to maintain a steep gradient between the $Ca^{2+}$ intracellular concentration and the one in the ER store. A control system is thus necessary to replenish the $Ca^{2+}$ reservoir when the ER $Ca^{2+}$ levels have dropped (Putney, 2005; Ambudkar et al., 2017). This mechanism is termed as Stored-operated Calcium Entry (SOCE) and was first described by Putney in 1986. The physiological activation trigger for this mechanism is the $Ca^{2+}$ release from the ER driven by inositol 1,4,5-triphosphate (IP3), upon stimulation (Ambudkar et al., 2017).

A specific ER transmembrane protein, STIM1, is the only mechanism by which ER $Ca^{2+}$ depletion can be sensed by the cell. Such diminishment in $Ca^{2+}$ levels inside the lumen can be triggered upon ER stress.

$Ca^{2+}$ normally binds to STIM1 EF hand, suppressing its activity (Liou et al., 2005; Zhang et al., 2005). However, when this does not occur, STIM1 oligomerizes and translocates to ER-plasma membrane junctions , where it

interacts with different lipids (Walsh et al., 2009; Korzeniowski et al., 2009). At that point, the $Ca^{2+}$ channel Orai1, which is initially distributed across the plasma membrane, is recruited by the ER-plasma membrane-junction-localized STIM1 oligomers. When bound to STIM1, ORAI1 is activated and can finally drive the influx of $Ca^{2+}$ inside the cell, precisely into the cytosol (Zhou et al., 2010; Calloway et al., 2010; Carrasco & Meyer, 2011). This electrical influx is a non-voltage activated and inwardly rectifying current, known as $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) (Golovina et al., 2001). Therefore, SOCE activity depends on STIM1 sensing function (Hooper et al., 2013).

Notably, SOCE activation determines a $Ca^{2+}$ influx in the cytosol, not in the ER. In fact, the ER $Ca^{2+}$ re-uptake is mediated by the ATPases SERCA pumps (Andersen & Vilsen, 1998; MacLennan, 2000; Vandecaetsbeek et al., 2009; Brini et al., 2013; Valentim et al., 2022).

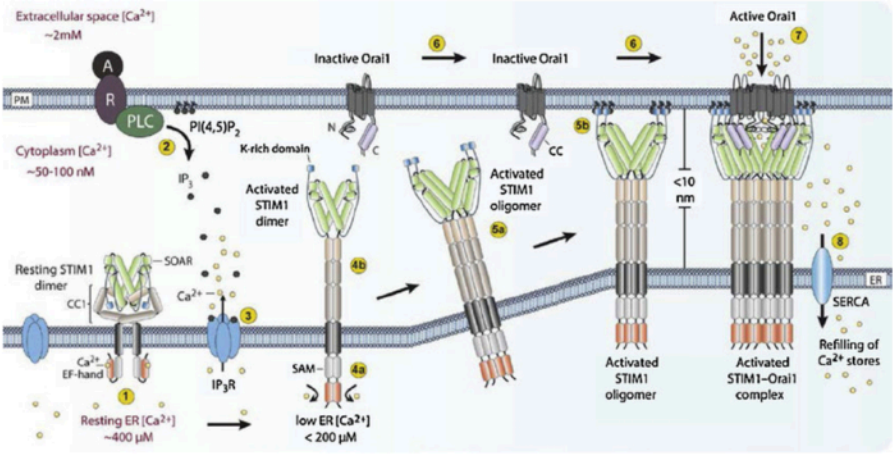



**Figure 15.** SOCE activity depends on STIM1 sensing function. Figure taken from Bhardwaj et al., 2016.

#### *3.2.1.4.* ***Mitochondrial impairment***

A well-known and widely studied common feature shared by neurodegenerative diseases is mitochondrial dysfunction (Gao et al., 2017). Mitochondria are the power plants of cells and therefore, are involved in several processes implicated in maintaining cell homeostasis; such as energy production, ROS production, apoptosis, calcium signaling. The disruption of any of these processes is able to impair cellular functionality and this is particularly true for neurons: indeed, mitochondrial dysfunction, like energy failure and production of ROS, have been linked to neurodegeneration (Rey et al., 2022).

Mitochondrial defects have been associated with ALS motor neurons death as well. As a matter of fact, several studies in ALS models have shown impaired ATP production and defects in oxidative metabolism (Keep et al., 2001; Mattiazzi et al., 2002; Menzies et al., 2002; Knott et al., 2008). ALS mitochondria seem to have an impaired $Ca^{2+}$ homeostasis and increased level of ROS production (Beal et al., 1997, 2002); such ROS increase plus an overload of mitochondrial calcium, have been associated to glutamate-receptor mediated neurotoxicity in spinal motor neurons from transgenic ALS animals (Carriedo et al., 2000; Muyderman & Chen, 2014).

# Aim of the research

Air pollution has been recognized as the single biggest environmental threat to human health and well-being (WHO, Air Quality Guidelines, 2021) and indeed, is believed by many to be one of the greatest scourges of our century, for its impact on human life. Besides affecting our health and well-being, it has an active role in affecting the society and the economy of our world and, at larger scales, the environment and ecosystem - including climate.

Among all pollutants, particulate matter is a heterogeneous class made up of particles of different sizes, which has the infamous highest burden of disease worldwide. Despite this, not all PM particles are regulated, nor even monitored: this is the case of aerosols with an aerodynamic diameter of 0.1 μm or less ($PM_{0.1}$/UFP), which represent the dominant fraction of aerosols dispersed into the air (Hofman et al., 2016).

The capacity of UFPs to get through human body barriers such as the placental and blood brain barrier (Oberdörster et al., 2004; Bovè et al., 2019) makes UFPs a potential threat to human health. However, knowledge about UFP emissions and their effects is still at its infancy.

Therefore, the general purpose of the present doctoral study is to evaluate the toxicity driven by the finest fraction of particulate matter (UFPs) in *in vitro* and *in vivo* models.

To this end, UFPs nanoparticles were produced in a lab-scale manner to yield particles having high similarities in size and microscopic structure with particles emitted by diesel exhausts (Mahamulkar et al., 2016; Meloni & Palma, 2020), therefore providing a model for anthropogenic diesel exhaust emissions. Notably, the leading source of UFPs emission, in urban environments, is mainly due to vehicles engines and traffic-related activities (Harrison et al., 2011; Kwon et al., 2020).

More precisely, this doctoral study focuses, *in vitro*, on finding the cellular mechanisms through which UFPs exert their detrimental effects on the nervous tissue, specifically on motor neurons. Secondly, it aims at establishing such mechanisms as pathological pathways shared with the neurodegenerative disease ALS - a motor neuronal disease where the role of environmental exposure has been extensively addressed. *In vivo*, the aim is to explore the toxicity of UFPs in a more complex organism and therefore to characterize a toxicological *in vivo C.elegans* model for UFP-toxicity.

# *3.*

# Methodology

## 3.1. **Drugs and Chemicals**

### *3.1.1.* ***Pharmacological tools, chemicals and reagents***

Numerous chemicals were used to performed the assays and techniques utilized throughout this doctoral research. Cell-culturing media, sera, antibiotics and media supplements, plus Bacto™ Peptone, are Gibco (ThermoFisher Scientific Inc., Waltham, USA) products. ECL reagents and nitrocellulose were purchased at Amersham (Cytiva, Danaher, Washington DC, USA). Pharmacological tools such as thapsigargin, *L*-BMAA, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 5'-Fluoro-2-deoxyuridine (FUdR), (−)-Tetramisole hydrochloride, cholesterol and Miller's LB Broth were purchased at Sigma-Aldrich (Merck, Darmstadt, DE); as well as the ATP bioluminescent assay kit, poly-D-lysine/poly-L-lysine and Ara-C for cell culturing. The molecular probe Fura-2/AM, Fluo-3/AM, pHrodo™ Green E. coli BioParticles™ Conjugate for Phagocytosis and the fluorescent dye TMRE are Molecular Probes (Invitrogen, ThermoFisher Scientific Inc.,

Waltham, USA) products and the 2-(2-Oxo-2-(2,2,4-trimethyl-3,4-dihydroquinolin-1(2H)-yl)ethyl)isoindoline-1,3-dione (ML-SA1) and DMSO are Millipore's (Merck, Darmstadt, DE). The CytoPainter ER Staining Kit was purchased at Abcam (Danaher, Washington DC, USA), whilst the Proteome Profiler Mouse Apoptosis Array ARY031 at R&D Systems (Bio-techne, Minneapolis, USA). Agar was a Melford product (Melford Laboratories Ltd., Ipswich, UK). Moreover, several primary antibodies, reported in **Table 1.**, were employed in the present study.

## 3.2. **PM isolation and sampling**

The isolation of PM was performed in a controlled combustion laboratory apparatus consisting of a sooting ethylene-oxygen premixed laminar flame (equivalence ratio, $\Phi = 1$). The flame was stabilized by a flat McKenna burner (Holthuis & Associates, CA, Sebastopol, California USA) consisting of a sintered stainless steel plug (diameter = 60mm) positioned centrally within an outer sintered bronze ring; where a shielding gas flowed to avoid air entrainment. The fuel and the oxidant required for the combustion were placed on the center of the burner and the cold gas velocity kept constant at 4 cm/s, at atmospheric pressure. Temperature was measured inserting a fine wire, coated in silica, into the flame for a few hundreds of milliseconds, to avoid soot deposition. (Apicella et al., 2002; Marcella et al., 2022).

PM was thermophoretically collected by its deposition on a horizontal glass plate (75x25x1mm), which was placed into the flame at 5-7 mm of height above the burner and rotated through a gear motor (rotation speed = 1.4 gears/s). The insertion/deposition time was set at 60ms/lap to prevent the glass plate from heating; whilst the deposition time for each sample, after

preliminary tests, was proven o be unable to affect soot properties at 25s and thus that time was set as duration time (Russo et al., 2020).

Once deposited onto the glass surface, the carbon PM was scratched and extracted with dichloromethane (DCM). This step was critical to separate organic carbon (OC) from solid carbonaceous ultrafine particles ($PM_{0.1}$, single particle diameter < 100 nm) , which are insoluble in DCM (Russo et al., 2020). $PM_{0.1}$ (concentration of 100 parts per million [ppm] = 100mg/1L DMSO) was collected on filters and further filtered on 20 nm pose size Anotop filters (Whatman), after being solubilized in dimethyl sulfoxide (DMSO), yielding particles with a diameter < 100 nm and > 20 nm (NP100) and nanoparticles with a diameter < 20 nm (NP20) (Marcella et al., 2022). This latter fraction constituted the 25% in weight of the total $PM_{0.1}$, thus making the approximate concentration of 25 ppm = 25mg/1L DMSO.

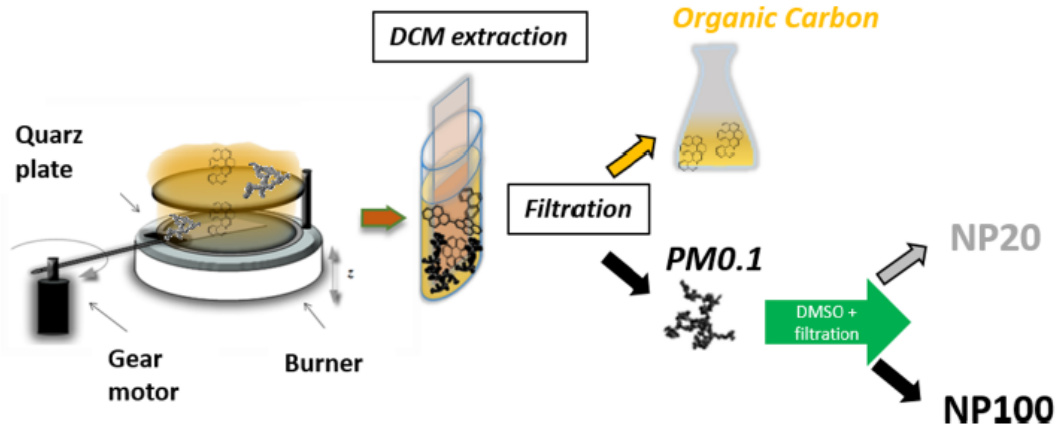



**Figure 1**. NP20 and $PM_{0.1}$ production. Adapted from Marcella et al., 2022.

## 3.3. Model organisms

In the present study, different model organisms were used to explore UFP-toxicity, with the aim of achieving a more comprehensive view of toxicological effects on different levels of cellular complexity. Thus, the models employed span from single neuronal cells to multicellular organisms, such as *Caenorhabditis elegans* (*C.elegans*) and *Mus musculus*.

### *3.3.1.* ***In vitro models***

#### *3.3.1.1.* ***Clonal cells***

Mouse motor neuron-like cells (NSC-34) (Cashman, 1992) were maintained onto 25cm flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C, 5% $CO_2$. Cells were grown onto poly-L-lysine pre-coated wells, petri dishes or glass coverslip in experimental conditions. Once adhered, cells were administered diluted $PM_{0.1}$ and NP20 into the supplemented DMEM.

#### *3.3.1.2.* ***Primary cells***

Rat cortical neurons were obtained from the cortex of 14/16-day-old Wistar embryos (Charles River Laboratories, Inc., Wilmington, USA). Briefly, gravid rats were anesthetized and decapitated and afterwards, embryos were collected and dissected. Isolation of cortex samples was performed in $Ca^{2+}$/$Mg^{2+}$ free PBS (Phosphate-Buffered Saline) containing 30 mM glucose to prevent cells aggregations. Tissues were incubated with papain for 10 min at 37°C to promote tissue breaking down, followed by

manual trituration in Earle's Balanced Salt Solution (EBSS) containing DNase (0,16 U/ml), bovine serum albumin (BSA) (10 mg/ml) and ovomucoid (10 mg/ml).

Cells were then collected and plated on multiwells or Petri dishes pre-coated with poly-D-lysine (20 μg/ml) and grown in Minimum Eagle's medium/F12 containing glucose, 5% fetal bovine serum (FBS), 5% horse serum (HS), glutamine (2 mmol/l), penicillin (50 U/ml), and streptomycin (5 ug/ml). Ara-C (cytosine arabinoside, 10 μM) was added within 72 hours of plating to limit non-neuronal cell growth. Cells were kept at 37°C, 5% $CO_2$ and experiments performed after seven days.

All procedures to isolate cortical neurons were performed according to protocols approved by the Ethical Committee of the "Federico II" University of Naples and by Italian Health Ministry to minimize animal pain and distress.

