Universitat Autònoma de Barcelona

New end-points to assess nanomaterials exposure effects

Sandra Ballesteros Ribera

Universitat Autònoma de Barcelona

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Dissertation respectfully submitted by

Sandra Ballesteros Ribera

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Under the supervision of Dra. Alba Hernández Bonilla and Dr. Ricard Marcos Dauder

Dra. Alba Hernández Bonilla	**Dr. Ricard Marcos Dauder**	**Sandra Ballesteros Ribera**

ABSTRACT

Nanomaterials (NMs) are considered emerging pollutants that are increasingly detected in different environmental matrices, with potential risks for human health and the ecosystems. In this sense, the focus of this Thesis has been directed to provide new approach methodologies for hazard assessment of NMs via advanced *in vitro* and *ex vivo* models, as well as novel biomarkers.

From our first study, a novel approach was developed to understand the risk of polystyrene nanoparticles (PSNPs) exposure for humans, as a model of micro-nanoplastics (MNPLs). Thus, *ex vivo* whole blood samples from 5 donors were exposed to several doses of PSNPLs and different end-points were evaluated in diverse subsets of white peripheral blood cells (WBCs). The results showed sharp differences in PSNPLs internalization with very limited uptake in lymphocytes and high uptake in monocytes. Moreover, the genotoxic DNA damage evaluation revealed a specific cellular sensitivity, being polymorphonuclear cells (PMNs), and monocytes those cells with the most significant levels of genotoxic damage. Additionally, PSNPLs exposure triggered changes in the whole blood secretome, with a significant increase in the expression of cytokines related to the inflammatory, immune, and stress response, as well as cell proliferation.

In the second study, the before-mentioned whole blood *ex vivo* model was used to evaluate the impact of three different graphene-based nanomaterials (GBNMs) at the level of the blood secretome. For that purpose, a large panel of cytokines was analysed, and the results showed important cytokine expression changes, most of them related with the immune and inflammatory response. At the same time, the indirect soft-agar assay, was used to unravel the functional consequences of these cytokine changes. The results showed that the GBNMs-altered secretome can inhibit the anchorage-independent cell growth capacity of HeLa cells, used as a model cell-line.

In the third study, the cell-transforming properties of nanoceria were confirmed through a long-term low-dose *in vitro* model. Stem-like properties, anchorage-independent growth, and invasion abilities were analysed as they are considered important oncogenic features driven by NMs exposure. Also, their potential interactions with cigarette smoke condensate (CSC), as a model of environmental carcinogenic pollutant were confirmed, showing a positive interaction in the induction of cell transformation. Besides, a battery of microRNAs related to the acquisition of the tumoral phenotype was assessed, revealing that cerium dioxide nanoparticles (CeO_2NPs) and the co-exposure produced potential toxicity at the transcriptome level.

Finally, our fourth study evaluated the potential epigenetic consequences of long-term exposure to titanium nanoparticles (TiO_2NPs) and multi-walled carbon nanotubes (MWCNT), specifically the microRNAs expression changes. The analysed microRNA battery revealed a big impact on the expression profiling in cells exposed to both nanomaterials. Moreover, from our initial battery, a small set of five microRNAs were selected as potential biomarkers of effect after NMs' exposures. This set was tested in BEAS-2B and MEF cells previously long-term exposed to different NMs, showing positive effects in all the tested samples, confirming the suitability of this battery.

ABBREVIATIONS LIST

AgNPs	Silver nanoparticles
CAFs	Cancer-associated fibroblasts
CeO_2NPs	Cerium dioxide nanoparticles
CSCs	Cancer stem cells
CSC	Cigarette smoke condensate
CM	Conditioned media
CoNPs	Cobalt nanoparticles
CTAs	Carcinogenic transforming assays
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EPs	Emerging pollutants
GBNM	Graphene-based nanomaterial
GO	Graphene oxide
GNPs	Graphene nanoplatelets
GNRs	Graphene nanoribbons
HBM	Human biomonitoring studies
MEF	Mouse embryonic fibroblast
MNPLs	Micro/nanoplastics
MOAs	Mechanisms/modes of action
MSiNPs	Mesoporous silica nanoparticle
MWCNTs	Multi-walled carbon nanotubes

NAMs	New approach methodologies
NMs	Nanomaterials
NPs	Nanoparticles
PC	Protein corona
PS	Polystyrene
PSNPLs	Polystyrene nanoplastics
PBMC	Peripheral blood mononuclear cell
ROS	Reactive oxygen species
TiO_2NPs	Titanium dioxide nanoparticles
ZnONPs	Zinc oxide nanoparticles

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1. INTRODUCTION

1. INTRODUCTION

1.1. EMERGING POLLUTANTS

Environmental pollution has become one of the most important challenges to combat nowadays. Diverse anthropogenic activities (industry, urbanization, transport, agriculture, health care activities, …) have led to the degradation of the environmental quality, entailing risks for flora, fauna, and humans (Vasilachi et al., 2021). In this context, it has been identified the presence of an increasing number of pollutants and their derived by-products, which are still not widely monitored in the environment, named emerging pollutants (EPs) (Geissen et al., 2015). They are raising the level of concern because their occurrence, fate, behaviour, adverse ecotoxicological and health effects are often poorly understood. This lack of scientific knowledge about their potentially harmful effects makes it impossible to carry out a risk evaluation, necessary to establish regulatory measures. As a result, human beings are at risk of suffering health problems derived from the exposure to emerging pollutants, coming in contact daily with them through the air, water, or food, among others.

EPs can be classified into a few general categories (Figure 1): pharmaceutical products (antibiotics, analgesics, anti-inflammatory drugs, hormones, etc.), personal care products (sunscreen agents, cosmetics, hair dyes, shampoos, body lotions, etc.), pesticides (insect repellents, herbicides, etc.), disinfection by-products (antiseptics, surfactants, etc.), industrial products (flame retardants, industrial additives and chemicals, gasoline additives, manufactured nanomaterials, etc.) (Ebele et al., 2017; Vasilachi et al., 2021). Although many of these substances may only exist in the environment at very low concentrations, they accumulate into the human body, the environmental compartments, and ecosystems generating new questions about long-term and low-level exposure associated risks.

1.2. NANOPARTICLES AS EMERGING POLLUTANTS AND THEIR POTENTIAL HEALTH EFFECTS

Among the different emerging pollutants, nanoparticles (NPs) are increasingly present in the different environmental matrices due to anthropogenic sources (Oberdorster et al., 2005). Thereby, the rapid development of NPs' industry and commercialization has raised concerns about their potentially harmful effects on human health (Seaton et al., 2010). Epidemiological studies have shown a strong correlation between exposure to NPs and the increased incidence of cardiovascular, pulmonary, or neurodegenerative diseases (Lu et al., 2014; Mushtaq et al., 2015; Kan et al., 2018). Other studies have reported some

evidence of their toxicity. For instance, the study of Ng et al. (2017) reported that zinc oxide nanoparticles (ZnONPs) induced significant oxidative stress-related cytotoxicity and genotoxicity in human lung fibroblasts both *in vitro* and in *Drosophila melanogaster in vivo* studies. However, there are several factors still unknown that need to be evaluated to predict the possible health risks (routes of exposure, NPs' changes once they enter inside the body, NPs' interaction with other biological systems, the magnitude and duration of the exposure, their persistence, the inherent toxicity, and the susceptibility or health status). To detect all these uncertainties, nanotoxicology has been developed as a new field of study that claims to implement the safety evaluation of engineered NPs to detect possible hazards derived from the exposure (Buzea et al., 2007; Subhashini et al., 2017).