#### *3.3.1.3.* ***L-BMAA exposure as a model of neurodegeneration***

β-N-methylamino-L-alanine (BMAA) is a non-proteingenic amino acid isolated for the first time from seeds of the plant Cycas circinalis (Vega & Bell, 1967). In the present study, in vitro models of ALS were obtained through cellular exposition to the isomer *L* of BMAA (*L*-BMAA, 300 μM / 48 h).

BMAA is an excitotoxin biosynthezised by several species - namely cyanobacteria, dinoflagellates and diatoms (Candeias et al., 2024), which was first linked to the higher incidence of the amyotrophic lateral sclerosis/ Parkinson's Dementia Complex (ALS/ PDC) in the pacific island of Guam, upon consumption of cycas flour and flying foxes by the Chamorros

population (Cox et al., 2003; Lopicic et al., 2022). It has been shown that exposition to this toxin is a worldwide phenomenon, especially following events of biomagnification. On top of that, BMAA has been detected in different mammals such as dolphins and in human brain tissues of ALS and AD patients in North America (Lopicic et al., 2022; Schneider et al., 2020). In fact, it is well long-known notion that such toxin is able to cross the BBB (Duncan et al., 1991) and enter the human brain. Here, new evidences indicate that it may exert a greater neurotoxic effect by allegedly being converted into another isomer. (Schneider et al., 2020).

Not surprisingly, the association between BMAA and the development of sporadic neurodegenerative diseases is well-established in literature (Candeias et al., 2024) and, therefore, *L*-BMAA was used as an *in vitro* model of neurodegeneration. The concentration of 300 μM that was employed for our scientific purposes was previously reported by Petroziello et al., 2017 to be able to lead to ER and oxidative stress in NSC-34 cells.

#### *3.3.1.4.* ***In vitro assays***

##### *3.3.1.4.1.* ***Time-Lapse and High-Content Microscopy***

As reported in Sapienza et al. (2022), Microscopy experiments were conducted with the Operetta High-Content Imaging System (PerkinElmer, Waltham, US). Cells were cultured in Falcon® 24-well Clear Flat Bottom. For time-lapse experiments, cells were stimulated with $PM_{0.1}$ and NP20 and cultured for 6 h. Within this time window, digital contrast images of 15 fields per well were captured every 10 min with a 10× objective. To quantify cell tracking features, brightfield snapshots were taken at 6 fields per well. Subsequently, the PhenoLOGIC machine learning software (PerkinElmer, Waltham, US) was employed to analyze kinetic proprieties as current X /Y

displacement, mean X/Y displacement per well, mean displacement per well and current speed.

#### *3.3.1.4.2.* ***Endocytosis Assay***

As reported in Sapienza et al. (2022), NSC-34 motor neurons (1 × 105 cells per well) were seeded into poly-*L*-lysine-coated imaging-compatible plates (BD, Falcon) 1 day before the assay. Once adhered (37 °C, 5% $CO_2$), cells were stimulated with $PM_{0.1}$ and NP20. Cell culture medium was then removed and the pHrodo™ Green E. coli BioParticles™ Conjugate suspension was added. The plate fluorescence was analysed in an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, US). Data were expressed as Relative Fluorescence Units (RFU) measured up to 3 h, with 1 h span, at an excitation wavelength of 509 nm and emission of 533 nm.

#### *3.3.1.4.3.* ***Western Blotting***

As reported in Sapienza et al. (2022), after being treated, cells were lysed through an ice-cold buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.2% SDS, 0.5% NP-40, 1 mM EGTA, 1 mM PMSF, 1 mM $Na_3VO_4$, 1 mM NaF, and a protease inhibitor mixture (Roche Diagnostics, Monza, IT). The protein content was determined by the Bradford method (Bradford, 1976). For each sample, 10-30 μg of total proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then electrotransferred onto 0.2 μm nitrocellulose membranes. The membranes were blocked for 1 h in a bovine serum albumin-based buffer (3% BSA in TBS containing 0.1% Tween®20) and primary antibodies incubated overnight (**Table 1.**).

*3.3.1.4.4.* ***Electrophysiological Recordings***

As previously reported in Sapienza et al. (2022) and in Sapienza et al. (2024), for spontaneous membrane potential recordings by current clamps, NSC-34 cells were bathed in a extracellular Ringer solution containing: 126 mM NaCl, 1.2 mM $NaHPO_4$, 2.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose and 18 mM $NaHCO_3$ at pH 7.4 (NaOH). The pipette solution contained: 140 mM K-Gluconate, 2 mM $MgCl_2$, 2 mM $Na_2ATP$, 0.3 mM NaGTP, 10 mM HEPES (pH 7.2 with KOH). The electrical activity was acquired in gap-free modality using a Digidata 1322A interface (Molecular Devices). Data were acquired and analysed using the pClamp software (version 9, Molecular Devices). Spontaneous membrane potentials were measured in NSC-34 cells under control conditions and in the presence of $PM_{0.1}$ and NP20. Sustained high-quality whole-cell recordings could be maintained for > 5 min, thus confirming the viability of cells.

The $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) was recorded by patch-clamp in whole-cell configuration with a 100 ms voltage ramp protocol (from +90 to −120 mV) from −15 mV holding potential every 10 s. The external solutions contained (in mM) 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4); whereas the pipette solution contained (in mM) 145 CsCl, 8 NaCl, 10 $MgCl_2$, 10 HEPES, 10 EGTA, and 2 Mg-ATP (pH 7.2). Since the CRAC channel and the *L*-type $Ca^{2+}$ channel are both highly selective for $Ca^{2+}$, Nimodipine was added to block the *L*-type voltage-dependent $Ca^{2+}$ channels. Furthermore, spermine chloride was also used to block $Mg^{2+}$-inhibitable currents, whilst Tetraethylammonium and Tetrodotoxin were added to the external solution to block tetraethylammonium-sensitive $K^+$ and tetrodotoxin-sensitive $Na^+$ currents, respectively. $I_{CRACs}$ were normalized to membrane capacitance, measured as pA/pF.

#### *3.3.1.4.5.* ***[Ca2+]i Measurement by Single-Cell Video Imaging***

As previously reported in Sapienza et al. (2022) and in Sapienza et al. (2024), NSC-34 motor neurons, plated on glass coverslips, were loaded with 10 μM Fura-2/AM at 37 °C. After 30 min, coverslips with cells were mounted in a perfusion chamber (Medical System) posed onto an Axiovert200 microscope (Carl Zeiss Microscopy) equipped with a FLUAR $40_X$ oil objective lens and a Xenon lamp. The experiments were carried out with MicroMax 512BFT cooled CCD camera (Princeton Instruments), LAMBDA10–2 filter wheeler (Sutter Instruments) and Meta-Morph/ MetaFluor Imaging System software (Universal Imaging). Fura-2/AM fluorescence intensity was measured every 3s. Ratiometric values were automatically converted by the software to $[Ca^{2+}]_i$ using a calibration curve. TRPML1 activity was studied by perfusing neurons with ML-SA1 (10 μM) in Krebs–Ringer saline solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH, pH 7.4) and calculated as percentage of $[Ca^{2+}]_i$ increase over basal values. SOCE was measured depleting ER $Ca^{2+}$ stores with the SERCA pump inhibitor thapsigargin (TG, 1 μM) in Krebs-Ringer saline solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM $MgCl_2$, 10 mM *d*-glucose, and 10 mM HEPES-NaOH, pH 7.4 + 0.5 mM EGTA). ER $Ca^{2+}$ depletion is known to stimulate a rapid coupling between the ER $Ca^{2+}$ sensor STIM1 and the plasma membrane channels involved in $Ca^{2+}$ store refilling (i.e.: ORAI channels). After ER depletion, the subsequent reintroduction of 3 mM $Ca^{2+}$ allows SOCE detection as a $[Ca^{2+}]_i$ increase.

*3.3.1.4.6.* ***Cell viability (MTT assay, mitochondrial membrane potential and intracellular calcium level)***

Methods for assessing cell viability were reported in Sapienza et al., 2024. In order to assess cell viability, neuronal cells were incubated with 3[4,5-dimethylthiazol-2-yl]−2,5-diphenyl-tetrazolium bromide (0.5 mg/ml in PBS) for 1 hour at 37°C as reported in Secondo et al., (2007). The reduction of tetrazolium salts by dehydrogenases with primarily mitochondrial localization was detected spectrophotometrically at 570 nm, once solubilized in DMSO. Therefore, the MTT assay was used to calculate the quantity of cells with active metabolism (Surin et al., 2017).

Mitochondrial membrane potential was measured in the "Redistribution mode" using the lipophilic and cell-permeable dye tetramethyl rhodamine ethyl ester (TMRE). After $PM_{0.1}$ or NP20 treatment, motor neurons were loaded with TMRE (20 nM/30 min) and washed, at the end of the incubation. Mitochondrial membrane depolarization was measured as a decline in mitochondria-localized fluorescence intensity. Intracellular calcium level was detected simultaneously with Fluo-3AM. Confocal images were obtained with a Zeiss inverted 700 confocal laser scanning microscopy using a Xenon laser at the illumination minimum output of 0.5%.

*3.3.1.4.7.* ***ER Staining***

As previously reported in Sapienza et al. (2024), the probe of the CytoPainter ER Staining Kit was incubated according to the manufacturer protocol on NSC-34 cells, plated on pre-coated multiwells. Live images were then acquired with an Axiovert 5 (Carl Zeiss) and processed with the

software Labscope (ZEISS Labscope, www.zeiss.com). Images were analysed through ImageJ (Fiji software).

#### *3.3.1.4.8.* ***Quantification of ATP content***

As previously reported in Sapienza et al. (2024), ATP content was measured as an index of mitochondrial and cell status. After boiling samples in 100 mM TRIS + 4 mM EDTA (pH 7.75), ATP level was measured by a bioluminescent assay using a standard luminometer.

#### *3.3.1.4.9.* ***Proteomic analysis of apoptotic proteins***

As previously reported in Sapienza et al. (2024), cell lysates were used to determine the effect of $PM_{0.1}$ and NP20 on apoptosis-related proteins. The proteomic profile of motor neuronal cells was evaluated using a Proteome Profiler Mouse Apoptosis Array (R&D System, ARY031). Apoptosis-related proteins were thus visualized by chemiluminescence and protein levels were quantified relatively to their respective controls, using ImageJ (Fiji software).

#### *3.3.1.4.10.* ***In vitro statistical analysis***

Data were expressed as mean ± SE. Statistical analyses were performed with One-way ANOVA with Newman-Keuls's/Bonferroni post-hoc test. Unpaired *t*-test was used for two groups comparison, using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p$ values $< 0.05$.

### *3.3.2. **In vivo models***

#### *3.3.2.1. **G93A SOD1 mouse mutants as a model of neurodegeneration***

The SOD1-G93A mice (JAX Stock #002726, Jackson Laboratories Bar Harbor, ME, USA) carry a human SOD1 gene having a single amino acid substitution of glycine to alanine at the position 93 (Gurney et al., 1994). This represents one of the most-used ALS animal model (Todd and Petrucelli et al., 2022) and, according to the ALS Therapy Development Institute, is the best model in terms of ALS phenotype and drug screening; as it mimics motor neurons loss and pathological mechanisms such as cytoplasmic inclusions, neuromuscular junction injury, inflammation and gloss in the spinal cord and, around the third/fourth month of life, motor difficulties and limb paralysis - thus highly resembling humans ALS patients (Zhu et al., 2023). The SOD1-G93A is normally viable and fertile. Experiments were conducted following the international guideline for animal research and after approval by the animal care committee of the University of Naples “Federico II” and the Italian Ministry of Health. All experiments were performed to minimize animal suffering and distress, as well as to reduce the number of animals included in the study. Spinal cord and cortex tissues were extracted as reported in Anzilotti et al., (2021).

#### *3.3.2.2. **C. elegans***

*C.elegans* is a widely alternative *in vivo* model for environmental toxicity studies (Long et al., 2023). This model was developed thanks to the intuition of the Nobel prize winner Sydney Brenner, who a little over 50 years ago, found the nematode C.*elegans* as a model that would allow him to study the molecular basis of animal behaviour and development. Today, this 1 millimeter long worm, is a well-established model in numerous fields

and its use has led to many important discoveries (Greener, 2021). Among the many, the nobel prize-worth discoveries of apoptosis genes in 1983 (Hedgecock et al. 1983; Ellis and Horvitz 1986; Yuan and Horvitz 1992; Yuan et al. 1993; Conradt and Xue 2005), the Introduction of GFP as a biological marker in 1994 (Chalfie et al. 1994) and the use of RNA interference (RNAi) to silence genes in 1998 (Fire et al. 1998).

The versatility of this model has led its application to be spread and adopted by researchers all over the globe. Indeed, features like the small size, the short life-cycle, the transparency allowing visualization of the internal organs, its well-annotated genome (which, of note, possesses homologs of about two-thirds of human disease genes), the ease of culturing and handling, the low maintenance cost and the less concern related to biosafety and ethics (Greener, 2021; Corsi et al., 2015; Long et al., 2023; Zhang et al., 2020b) have made this animal a relatively simple and effective model to tackle research questions.

The fields of application are numerous and diverse - such as the research fields of molecular basis of human diseases and pathologies (Markaki & Tavernarakis, 2020), rare disease modeling, drug discovery and pharmacological screenings (Kropp at al., 2021; Greener et al., 2021), aging and aging-associated neurodegenerative diseases (Van Pelt & Truttmann, 2020) and metabolism and nutrition (Yue et al., 2021; Chakravarty, 2022). Lastly, C.*elegans* has proven to be a powerful model for studying neurotoxicity; whilst for generic environmental toxicity studies, although relevant, the potential of C.*elegans* is still largely under-explored. (Queirós et al., 2019). However, this latter area has gained more and more attention from the public and interesting nano-toxicology and ecotoxicology studies have been conducted to deepen the knowledge on the fate of anthropogenic substances in the human body and environment - as their exposure can also

be chronic or even multigenerational; a condition which can be studied with C.*elegans* short life-cycle and large progeny. For instance, studies on the reproductive effects of common nanomaterials have been done on C.*Elegans* (Yao et al., 2022).

To conclude, C.*elegans* is an invaluable tool for studying the toxicological effects of compounds and a model organism that can easily bridge the gap between *in vitro* and i*n vivo* studies, allowing experimenters to expand the level of complexity of the effects addressed.