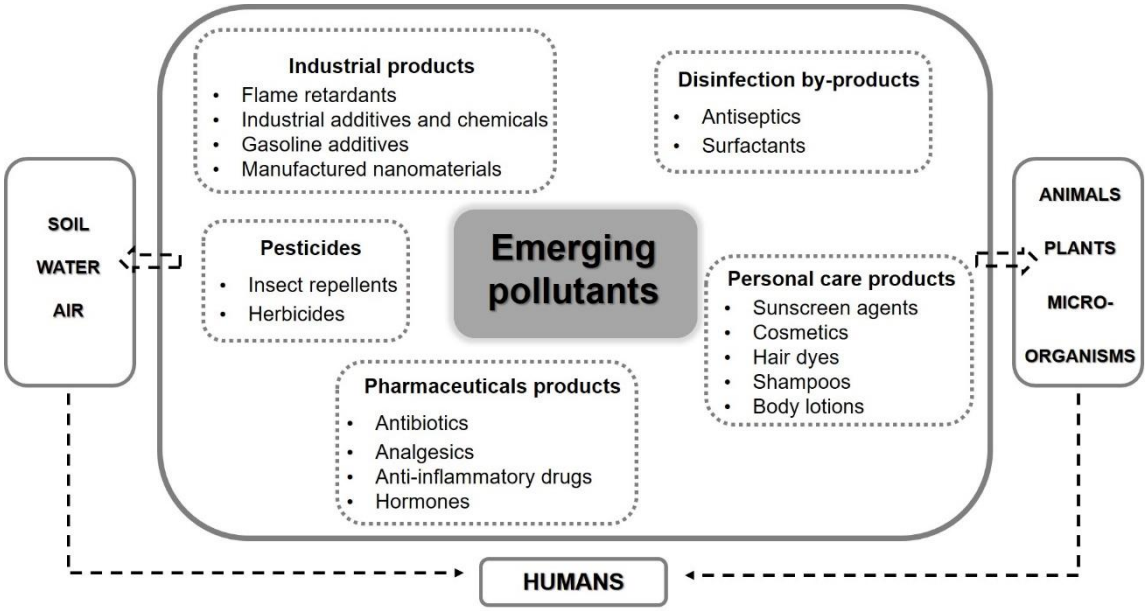

Figure 1. Categories of EPs present in different environmental compartments (soil, water, and air) that impact on animals, plants, microorganisms, and humans. (Adapted from Vasilachi et al., 2021).

1.3. POTENTIAL HUMAN EXPOSURE TO NANOMATERIALS

The potential human exposure to nanomaterials (NMs) as products based on NPs, can be classified into three categories: occupational exposure, environmental exposure, and consumer's exposure. The most explored one has been occupational exposure because workers are expected to be exposed to the highest NMs doses. This exposure mainly results from the research, synthesis, commercial production, packaging, and transport of NM-based products. One advantage of these environments is that their release, exposure, and worker safety measures can be almost fully controlled. On the other hand, although not in the same quantities, consumers are also exposed to NPs. Various products daily consumed by the general population, including health supplements, cosmetics, sunscreens, clothing, electronics, and spray cleaners, contain NPs. These products can

be ingested, absorbed through the skin, or inhaled, increasing the potential exposure to NPs (Kuhlbusch et al., 2018). However, the most challenging part is to control the environmental exposure of the general population. Vehicular pollution is the primary source of atmospheric NPs in urban areas, but also indoor activities such as garment washing or dusting, release huge quantities of NPs to the atmosphere. Unfortunately, information on the full extent and consequences of NPs exposure are not yet available and therefore it is certainly important to carry out a precise scrutinization to evaluate a risks-benefits balance of the exposure (Nowack et al., 2017).

1.4. NANOMATERIALS BODY ENTRY ROUTES

There are different routes of exposure to NMs and, in this sense, it is important to know the NM's exposure route since its biological effect will partly depend on the entry route into the human body (Meng et al., 2018). In general, the main ways to enter into the human body are the skin (dermal uptake), the lungs (inhalation uptake), and the gastrointestinal barrier (oral uptake) (Figure 2).

Firstly, the skin and the mucose form the main external barriers which are covered by epithelia to prevent the penetration of foreign substances. These layers have low permeability thanks to the epithelial cells that form intercellular junctions, cellular differentiation, and mucus secretion. However, some NPs can penetrate the outer stratum corneum layer of the skin, while others can permeate into the deeper dermal layer, reaching the blood vessels located in the connective tissue. Once there, they are distributed through blood, interacting and accumulating in different organs, such as the liver, kidney, or even the brain, disrupting their normal activity in most cases (Yildirimer et al., 2011; Raftis and Miller, 2019).

Secondly, inhalation is an extended route of unintentional exposure to pollutants among which NMs are emergent particulate air pollutants. The shape, size, and chemical composition of NPs will greatly vary their ability to penetrate and deposit throughout the respiratory tract. Lungs can be the first target organ to be affected by NPs and they can even also be the gateway for the systemic distribution of NPs in the body. At the level of bronchioles, NPs accumulate in the *interstitium* where they can reside for a long time, as has been shown in the lung parenchyma of women living in polluted cities (Churg et al., 2003). Further, at the level of alveoli, where the distance to capillaries is shorter, NPs cross the air-blood barrier to reach systemic circulation and secondary organs (Puisney et al., 2018). For instance, the study of Miller et al. pointed out that up to three months after their inhalation, human volunteers had still traces of gold NPs in blood and urine (Miller et al., 2017).

Thirdly, the gastrointestinal barrier also constitutes one of the major routes for NMs exposure. It has a considerable surface area for potential interactions with ingested NPs. Translocation of particles through the intestinal barrier involves their diffusion through the mucus layer, contact with enterocytes and/or M-cells, and uptake following different pathways, where the most described is endocytosis through epithelial cells (Bergin et al., 2013). NPs translocation may lead to the deposition of NPs in the intestinal cells, inducing chronic inflammation and triggering inflammatory diseases such as Crohn's disease, or they may get into the bloodstream and be deposited in other organs of the body (Riasat et al., 2016).

In addition to the routes of exposure mentioned above, the parenteral route is a novel route of exposure used for a range of therapeutic (e.g., gene therapy or drug delivery) and diagnostic (e.g., contrast agents in magnetic resonance imaging (MRI)) applications in the biomedical field (Figure 2). Depending on the fate and the target, there are different routes of NMs administration: intravenous injection, intratumorally and subcutaneous injection, dermal, nasal, retinal, inhalation, and oral (Bourquin et al., 2018). When NPs are intravenously injected, they first encounter the blood, generating a complex system of biological interactions between blood and NPs. These interactions with blood plasma proteins contribute to induce changes in NPs' properties, which may lead to functional variations.

In general, regarding the risk of a certain compound, a fundamental factor is the number of people who are exposed to that compound. In the case, for example, of inhalation and/or ingestion, the number of people exposed is much greater than those who are exposed through the parenteral route and, therefore, this route would carry much less risk. Nevertheless, in individual terms, the risk for people treated by the parenteral route is very high, since they are exposed to higher amounts of the compound because there is not a prior "filter barrier". When NPs are injected into the blood, the concentrations will most likely be different from the concentrations of NPS that have been ingested, inhaled, or absorbed, precisely because when crossing the barrier there is a process of "clearance" so that the concentration reaching the blood becomes much lower than the original. Thereby, the understanding of the NMs' biological effects in the biomedical field is an urgent area of examination to ensure safety in their use.