##### *3.3.2.2.1.* ***Maintenance***

The N2-CGCM wild type strain was obtained from the Caenorhabditis Genetics Center (CGC), Minnesota, USA and maintained onto Nematode Growth Medium (NGM) agar plates and fed with the OP50 *E.coli* strain, obtained from the CGC (Stiernagle, 2006).

NGM agar was prepared by mixing Agar, Bacto Peptone and NaCl. After autoclaving, the flask was kept in 55°C water bath for 15 minutes to let it cool. 5mg/ml cholesterol in ethanol, 1 M $KPO_4$ buffer pH 6.0, 1M $MgSO_4$ were added in a second moment (Stiernagle, 2006). NGM agar was aseptically poured into petri dishes (Brenner, 1974) using a peristaltic pump so to dispense a constant amount of NGM agar in each plate. Plates were left at room temperature for 2-3 days to allow excess moisture to evaporate, before being seeded with OP50 *E.coli* strain.

A single colony of such strain was previously streaked, aseptically, on a LB agar plate (Byerly et al., 1976) from a starter culture. Then, a single colony from this plate was aseptically inoculated into LB broth media. Inoculated cultures were kept overnight shaking at 37°C to let them grow before seeding. Once seeded, *E.coli* OP50 lawns were allowed to grow at

20°C for 2/3 days before use and plates stored in an air-tight container. (Stiernagle, 2006). Worms were kept and fed onto OP50 seeded plates at 20°C for at least three generations prior to experiments to ensure full viability.

#### *3.3.2.2.2.* ***Brood Assay***

A brood assay was set up to evaluate the progeny of worms. To do this, egg lays were synchronized to yield L4 larvae, which are not yet sexually mature. Therefore, L4 worms were picked with a platinum wire mounted into the tip of a Pasteur pipet and placed singularly onto a seeded plate pre-treated for 24 hours with either 0,01% DMSO, $PM_{0.1}$ or NP20 (50ul/5ml agar), to allow the substance to penetrate and be absorbed by the agar. Such dilution was chosen after performing an OP50 growth-curve assay, to ensure that the compounds tested would not affect the growth of the bacterial lawn. The OP50 growth-curve assay consisted in a 8-min shake test and a growth curve linear shake test performed in a 96 multiwells plate and, subsequently, analysed through a microplate reader. Once adults, adult hermaphrodites start producing progeny for a period of 2-3 days, until their self-produced sperm is depleted. Therefore, worms were let laying eggs for 24 hours onto plates. To ensure that the compound tested would have no effect on the length of the egg-laying period, each worm was transferred onto a new plate for five days; the eggs that were laid were allowed to hatch and the number of larvae scored after 48 hours. Larvae were visualized using a dissecting stereomicroscope and scored through a grid and a cell-counter. The progeny of dead worms was excluded from the final count.

#### *3.3.2.2.3.* ***Body size assay***

Ten young adults were picked and placed onto a 35mm pre-treated place and let laying eggs for 2 hours. At the end, young adults were removed from the plate and eggs were allowed to hatch. Every 24 hours images of the progeny were taken with a Leica dissecting microscope to evaluate the size of the progeny.

#### *3.3.2.2.4.* ***Lifespan and survival assay***

Synchronous populations of L1 were obtained and placed onto 60 mm plates seeded with *E.coli* OP50 and pre-treated with 0.01% NP20, DMSO or $PM_{0.1}$. 50 μM of 5'-fluorodeoxyuridine (FUdR) was added to sterilize Caenorhabditis elegans (Anderson et al., 2016). Every two-three days live and dead worms were counted. Worms were scored as dead when not responding to gentle prodding. Worms crawling out from plates or dead from causes other than age, where censored. The longevity assay was entirely carried out at 20°C.

#### *3.3.2.2.5.* ***In vivo statistical analysis***

Statistical analyses were performed with One-way ANOVA with Newman-Keuls's/Bonferroni post-hoc test and correction, or with Unpaired *t*-test, which was used for two groups comparison. For survival plots, data were analysed through a Log-rank (Mantel-Cox) test. Analyses were carried out using GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p$ values $< 0.05$.

**Table 1**. List of antibodies used.

| | RRID | Ab Source | Ab Type | Dilution used | Supplier | Cat. Number |
|---|---|---|---|---|---|---|
| **GRP78 (BiP)** | AB_10695864 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 3183 |
| **Caspase-12** | AB_2069200 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 2202 |
| **CHOP** | AB_2089254 | Mouse | Monoclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 2895 |
| **Anti-SERCA2 ATPase** | AB_2061425 | Mouse | Monoclonal | 1:1000 | Abcam (Cambridge, UK) | ab2861 |
| **Anti-SERCA3 ATPase** | AB_2756568 | Rabbit | Polyclonal | 1:1000 | Alomone Labs (Jerusalem, Israel) | ACP-014 |
| **Anti-STIM1** | AB_2039893 | Rabbit | Polyclonal | 1:1000 | Alomone Labs (Jerusalem, Israel) | ACC-063 |
| **β-Actin-Peroxidase** | AB_262011 | Mouse | Monoclonal | 1:10000 | Sigma-Aldrich (Milan, Italy) | A3854 |
| **Caspase 9 (cleaved Asp330)** | AB_2886615 | Rabbit | Polyclonal | 1:1000 | GeneTex (Irvine, CA, USA) | GTX132331 |
| **RyR3** | AB_2040186 | Rabbit | Polyclonal | 1:1000 | Alomone Labs (Jerusalem, Israel) | ARR-003 |
| **Anti-IP3R-3** | AB_397705 | Mouse | Monoclonal | 1:500 | BD Biosciences (CA, USA) | 610313 |
| **LC3B** | AB_11176277 | Rabbit | Polyclonal | 1:1000 | GeneTex Inc. (Irvine, CA, USA) | GTX127375 |
| **p62/SQSTM1** | AB_10011069 | Rabbit | Polyclonal | 1:1000 | Novus Biologicals (Littleton, CO, USA) | NBP1-48320 |
| **TPC2** | AB_10918019 | Rabbit | Polyclonal | 1:1000 | Alomone Labs (Jerusalem, Israel) | ACC-072 |
| **TRPML1** | AB_10915894 | Rabbit | Polyclonal | 1:1000 | Alomone Labs (Jerusalem, Israel) | ACC-081 |
| **Anti-p-AMPKα** | AB_330330 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 2531 |
| **Anti-AMPKα** | AB_330331 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 2532 |
| **Cleaved Caspase-3 (Asp175)** | AB_2341188 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 9661 |
| **Cytochrome C** | AB_2090454 | Rabbit | Polyclonal | 1:1000 | Cell Signaling Technology, Inc. (Danvers, MA, USA) | 4272 |

# Presentation of results

## 4.1. UFPs effects *in vitro*

### *4.1.1. NP20 and $PM_{0.1}$* Struct*ural characterization and access to cells*

$PM_{0.1}$ and NP20 were produced as described in the methodology section and their structural characterization performed through transmission electron microscopy (TEM) and high-resolution TEM (HRTEM), respectively (**Figure 1A,B**).

First among everything, once particles had been characterized, it was assessed whether nanoparticles were up-taken into cells, upon exposure to UFPs. Cellular uptake was determined by the pHrodo™ Green dye, a nonfluorescent dye at neutral pH which is able to emit increasing fluorescence as the pH becomes more acidic. In fact, the acidification of endocytic compartments is a hallmark of endocytosis and in this experiment, a significant increase in fluorescence emission was registered in NSC-34 cells exposed to $PM_{0.1}$ and NP20, starting from 3 hours (**Figure 1C**). This strong endocytic event was expressed as relative fluorescence units (RFU) and measured up to 6 hours. To further strengthen this result, brightfield images depicting cells exposed to both pollutant fraction showed round bodies which are reminiscent of endocytic vesicles (**Figure 1D**).

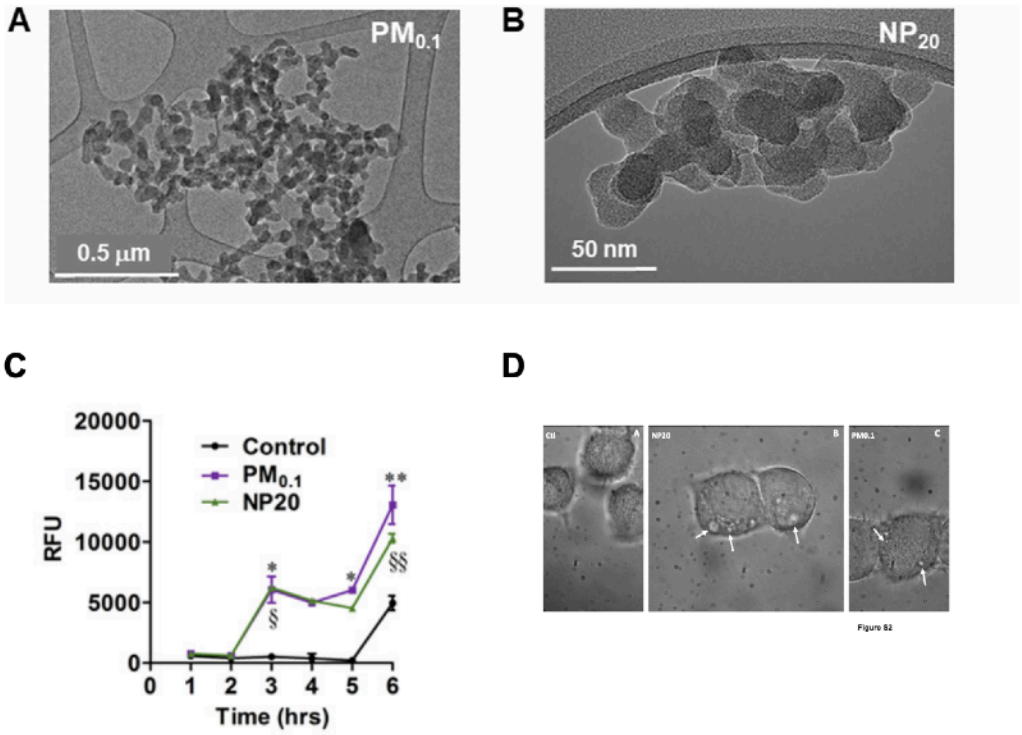



**Figure 1**. $PM_{0.1}$ and NP20 structural characterization and cellular uptake. (**A**) TEM image of $PM_{0.1}$ and (**B**) HRTEM image of NP20. (**C**) Endocytosis in NSC-34 motor neurons measured by pHrodo™ Green *E. coli* BioParticles™ Conjugate (37 °C, 5% $CO_2$). Data were expressed as Relative Fluorescence Units (RFU) measured up to 6 h with 1 h span at an excitation wavelength of 509 nm and emission at 533 nm. NSC-34 (1 × 105 cell/well) were stimulated (37 °C, 5% $CO_2$) with $PM_{0.1}$ (2.86 ppm), NP20 (0.71 ppm). * $p < 0.05$ vs. previous time points for the same treatment and same time point as control; § $p < 0.05$ vs. previous time points for the same treatment and same time point of Control; ** $p < 0.05$ vs. previous time points for the same treatment and control; §§ $p < 0.05$ vs. previous time points for the same treatment and control. (**D**) Brightfield images of endocytosis in NSC-34 motor neurons (150 x 103 cells/well) exposed for 3 hours to $PM_{0.1}$ (2,86 ppm) and NP20 (0,71 ppm).

### *4.1.2.* ***Kinetic and electrophysiological properties of NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20.***

Current-clamps experiments were performed to determine the membrane potential of NSC-34 motor neurons exposed to ultrafine particles. Results showed that the NP20 fraction, but not $PM_{0.1}$, was able to shift the membrane potential towards more depolarizing values (**Figure 2C**).

The kinetic of NSC-34 motor neurons was then investigated through cell tracking experiments, as stimuli can affect cells movement in space. Therefore, NSC-34 cells were exposed to $PM_{0.1}$, NP20 and to medium alone as a control, over 6h at 37°C; cells start points were each set at 0 on the x axis and at 0 on the y axis (**Figure 2A**). To ensure that the effects observed were not an effect of speed, the mean speed per well was measured, yielding similar values in all conditions, including controls (**Figure 2B**). Only NP20 seemed to alter NSC-34 cells displacement along the x axis, measured as current displacement (**Figure 2A(a)**) and mean displacement in X axis per well (**Figure 2A(b)**): more precisely, motor neuron-like cells stayed closer to their point of origin, compared to untreated cells, although speed was not altered (**Figure 2A,B**). Nevertheless, NP20 did not affect cells movement on the y axis (**Figure 2D**).

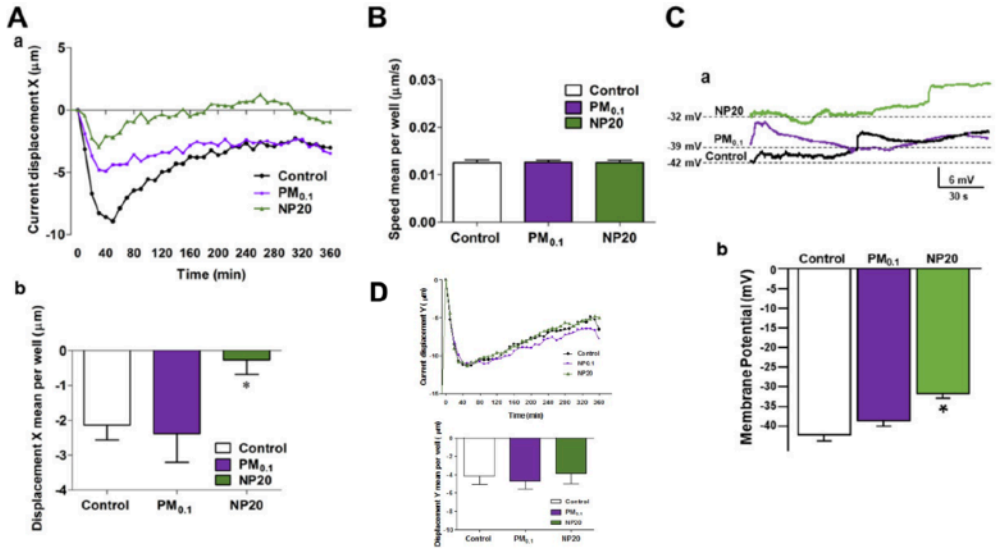



**Figure 2**. Effects of $PM_{0.1}$ and NP20 on the kinetic and electrophysiological properties of NSC-34 motor neurons. Time-lapse measurement were performed with high-content microscopy (Operetta High-Content Imaging System, PerkinElmer), per well (**A**,**B**) Tracking characteristics (current displacement in X axis in A(a), mean displacement in X axis per well in A(b). (**B**) Mean speed per well. NSC-34 (150 × 103 cells/ well) were stimulated (6 h, 37 °C) with DMEM alone (Control), $PM_{0.1}$ (2.86 ppm) and NP20 (0.71 ppm). (**C**) Resting membrane potentials recorded of at least 5 motor neuron-like cells treated either with $PM_{0.1}$, NP20. In (a) superimposed current-clamp traces registered and relative quantification in (b). Each experimental group comprehended 5 cells. * $p < 0.05$ vs. control and $PM_{0.1}$. (**D**) Tracking characteristics of displacement in Y axis (current displacement and mean displacement in Y axis per well.

### *4.1.3.* ***Cell viability and mitochondrial dysfunction***

Once established that the kinetic and electrophysiology properties of cells exposed to NP20 were altered and that UFPs were confirmed to be incorporated in cells, the molecular mechanisms underlying UFPs-related effects were evaluated.

Firstly, the mitochondrial bioenergetic was investigated through several mitochondrial parameters and pharmacological tools. With a brief pulse of FCCP, an oxidative phosphorylation disruption was induced in motor neuron-like cells exposed for 12 and 24 hours to UFPs, as well as controls: this led to a time-dependent reduction in FCCP-induced $[Ca^{2+}]_i$ increase in treated cells, compared to untreated cells (**Figure 3A(a–d)**), suggesting a significant reduction in mitochondrial $Ca^{2+}$ level in NSC-34 exposed to the two air pollutants and a consequent $Ca^{2+}$ homeostasis dysfunction. However, this reduction was not detectable at earlier times (**Figure 3A(d)**).

Subsequently, another correlate of an oxidative phosphorylation dysfunction was examined, that is its product: ATP. The level of the latter, was indeed significantly reduced in cells upon chronic exposure to $PM_{0.1}$ and NP20 (**Figure 3B**). To reinforce this finding, the expression of cytochrome C was higher in motor neuron-like cells exposed to nanoparticles after 48 hours, thereby suggesting a detrimental release from damaged mitochondria (**Figure 3B(insert)**). These results, together with an increase in ROS generation in cells exposed to both $PM_{0.1}$ and NP20 (**Figure 3C**), indicate an impairment of mitochondrial function and a reduced mitochondrial viability.

Furthermore, the mitochondrial membrane potential (ΔΨm) is essential for the maintenance of cellular health and viability as it is a pivotal component in the process of energy storage during oxidative phosphorylation and in mitochondrial homeostasis - as it is involved in the selective elimination of dysfunctional mitochondria (Zorova et al., 2018). A sustained alteration of this membrane potential may induce unwanted loss of cell viability and therefore, to further strengthening our results, the mitochondrial membrane potential (ΔΨm) was assessed and its depolarization measured as a significant decline in mitochondria-localized fluorescence intensity in NSC-34 cells exposed to UFPs for 24 hours, compared to controls (**Figure 3E**). Moreover, intracellular $Ca^{2+}$ level was evaluated in parallel in the same cells, showing a striking increase in its level in cells exposed to the two fractions of particulate matter (**Figure 3E**). Taken together, these results suggest that cell viability is fully compromised upon long-term exposure.

Cell viability was also assessed in NSC-34 motor neuron-like cells and primary cortical neurons, measuring mitochondrial metabolic activity through MTT assays (**Figure 3D,F**). Both cell populations displayed a progressive reduction in mitochondrial activity and, thus, a reduction of cells with active metabolism at different concentrations of $PM_{0.1}$ and NP20, with motor neuron cells exhibiting a more marked decrease, compared to cortical neurons (**Figure 3D**).

In conclusion, the experiments above indicate a UFP toxic effect on mitochondria, leading to their impairment and to a reduce cellular viability. Of note, mitochondrial defects have been associated with ALS motor neurons death, such as impaired mitochondrial $Ca^{2+}$ homeostasis, defects in oxidative metabolism, increased level of ROS production and impaired ATP

production (Keep et al., 2001; Mattiazzi et al., 2002; Menzies et al., 2002; Knott et al., 2008 ; Beal et al., 1997, 2002).

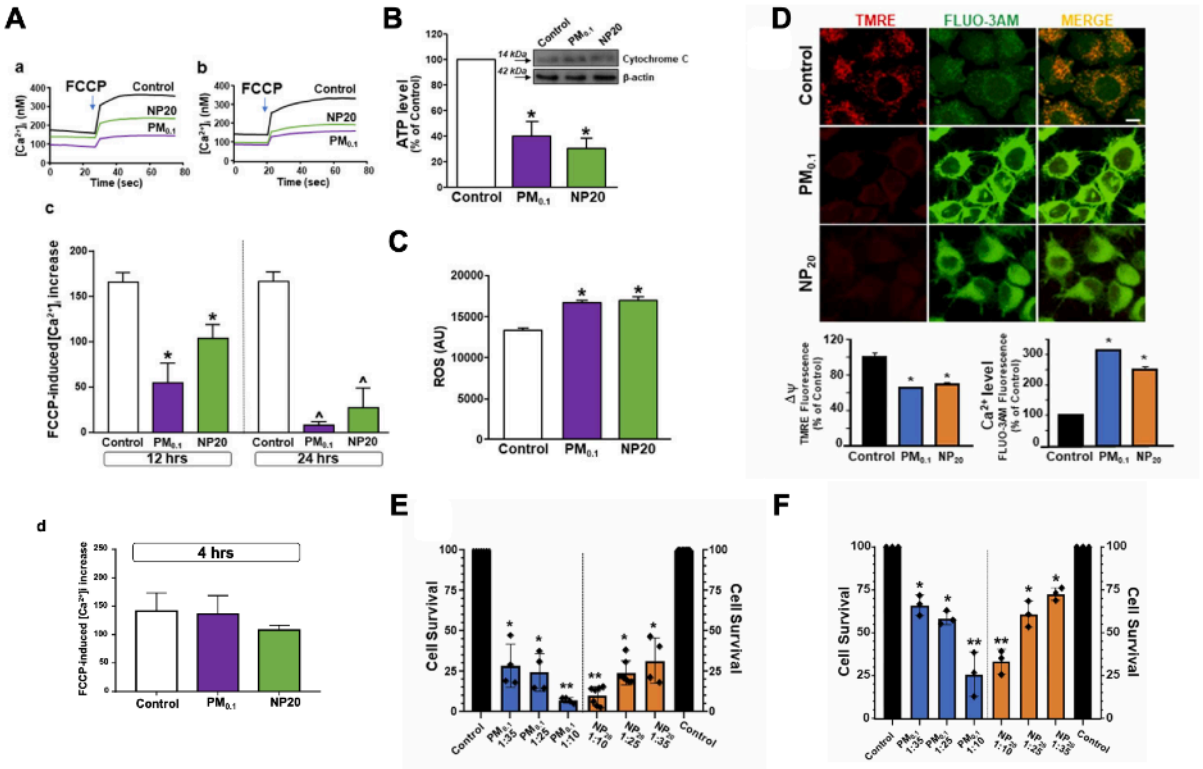



**Figure 3**. Mitochondrial dysfunction and cellular viability following exposure to $PM_{0.1}$ and NP20. (**A**) Superimposed traces representing the effect of FCCP on $[Ca^{2+}]_i$ after treatment with $PM_{0.1}$ and NP20 for 12 h (a) or 24 h (b) and relative quantification as $[Ca^{2+}]_i$ increase (c). (d) Relative quantification as $[Ca^{2+}]_i$ increase after 4 hour exposures. For each experiment at least n = 15 cells were detected. * $p < 0.05$ vs. its respective control at 12 h; ^ $p < 0.05$ vs. its respective control at 24 h. (**B**) Bar graph representing the level of ATP in NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20 for 48 h. * $p < 0.05$ vs. control. Western blotting bands depict cytochrome C released in cytoplasm after treatment. (**C**) Bar graph representing ROS level measured as arbitrary units in NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20 for 48 h. Experiments were repeated at least three times. (**D**) Confocal images measuring cell viability of TMRE- and Fluo-3AM-loaded NSC-34 cells exposed to $PM_{0.1}$ and NP20 for 24 hrs. Below: quantification of mitochondrial membrane potential as $\Delta\Psi$ N = 40 cells) and intracellular $Ca^{2+}$ level in the same cells as measure of cell viability. * $p < 0.05$ vs. respective controls. (**E,F**) Measurement of cell viability in NSC-34 motor neurons (**E**) and mature primary cortical neurons (**F**) exposed to $PM_{0.1}$ and NP20 at various concentrations (for reference 1:35 dilution corresponds to 2.86 ppm and 0.71 ppm for $PM_{0.1}$ and NP20, respectively). Cell viability was measured as mitochondrial activity, since cell suffering corresponds to the reduction in mitochondrial activity. * $p < 0.05$ vs. controls, ** $p < 0.05$ vs. previous concentrations in each group.

### *4.1.4. **UFPs effects on the ROS sensor TRPML1 and on autophagy in NSC-34 Motor Neurons***

Given the increased level or ROS, the lysosomal channel TRPML1 - a specific cellular sensor of ROS required for lysosome adaptation to mitochondrial damage (Zhang et al., 2016), was examined in terms of TRPML1 expression and function. Chronic exposure to $PM_{0.1}$ and NP20 triggered the increase in expression of TRPML1, but interestingly not in TPC2 (**Figure 4B,C**).

The lysosome has emerged as an important $Ca^{2+}$ cellular store and as a signaling hub of the cell. Therefore, many defects in lysosomal $Ca^{2+}$ homeostasis have been observed and associated in several neurodegenerative diseases, such as ALS (Tedeschi et al., 2019a). TRPML1 $Ca^{2+}$ releasing function and lysosomal $Ca^{2+}$ homeostasis were also altered: perfusion with the specific TRPML1 agonist -ML-SA1, induced a swift $[Ca^{2+}]_i$ increase in NSC-34 cells exposed for 48 hours to UFPs (**Figure 4A(a,b)**). Treatment with the antioxidant molecule Trolox significantly hampered TRPML1 hyperactivation and ML-SA1-induced $[Ca^{2+}]_i$ increase (**Figure 4A(a,b)**), thus restoring TRPML1 resting activity.

As TRPML1 is a regulator of the autophagic flux (Medina et al., 2015; Wang et al., 2015), a potential alteration of the latter was examined through the expression of two common autophagic markers - LC3-II and p62. Results showed that both markers were increased after 48 hours of exposure to both $PM_{0.1}$ and NP20 (**Figure 5A(a–d)**), suggesting an engulfment of the autophagic flux. Defects in clearing mechanisms are common in neurodegenerative pathologies like ALS, as aggregates of proteins and cellular inclusions have been observed (Cipolat et al., 2016 ; Song et al., 2012).

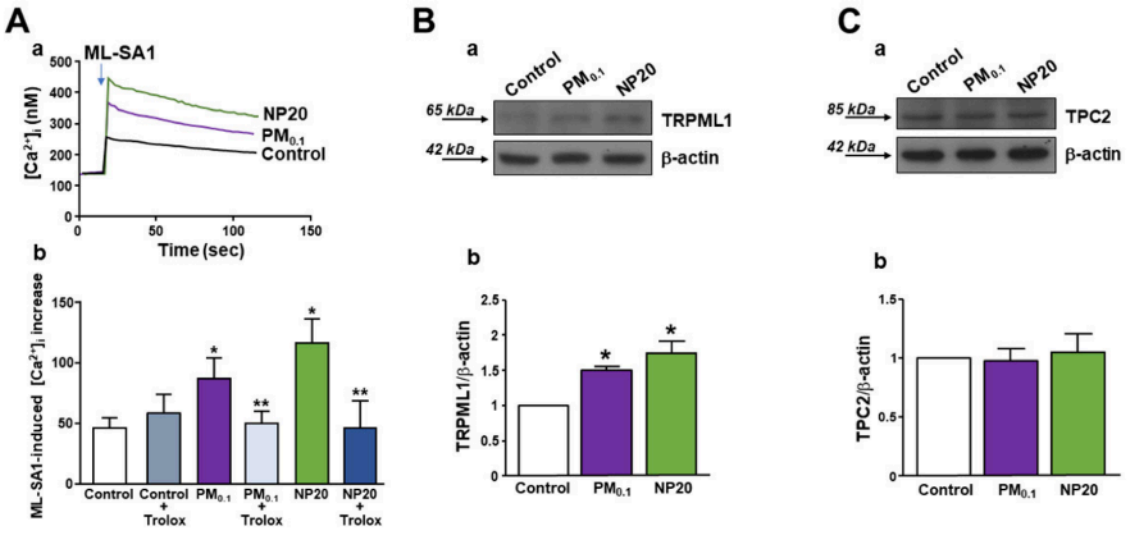



**Figure 4**. Effect of $PM_{0.1}$ and NP20 on expression and activity of the lysosomal channel TRPML1. (**A**) Superimposed representative traces (a) and quantification (b) of the effect of ML-SA1 (10 μM) on $[Ca^{2+}]_i$ in NSC-34 motor neurons exposed to $PM_{0.1}$ (2.86 ppm) and NP20 (0.71 ppm) (48 h) in the presence or absence of Trolox (100 μM). This latter drug was added every 24 h. All the experiments were repeated three times on almost 20 cells for Control, Control+Trolox, Control+$PM_{0.1}$, Control+NP20 and 25 cells for $PM_{0.1}$+Trolox and NP20+Trolox. * $p < 0.05$ vs. Control; ** $p < 0.01$ vs. $PM_{0.1}$ or NP20. (**B**) Representative western blotting band of TRPML1 expression in NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20 (48 h) (a) and quantification (b). All the experiments were repeated three times on different preparations. * $p < 0.05$ vs. control. (**C**) Representative Western blotting of TPC2 expression in NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20 (48 hrs) (a) and quantification (b). All the experiments were repeated three times on different preparations.

The activation of AMPK (p-AMPK) is able to initiate various metabolic processes - among these, autophagy (Li & Chen, 2019). In our model, AMPK protein expression was significantly increased in motor neuron-like cells upon exposure to $PM_{0.1}$ and NP20 (**Figure 5B(a–c)**). It is worthy mentioned that AMPK activation can also be due to AMP/ADP, rather than ATP, consistently with our previous results which highlight a bioenergetic cellular failure.

Again, the treatment with Trolox was able to restore the autophagic flux in the $PM_{0.1}$ condition, as LC3-II protein expression was significantly reduced in $PM_{0.1}$- treated cells (**Figure 5C(a,b)**).

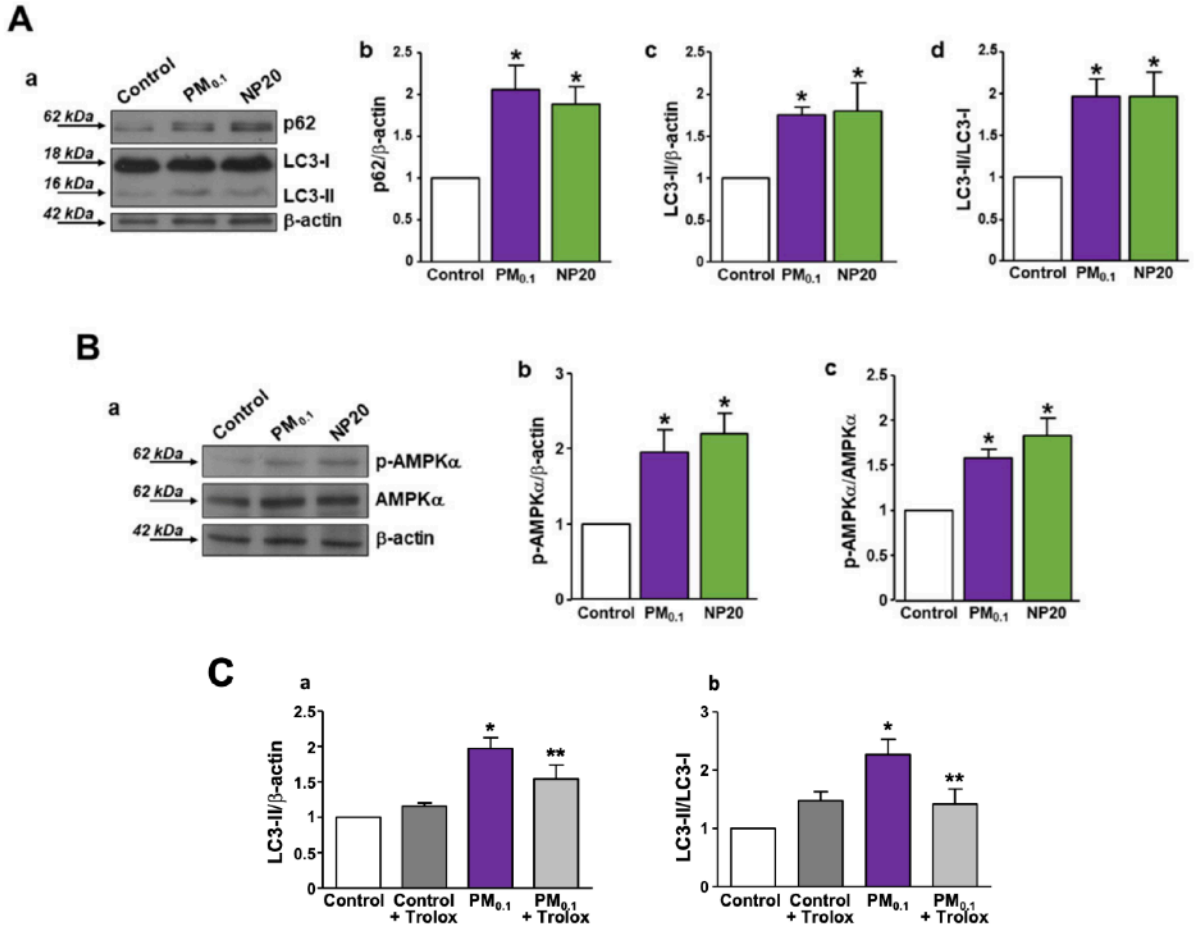



**Figure 5**. Effect of $PM_{0.1}$ and NP20 on p62, LC3 and AMPK protein expression in NSC-34 motor neurons. (**A**) Representative Western blotting (a) and quantification (b–d) of p62 and LC3-II expression, respectively, in NSC-34 motor neurons exposed to $PM_{0.1}$ (2.86 ppm) and NP20 (0.71 ppm) (48 h). Each bar represents the mean ± S.E. of data obtained from three different experimental sessions. * $p < 0.01$ vs. each respective Control. (**B**) Representative Western blotting (a) and quantification (b,c) of p-AMPKα and AMPKα expression in NSC-34 motor neurons exposed to $PM_{0.1}$ and NP20 (48 h). Each bar represents the mean ± S.E. of data obtained from three different experimental sessions. * $p < 0.01$ vs. each respective Control. (**C**) Representative quantification of LC3-I/LC3-II expression in NSC-34 motor neurons exposed to $PM_{0.1}$ (48 hrs) in the presence of trolox. Each bar represents the mean ± S.E. of data obtained from three different sessions. * $p < 0.01$ vs control, ** $p < 0.01$ vs $PM_{0.1}$ alone.

### *4.1.5. **Disruption of ER $Ca^{2+}$ homeostasis and ER morphology***

Given the important role of the ER in maintaining the cellular homeostasis and its involvement in several neurodegenerative diseases, its $Ca^{2+}$ levels and morphology were evaluated.

By staining with a specific fluorescent dye the organelle in live-cell imaging experiments, significant alterations of ER morphology were found in cells exposed to both UFP fractions: indeed, cells exposed to the two air pollutants showed an increase of non-tubular ER, compared to control cells, exhibiting highly tubular and structured ER (**Figure 6A,B**). Furthermore, quantitative fluorescence analysis of dye-tagged ER indicated a notably increase in the basal fluorescence in cells exposed to $PM_{0.1}$ and NP20, compared to cells in control conditions (**Figure 6C**).

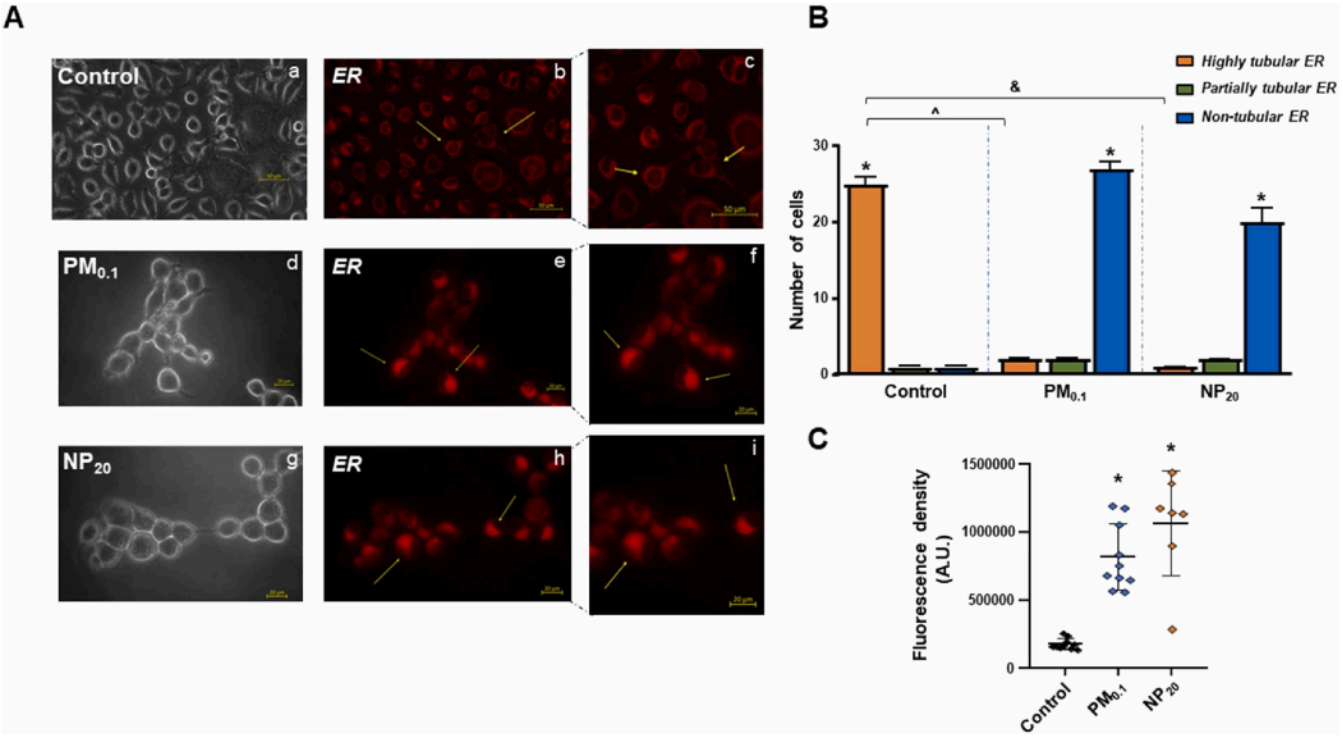



**Figure 6**. $PM_{0.1}$ and NP20 induced ER morphological changes in NSC-34 cells. (**A**) ER morphology in live motor neuronal cells stained with a ER specific marker, under control conditions (b, c) or exposed to $PM_{0.1}$ (1:35 dilution corresponding to 2.86 ppm/48hrs) (e, f), and NP20 (1:35 dilution corresponding to and 0.71 ppm/48hrs) (h, i). Relative bright field images are reported in a, d and g, respectively. (**B**) Quantification of ER morphology in A (b, e and h) as "*Highly tubular ER", "Partially tubular ER" or "non-tubular ER"* under control conditions or exposed to $PM_{0.1}$ and NP20. * $p < 0.05$ vs. all in each group (**C**) Fluorescence density of ER staining in A (b, e and h) measured as arbitrary units in at least 7 fields of each image. Data are represented as means±SE of 3 separate experiments * $p < 0.05$ vs. control (vehicle-treated).

The ER $Ca^{2+}$ level was examined through time-course functional experiments, which showed that $PM_{0.1}$ exposure was able to drastically reduce the levels of $Ca^{2+}$ inside the ER already at 12 hours of exposure and to maintain this detrimental reduction thereafter (**Figure 7A(a,c)**). On the contrary, the effect of NP20 seemed to be slightly slower, leading to a drastic reduction only at 24 hours of exposure (**Figure 7A(a-c)**). In this experiment, ER $Ca^{2+}$ stores were depleted with the SERCA pump inhibitor thapsigargin (TG, 1 μM). ER $Ca^{2+}$ depletions is known to stimulate a swift coupling between the ER $Ca^{2+}$ sensor STIM1 and the plasma membrane channels to replenish the ER $Ca^{2+}$ stores, in the so-called activation of the store operated calcium entry (SOCE) response (Putney, 2013). Therefore, after ER $Ca^{2+}$ depletion is forced with TG, the reintroduction of $Ca^{2+}$ into the cell cytosol elicits a $[Ca^{2+}]_i$ increase, which allows for SOCE detection.

At 12 hours, SOCE significantly increased following $PM_{0.1}$ and NP20 exposure. However, at 24 hours, the two fractions displayed opposite behaviour, with, on the one hand, SOCE drastically reducing after 24 hours in the case of cells exposed to $PM_{0.1}$ (**Figure 7B(a,c)**) and on the other, with SOCE stably maintaining its activation significantly higher than controls after 24 hours of exposure (**Figure 7B(b,c)**). In the latter condition, a mismatch between the increased SOCE response activation and the extremely low ER $Ca^{2+}$ level was evident, pointing out to a compromised $Ca^{2+}$ handling machinery.

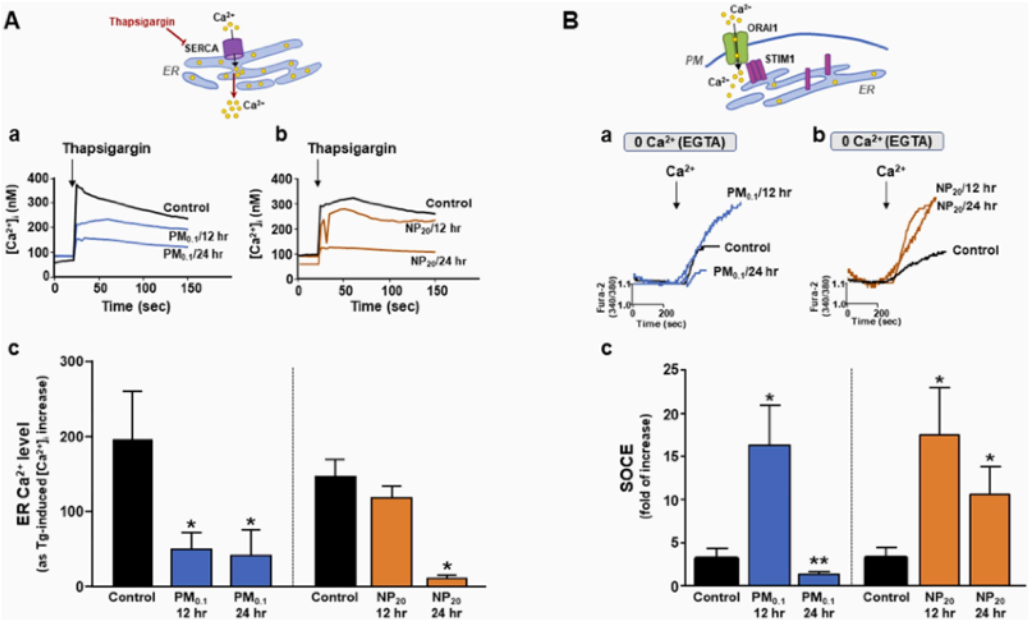



**Figure 7**. $PM_{0.1}$ and NP20 reduced $Ca^{2+}$ content in ER modulating store-operated calcium entry (SOCE) mechanism in motor neuron- like NSC-34 cells. (**A) (**a,b**)** Representative superimposed single-cell traces of the effect of 1 μM thapsigargin (Tg) on $[Ca^{2+}]_i$ in $Ca^{2+}$-free (0 $Ca^{2+}$; 1.5 mM EGTA) under control conditions (black traces in a and b) and after 12 and 24 hrs $PM_{0.1}$ (1:35, Blue traces in a) or NP20 (1:35, Orange traces in b). (c) Quantification of A. Data are means±SE of 3 separate experiments * $p < 0.05$ vs. respective control. (**B) (**a,b**)** Representative superimposed single-cell traces of SOCE under control conditions (black traces in a and b) and after 12 and 24 h $PM_{0.1}$ (1:35, Blue traces in a) or NP20 (1:35, Orange traces in b). To measure SOCE as $[Ca^{2+}]_i$, movement, neurons were exposed to thapsigargin in the absence of extracellular calcium and, then, re-exposed to 2 mmol/L $Ca^{2+}$. (c) Quantification of B. Data are means±SE of 3 separate experiments * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 12 hrs.

### *4.1.6. $PM_{0.1}$ and NP20-induced ER $Ca^{2+}$ level reduction was due to ER $Ca^{2+}$ handling machinery dysfunction*

The hypothesis of a compromised ER $Ca^{2+}$ handling system was further investigated starting with one of the key regulators of SOCE, that is STIM1: the only intraluminal ER $Ca^{2+}$ sensor. STIM1 expression was thus studied in motor neuron-like cells exposed to $PM_{0.1}$ and NP20 and it showed, in the first case, a reduction in its expression at 12 hours, followed by a sharp increase at 24 hours (Figure 8A). Conversely, NP20 exposure drove STIM1

expression to higher levels already at 12 hours and to an additional increase at 24 hours, compared to controls (Figure 8A).

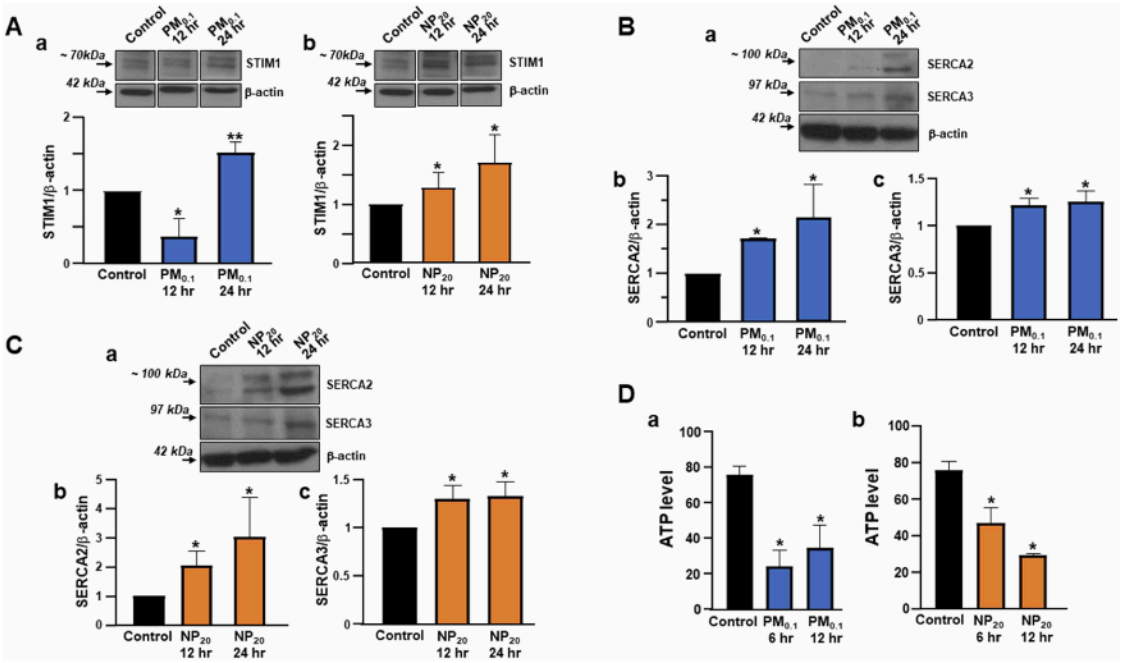



**Figure 8**. $PM_{0.1}$ and NP20 induced changes in ER proteins involved in $Ca^{2+}$ sensing and handling (i.e. STIM1, SERCA2, and SERCA3). (**A**) Western blots and quantification of STIM1 expression in NSC-34 cells after 12 and 24 hrs exposure to $PM_{0.1}$ in "a" (1:35 corresponding to 2.86 ppm) or NP20 in "b" (1:35 corresponding to 0.71 ppm). Data are means±SE of 4 separate experiments. * $p < 0.05$ vs. respective controls. ** $p < 0.05$ vs. 12 hrs PM. (**B, C**) Western blots and quantification of SERCA2 (B, a,b; C,a,b) and SERCA3 (B, a, c; C, a,c) expression in NSC-34 cells after 12 and 24 hrs exposure to $PM_{0.1}$ in "B" (1:35 corresponding to 2.86 ppm) or NP20 in "C" (1:35 corresponding to 0.71 ppm). Data are means±SE of 5 separate experiments. * $p < 0.05$ vs. respective controls. (**D**) ATP level in NSC-34 cells after 6 and 12 hrs exposure to $PM_{0.1}$ in "a" or NP20 in "b". Data are means±SE of 3 separate experiments. * $p < 0.05$ vs. respective controls.

After SOCE activation, cytosolic $Ca^{2+}$ entry in the ER is mediated by SERCA pumps. Therefore, the effect of UFPs on the ATPases SERCA2 and SERCA3, which are mainly expressed in the brain (Baba-Aissa et al., 1998, Brini & Carafoli, 2009), was examined. Results indicate an up-regulation of SERCA2 and SERCA3 expression, at 12 and 24 hours, in NSC-34 exposed to $PM_{0.1}$ or NP20 (Figure 8B,C). Because the ATP level of cells exposed to UFPs was severely curtailed already at early times (i.e: 6 hours, Figure 8D) and SERCAs require energy via ATP in order to pump $Ca^{2+}$ ions from the

cytosol to the ER lumen, this over-expression of SERCAs may be not representative of functional proteins or/and may be a compensatory mechanism to restore homeostasis after a detrimental reduction of ER $Ca^{2+}$, along with the over-expression of STIM1.

#### *4.1.7.* ***ER $Ca^{2+}$ replenishment dysfunction and ER stress***

SOCE activation and the related $Ca^{2+}$ release-activated current $I_{CRAC}$, were studied at long-term exposure times (48 hours). At this time point, ATP is drastically reduced in cells exposed to both NP20 and $PM_{0.1}$, as previously demonstrated (**Figure 3B**). Results show that the ER $Ca^{2+}$ handling machinery was completely dysregulated: SOCE activation was found to be strikingly low, compared to controls (**Figure 9A(b)**); while STIM1 was up-regulated (**Figure 9A(c)**). In accordance with the effect of $PM_{0.1}$ and NP20 on SOCE $[Ca^{2+}]_i$, patch-clamp experiments showed that $I_{CRAC}$ -measured at −120 mV, was drastically reduced compared to control currents (**Figure 9B**).

Moreover, SERCA2 and SERCA3 were significantly up-regulated, together with the ER release $Ca^{2+}$ channels RyR3 ad IP3R3, in cells exposed to $PM_{0.1}$ or NP20 for 48 hours (**Figure 9D(a-c)**). This up-regulation is quite interesting, as it could represent one of the main causative mechanisms of $Ca^{2+}$ loss and ER stress.

Taken together, the above experiments point out to a complete disfunction of ER $Ca^{2+}$ refilling and ER $Ca^{2+}$ homeostasis. Disturbances of the latter indeed, can lead to ER stress; a state which if chronically induced, can culminate in apoptosis. In our model, the ER in cells exposed to $PM_{0.1}$ and NP20 exhibited a sustained state of dysregulation and therefore, the expression of the active form of Caspase-12, a central protein guiding the initiation of ER stress-related cell death (Szegezdi et al., 2003), was

assessed. Indeed, the active form of Caspase-12 was significantly up-regulated by the $PM_{0.1}$ or NP20 48 hours exposure (**Figure 9E(a-c)**). To further prove that apoptosis was initiated, the expression of another caspase was studied; that is Caspase-9, involved in the ER stress-related apoptosis cascade (Morishima et al., 2002).

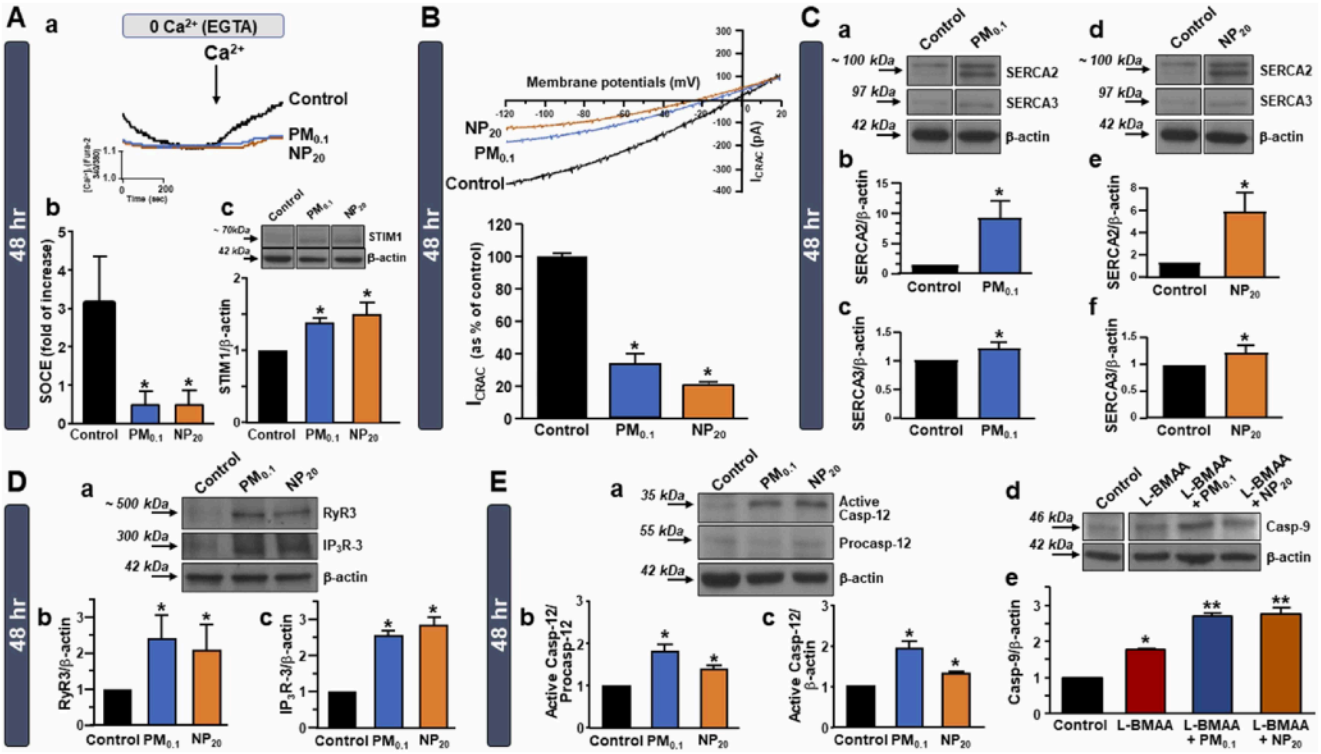



**Figure 9**. Long exposure to $PM_{0.1}$ and NP20 downregulated SOCE and $I_{CRAC}$ modifying ER machinery and activating ER stress. (**A** a,b) Representative superimposed single-cell traces of SOCE (at least 30 cells for each group) and quantification under control conditions (black trace) and after 48 hrs exposure to $PM_{0.1}$ 1:35 (Blue trace) or NP20 1:35 (Orange trace). (**A** c) Western blot and quantification of STIM1 expression in NSC-34 cells after 48 hrs exposure to $PM_{0.1}$ (1:35 corresponding to 2.86 ppm) or NP20 (1:35 corresponding to 0.71 ppm). Data are means±SE of 4 separate experiments. * $p < 0.05$ vs. respective controls. (**B**) Whole-cell recordings and quantification of $I_{CRAC}$ in NSC-34 motor neurons under control conditions and after exposure to $PM_{0.1}$ 1:35 (Blue trace) or NP20 1:35 (Orange trace). Data are means±SE of 3 separate experiments (5–8 cells for each group). * $p$ <0.05 vs control. (**C**) Western blot quantifications of SERCA2 (a,b,d,e) and SERCA3 (a,d,c,f) expression in NSC-34 cells after 48 hrs exposure to $PM_{0.1}$ (1:35 corresponding to 2.86 ppm) or NP20 (1:35 corresponding to 0.71 ppm). Data are means±SE of 5 separate experiments. * $p < 0.05$ vs. respective controls. (**D**) Western blotting and quantification of RyR3 and IP3R-3 in NSC-34 cells under control conditions and after exposure to $PM_{0.1}$ or NP20 (both at 1:35 dilution). Data are means±SE of 4 separate experiments. * $p < 0.05$ vs. respective controls. (**E**) Western blot quantification of active Caspase 12 and procaspase-12 in NSC-34 cells exposed to $PM_{0.1}$ or NP20 (both at 1:35 dilution/48hrs) (a-c) and Caspase-9 in NSC-34 cells exposed to *L*-BMAA alone or *L*-BMAA + $PM_{0.1}$ or NP20 (both at 1:35 dilution/48hrs) (d, e). Data are means±SE of 3 separate experiments. * $p$ <0.05 vs control. ** $p$ <0.05 vs *L*-BMAA and control.

This latter experiment was performed on cells treated with both a UFP fraction and the neurotoxin *L*-BMAA (used in in vitro model of ALS). Results showed an increase of Caspase-9 in cells treated with *L*-BMAA exacerbating with the additional exposure to UFPs, underlying the ability of UFPs to interfere with ALS mechanisms (**Figure 9E(d,e)**).

### *4.1.8.* ***$PM_{0.1}$ and NP20 were able to induce ER stress***

From the results above, $PM_{0.1}$ and NP20 seemed to be able to trigger ER stress and indeed, they induced a significant protein over-expression of the ER stress sensor BiP/GRP78 in both NSC-34 cells and primary cortical neurons (**Figure 10A,C**). Given that ER stress is implicated in ALS (Jeon et al., 2023), as a proof of concept, a significant increase in protein expression of the two ER stress markers BiP/GRP78 and CHOP was found in brain areas containing motor neurons (i.e: spinal cord and brain cortex) of ALS symptomatic G93A mice (**Figure 10B,D,** respectively). Furthermore, the profile of some proteins involved in oxidative stress and protective neuronal functions (i.e. catalase, BADL, XIAP), mitochondrial dysfunction and ER/ mitochondria cross-talk (i.e. Smack/Diablo), and ER stress (i.e. p53) was assessed with a proteomic analysis in NSC-34 motor neuron-like cells exposed to $PM_{0.1}$ and NP20. The proteomic profile of such cells mirrored the one of motor neurons exposed to β-N-methylamino-*L*-alanine (*L*-BMAA) (**Figure 10E**): the neurotoxin responsible for the Guamanian form of the disease named ALS/PDC (Petrozziello et al., 2017). Moreover, $PM_{0.1}$ was able to potentiate the increased expression of BiP, when cells were exposed to the neurotoxin *L*-BMAA (**Figure 10F**). All this data are suggestive of a common detrimental mechanism shared by UFP-related neurotoxicity and ALS neurodegeneration.

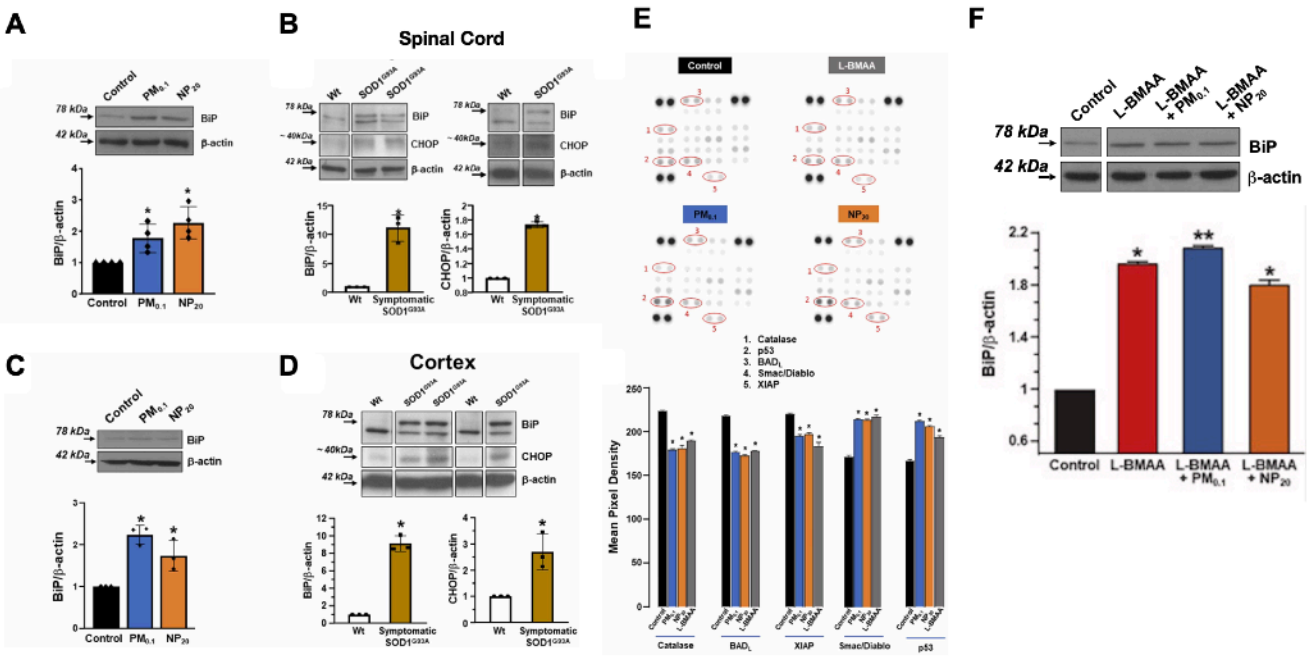



**Figure 10**. $PM_{0.1}$ and NP20 were able to induce ER stress. (**A, B**) Representative Western blotting quantification of BiP expression in NSC-34 motor neuron cells after 48 hrs exposure to $PM_{0.1}$(1:35 dilution corresponding to 2.86 ppm) and NP20 (1:35 dilution corresponding to and 0.71 ppm) (**A**) * $p < 0.05$ vs. respective control; (**B**) Representative western blotting and quantification of BiP and CHOP expression in Spinal Cord of Wt and SOD1G93A mice at 4.5 months of age (symptomatic ALS mice). * $p < 0.05$ vs. respective control. Double bands of BiP/GRP78 in Fig. F could be due to the presence of non-neuronal cells in mice brain lysates. Here, upper bands have been quantified. Data are means±SE of 4 (**A**) or 3 (**B**) separate experiments. * $p < 0.05$ vs. respective controls. (**C**) Representative Western blotting and quantification of BiP expression in primary cortical neurons after 48 hrs exposure to $PM_{0.1}$ (1:35 dilution corresponding to 2.86 ppm) and NP20 (1:35 dilution corresponding to and 0.71 ppm). * $p < 0.05$ vs. respective control; (**D**) Representative Western blotting and quantification of BiP and CHOP expression in brain cortex of Wt and SOD1G93A mice at 4.5 months of age (symptomatic ALS mice). * $p < 0.05$ vs. respective controls. Data are means±SE of 3 separate experiments. * $p < 0.05$ vs. respective controls. (**E**) Representative blots of proteomic immunoassay from NSC-34 cells under control conditions or exposed to *L*-BMAA (300 µM/48hrs), $PM_{0.1}$ (1:35 dilution corresponding to 2.86 ppm/48hrs), and NP20 (1:35 dilution corresponding to and 0.71 ppm/48hrs). Below: quantification as reported in **E.** Data are means±SE of 3 separate experiments * $p < 0.05$ vs. respective control (vehicle- treated). (**F**) Representative Western blotting and quantification of BiP in NSC-34 cells treated with the neurotoxin *L*-BMAA, *L*-BMAA+$PM_{0.1}$ or *L*-BMAA+ NP20. * $p < 0.05$ vs. control ; ** $p < 0.05$ vs. *L*-BMAA.

## 4.2. **UFPs effects *in vivo***

Several toxicological *C.elegans* endpoints were evaluated to understand whether the exposure to low level of UFPs in complex organisms was characterized by toxicity.

The effect of $PM_{0.1}$ and NP20 on OP50 growth was first investigated, to ensure that the effects observed in experiments were not due to an effect of UFPs on the bacterial lawn which worms feed onto (OP50 *E.coli*) (**Figure 12C**). Various dilution were tested and eventually the dilution of 1:100 from our stock solutions was chosen, to minimize the effects of UFPs on OP50 growth. In fact, although DMSO (only solvent), $PM_{0.1}$ and NP20- OP50 growth curves were statistically reduced compared to the control, there are no differences among them. Thus, the effect observed is only due to the solvent (DMSO, 1%).

Interestingly, a cellular accumulation of $PM_{0.1}$ was observed in the first tract of the intestine of a aged worm feeding onto a $PM_{0.1}$ treated plate, suggesting the absorption of particles through ingestion (**Figure 11**).

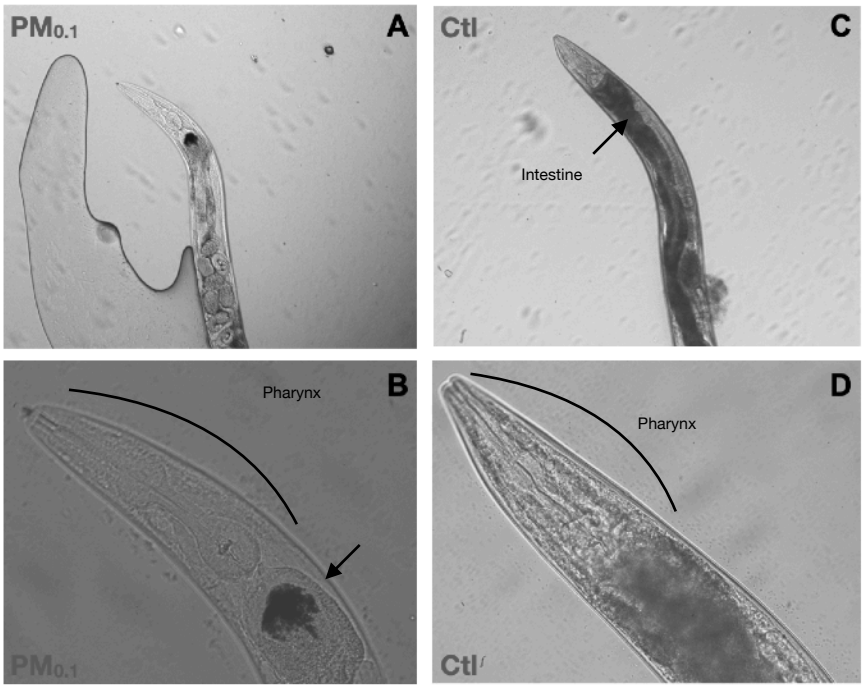



**Figure 11**. Brightfield images of *c.elegans* worms paralyzed with tetramisole hydrochloride (50mM). (**A**) aged worm fed on $PM_{0.1}$ treated plates, 10x magnification and (**C**) aged control worm fed on DMSO treated plates. (**B**) 40x magnification of A., the arrow indicates the accumulation of $PM_{0.1}$ in the intestine of the worm. Of note, the intestine appears less dark and less structured compared to the control, perhaps indicating an obstruction of the intestine. (**D**) 40x magnification of C, DMSO control.

Once this was done, the capacity of UFPs to affect worms lifespan and thus their survival, was investigated. Results indicate that both $PM_{0.1}$ and NP20 had an effect on survival, with a median survival of 18 days and 18 days for worms feeding onto plates treated with $PM_{0.1}$ or NP20, compared to control worms on DMSO-treated plates (median survival = 21 days) (**Figure 12A**).

Experiments on the reproductive aspect of *C.elegans* were subsequently conducted: as a first step, by determining the number of embryos produced (brood size), in hermaphrodites feeding onto bacterial lawns in plates treated with $PM_{0.1}$ or with NP20 (**Figure 12B**). In this case, only NP20 showed a significant effect, with a reduction of the number of larvae generated, compared to controls. Then, when assessing the body size of progeny fed onto treated plates, from their egg-hatch till their adulthood, there was a significant decrease in body size - in terms of area, in larvae in the DMSO, $PM_{0.1}$ and NP20 conditions at 48 hours, compared to controls (**Figure 12D**). However, DMSO adults reached sizes comparable to controls, after 72 hours, while worms in the $PM_{0.1}$ and NP20 conditions looked thinner and significantly differ from controls. Nevertheless, $PM_{0.1}$ worms did not differ statistically to DMSO worms. The size of NP20 worms were, on the contrary, significantly reduced compared to both controls and DMSO worms. NP20 were therefore able to impact the growth of worms, in terms of body size. It could be contested that this may be due to a delay in development, however, in a study of Haghani et al., (2019) *C.elegans* development seems not to be perturbed, although developmental exposure to UFPs does seem to reduce worm size. This is consistent with the experiments performed above, thus suggesting that the possibility of developmental delay should be ruled out.

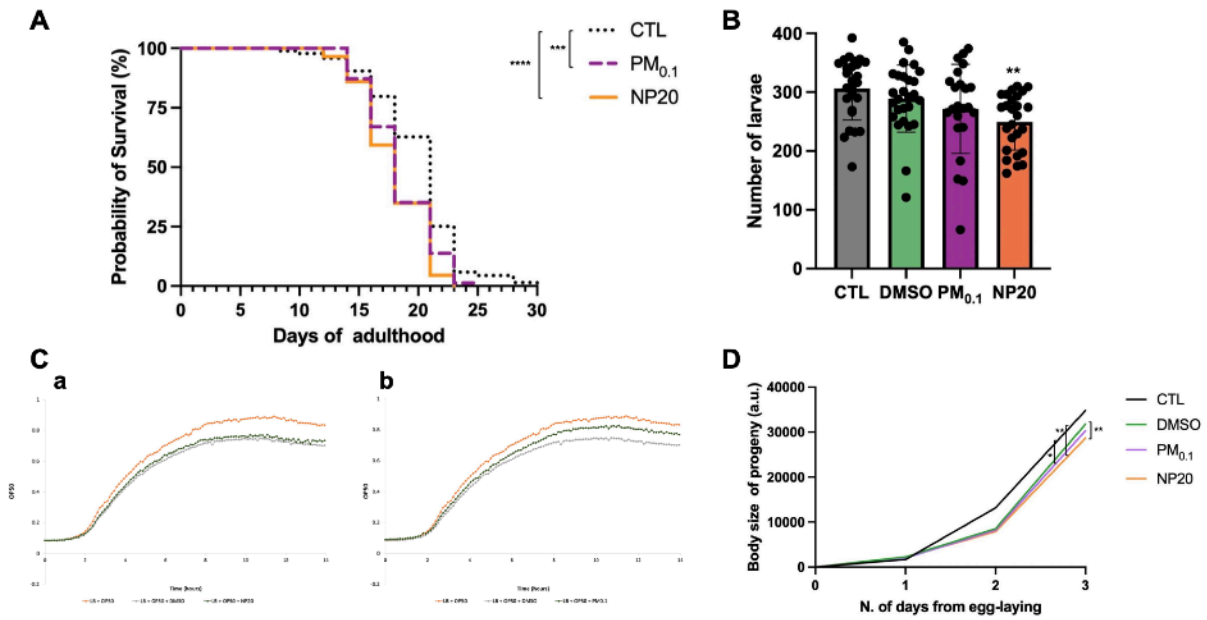



**Figure 12**. Toxicological endpoints measuring the effects of $PM_{0.1}$ and NP20 on *c.elegans* (100 ul/10000 ul of agar). (**A**) Representative survival plot of *c.elegans* worms treated with $PM_{0.1}$, NP20 and DMSO (vehicle-control) expressed in probability of survival per days of adulthood. Median survival were 21, 18 and 18 days for controls, $PM_{0.1}$ and NP20, respectively. Each experimental condition included approximately 100 worms. The experiment was repeated twice. *** $p$ value = 0.0006 ; **** $p$ value < 0.0001. (**B**) The brood size of worms under control, only vehicle (DMSO), $PM_{0.1}$, NP20 conditions was measured along the egg-laying period. Data of 3 different biological replicates (10 worms per condition in a single replicate) are represented. ** $p$ value < 0.005. (**C**) Representative OP50 E.coli growth curve showing in yellow the growth of bacteria in LB media, in grey the growth of bacteria in the presence of 1% DMSO (only vehicle) and in green the growth of OP50 bacteria in the presence of (a) 1% NP20 and (b) 1% $PM_{0.1}$. The experiment was repeated 3 times and data from all replicates show only a significant difference between control and the DMSO, $PM_{0.1}$ and NP20 conditions with a $p$ value < 0.05 in all conditions. (**D**) Body size of worms developed onto treated plates from egg-laying to adulthood measured each day for 3 days. Data are expressed as mean value of 3 different biological replicates under the same conditions. At day 2, worms in all conditions significantly differed from controls ($p$ value < 0.05). At day 3, only $PM_{0.1}$ and NP20 differed from controls (* $p$ value < 0.05 ; ** $p$ value < 0.01), moreover NP20 significantly differ from DMSO (** $p$ value < 0.01).

# Discussion

## 5.1. Discussion of results

The general purpose of the present doctoral study was to evaluate the toxicity driven by the finest fraction of particulate matter (UFP) in *in vitro* and *in vivo* models. As a matter of fact, particulate matter is currently the most pressing issue in air quality regulation worldwide due to its health-related burden (Fuzzi et al., 2015).

At this scope, UFP nanoparticles were produced in a lab-scale manner, to yield particles having high similarities in size and microscopic structure with particles emitted by diesel exhaust combustion (Mahamulkar et al., 2016; Meloni & Palma, 2020), therefore providing a model for anthropogenic diesel exhaust emissions. Specifically, the present work focused on the $PM_{0.1}$ and NP20 fractions, which were structurally characterized through high resolution/electron transmission microscopy.

Given the capacity of UFPs to get through human body barriers such as the placental and blood brain barrier (Oberdörster et al., 2004 ; Bovè et al., 2019), exposure to UFPs can, with all likelihood, lead to adverse health outcomes. Therefore, this doctoral study focused on finding the cellular mechanisms through which UFPs exert their detrimental effects on the nervous tissue, specifically on motor neurons - in terms of molecular processes. Secondly, it aimed at establishing such mechanisms as

pathological pathways shared with the neurodegenerative disease of ALS - a motor neuronal disease in which the role of environmental exposure has been extensively addressed.

The above *in vitro* aims where achieved through several experiments. To begin with, $PM_{0.1}$ and NP20 were showed to be swiftly up-taken by cells through endocytosis. The NP20 fraction was then able to affect the movement in space of motor neurons exposed to UFPs and to alter their electrophysiological properties.

Then, once UFPs got access to the cell, they were able to impair the functionality of several organelles, starting with the mitochondrion. Here, UFPs induced mitochondrial toxicity, which led to an alteration of $Ca^{2+}$ homeostasis and a bioenergetic failure. These events occurred early on - as ATP was already reduced at 6 hours. This mitochondrial toxicity culminated in a reduction of mitochondrial viability and a state of oxidative stress, characterized by an increased level of ROS.

Furthermore, this state of redox imbalance was sensed by the lysosomal $Ca^{2+}$ release channel TRPML1; which in turn resulted hyper-activated upon exposure to UFPs, contributing to a dysfunction of $Ca^{2+}$ homeostasis occurring in the lysosome. The TRPML1 channel is involved in lysosome adaptation mitochondrial damage (Zhang et al., 2016) and can promote autophagy through the activation of TFEB (Medina et al., 2015). Not surprisingly, autophagy was also affected upon UFPs exposure: its persistent activation - likely due, to a great extent, to the hyper-activation of TRPML1, was sufficient to determine an engulfment of its flux.

Autophagy is a neuroprotective pathway which can also be promoted by defects in mitochondrial bioenergetic functionality, through the activation of the AMPK pathway (Zhao et al., 2016b). Experiments showed that,

following UFPs exposure, AMPK expression was increased and this, together with an increased production of ROS, is in accordance with literature, which suggests that most of the mechanisms underlying environmental contaminants toxic effects, including coarser PM, are mediated by a ROS increase and AMPK activation (Orrenius et al., 2013).

Mitochondria and lysosomes were not the only organelles impaired: there are, indeed, close physical and functional interactions occurring between lysosomes and the ER (Kilpatrick et al., 2013). Of note, the ER $Ca^{2+}$ sensor STIM1 has been shown to be a key regulator of the lysosome/ ER coupling (Tedeschi et al., 2019b) and recent evidence has even found a physical interaction between STIM1 and the lysosomal DNA Damage Regulated Autophagy Modulator 1 (DRAM1) that tethers lysosomes to the ER (Wang et al., 2024)

Our experiments *in vitro* showed, indeed, a complete dysfunction of the ER $Ca^{2+}$ handling machinery - namely, the ER $Ca^{2+}$ replenishment mechanisms. In fact, the main actors of ER refilling were dysfunctional: STIM1 sensing the intraluminal ER $Ca^{2+}$ depletion and SERCA pumps in charge of the $Ca^{2+}$ replenishment. The ER was therefore characterized by a progressive ER $Ca^{2+}$ leak, following UFPs exposure.

The ER homeostasis was certainly compromised and, together with a disruption of ER morphology, this was able to lead to ER stress. Interestingly, new evidence show that there is an over-expression of the lysosomal protein DRAM1 triggered by mitochondrial stress, in models of neuromuscular diseases such as ALS. DRAM1 increased expression is thus able to disrupt the ER structure and trigger ER stress, in response to mitochondrial damage (Wang et al., 2024). Therefore, new studies targeting this protein may help to pin down pathomechanisms associated with UFPs exposure. Furthermore, a prolonged exposure to UFPs, was able to lead cells

to apoptosis through the activation of a cascade of caspases triggered by ER stress and, moreover, cell viability was greatly reduced.

Taken together, results *in vitro* show that the ultrafine fractions of PM examined in this research, namely $PM_{0.1}$ and NP20, exert neurotoxicity through several mechanisms targeting different cellular components and compartments: such as the plasma membrane, mitochondria, lysosomes, ER. UFPs were able to impair their functionality and eventually to have a detrimental effect on cellular homeostasis. Furthermore, the present study provided evidence that UFP-driven toxicity shared several pathomechanisms with neurodegenerative diseases, in particular with ALS.

Another important aim of this doctoral research was to explore the toxicity of UFPs in a complex organism and therefore, to characterize a toxicological *in vivo* model for UFP-toxicity (*C.elegans*). Results indicate that UFPs exert a toxic effect on *C.elegans*, reducing its lifespan. NP20 seemed to be the most detrimental fraction, as it was also able to impact *C.elegans* reproduction and body size of worms exposed to it, early on in their life.

Overall, the data collected indicate a clear UFP-driven toxicity at low dosages that affects both cellular models and organisms, with the smaller fraction within the UFP group - NP20, being more toxic than particles having an aerodynamic diameter < 100nm and > 20 nm. Notably, in light of the observation that NP20 effects in this study were underestimated, as the NP20 stock solution was less concentrated than $PM_{0.1}$ stock solution. This is in line with literature, which suggests that smaller particles are associated with greater effects (Schlesinger et al., 2006 ; Zhang et al., 2015).

**Figure 1**. Graphical representation of the effects of $PM_{0.1}$ and NP20 on mitochondria, lysosomes and autophagy.

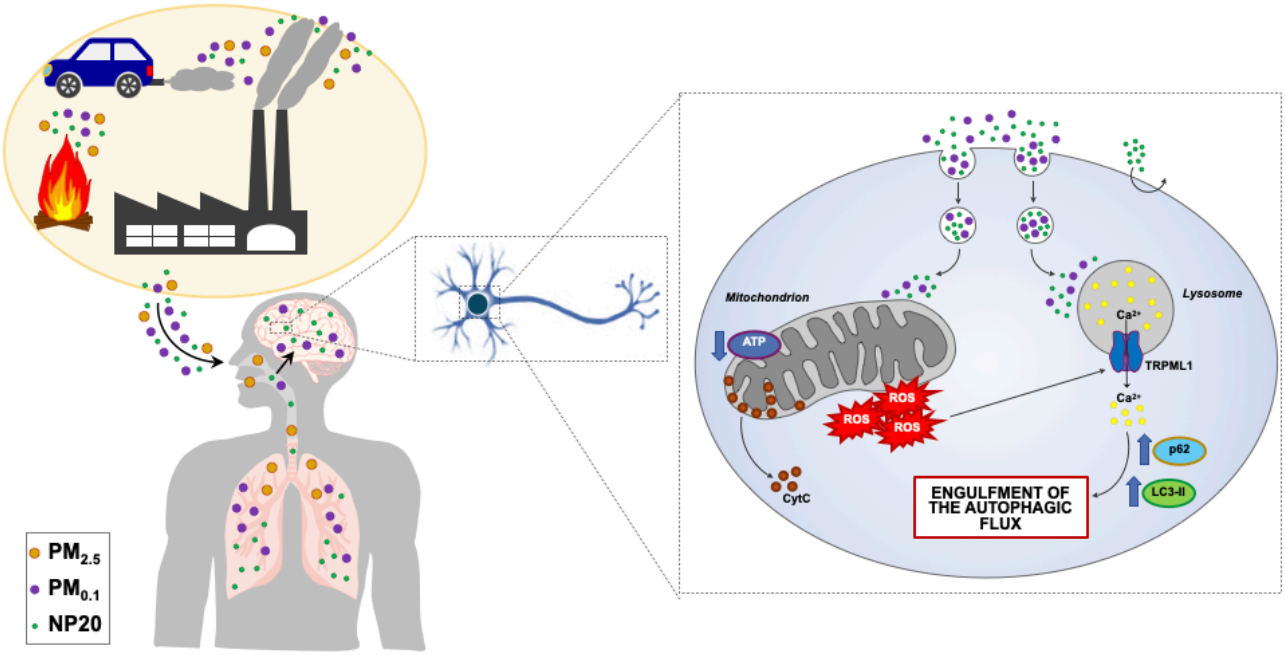
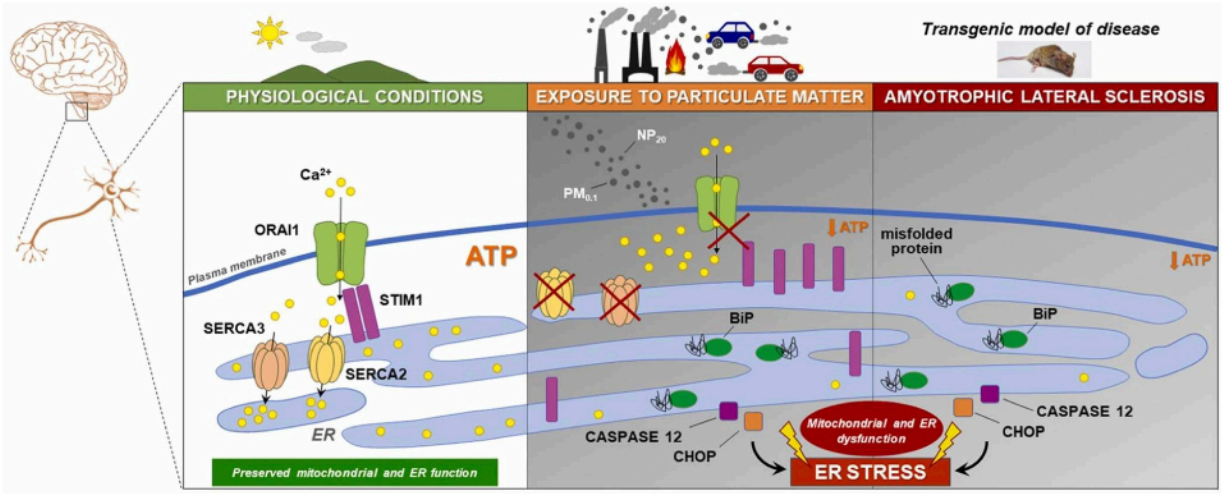



**Figure 2**. Graphical representation of the effects of $PM_{0.1}$ and NP20 inducing ER stress as a shared mechanism with ALS.



## 5.2. **Impact on society and future perspectives**

Air pollution has been recognized as the single biggest environmental threat to human health and well-being (WHO, Air Quality Guidelines, 2021) and indeed, is believed by many to be one of the greatest scourges of our century, for its impact on human life. Besides affecting our health and well-being, it has an active role in affecting the society and the economy of our world and, at larger scales, the environment and ecosystem - including climate.

Among all pollutants, particulate matter is a heterogeneous class made up of particles of different sizes, which has the infamous highest burden of disease worldwide. Despite this, not all PM particles are regulated, nor even monitored: this is the case of aerosols with an aerodynamic diameter of 0.1 μm or less ($PM_{0.1}$/UFP), which represent the dominant fraction of aerosols dispersed into the air (Hofman et al., 2016). In urban environments the leading source of UFPs emission is mainly due to vehicles exhaust and traffic-related activities (Harrison et al., 2011; Kwon et al., 2020). Importantly, UFPs real concentration in everyday exposure is unknown - although it is estimated to be way higher than we are aware of. Moreover, exposure to UFPs will massively increase in the next decades - to a great extent, due to the advent of new technologies (e.g.: e-cigarettes, new coal combustion technologies, modern technology fossil fuel-burning power stations etc.), which were shown to increase UFP emissions, despite the fact that some of these technologies were developed in an attempt to reduce the release of other pollutants/coarser particles.

UFP emissions should therefore be finely regulated and should represent a pressing issue of public concern. $PM_{0.1}$ and its sub-fractions are considered to be more dangerous than larger particles, as they are able to

cross all human barriers and reach all human district, besides having a strong impact on climate. Of note, recent extreme drought and flooding event may be in part driven by the recent increase in UFP emissions (Kwon et al., 2020). Current regulations and guidelines, nevertheless, primarily focus on fine PM ($PM_{2.5}$), leaving out ultrafine particles (UFPs) - particularly particles < 20 nm, which were extensively studied and showed to have greater toxic effects, in the present manuscript.

This doctoral study is therefore of high health-relevance, as it contributes to provide the basis, through the use of *in vivo* and *in vitro* exposure models, for studying and exploring the mechanisms and the effects of UFPs, still largely obscure. Furthermore, it provides evidence for smaller sub-fractions of UFPs to be more toxic than larger UFPs, in virtue of their size and to shed light on the cellular mechanisms by which UFPs exert their effects.

Thus, these results suggest a revising of current European regulations on exhaust particle emission - particularly on the cutoff size of 23 nm, as this should be further lowered.

Yet, research on UFPs is still in its infancy and more and more studies are needed to address several questions which remain unanswered, such as a more comprehensive investigation including condensable and solid UFPs, or experiments addressing long-term effects and even trans-generational effects of UFPs on human health.

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