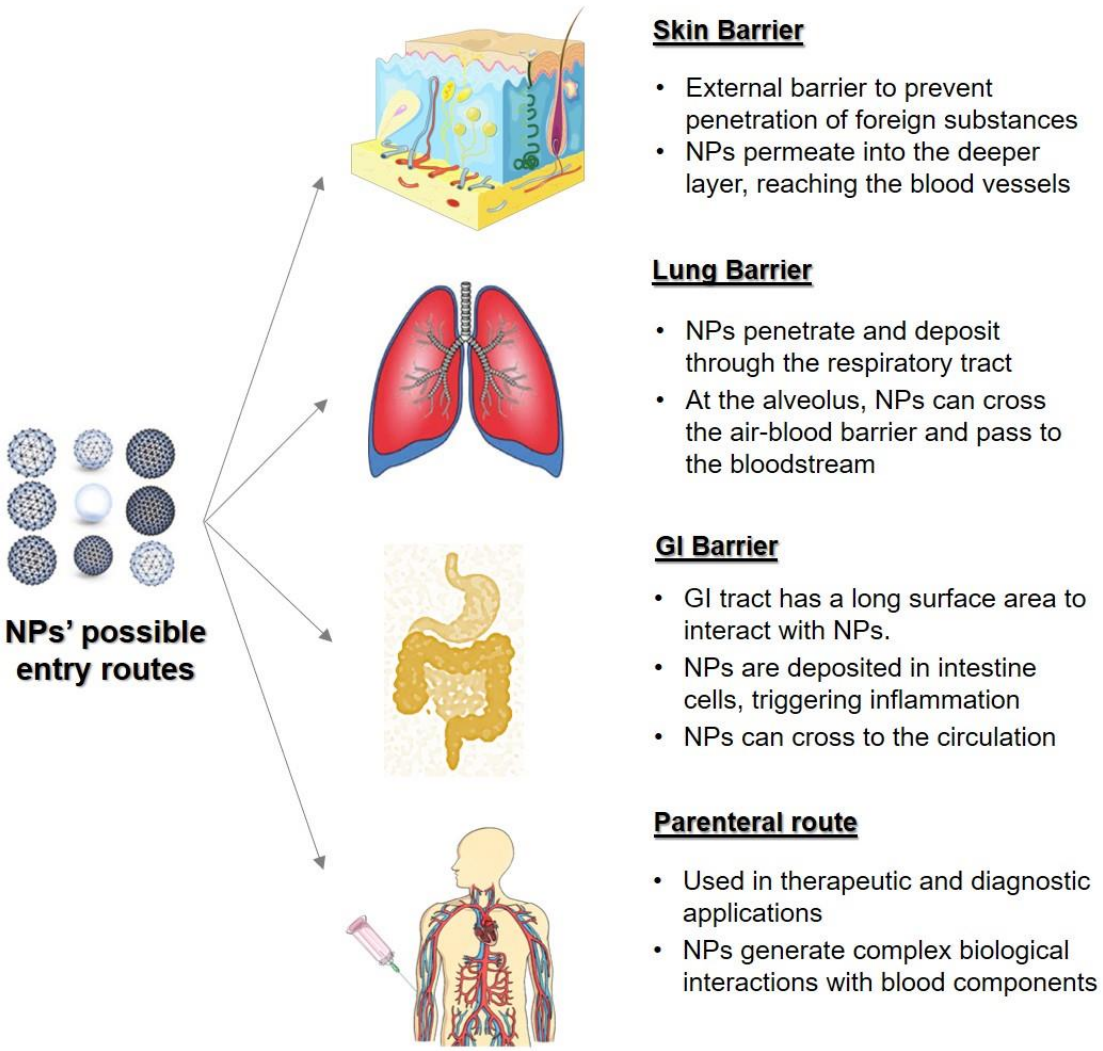

Figure 2. Main ways of entry into the human body. (Adapted from Naseer et al., 2018).

1.5. EXPERIMENTAL APPROACHES

To assess the exposure risk associated with NMs, it is necessary to have tools that give us reliable information on the consequences of this exposure. Today, the best tool available for risk assessment studies is human biomonitoring (HBM) studies (Figure 3). This is an important tool to generate human exposure estimates after identification, measurement, and characterization of NPs present in the air, water, soil, or other sources such as food, as well as in human biological samples of human groups with potentially high exposure levels. However, the complexity of these HBM studies, and mainly the difficulty to establish/quantify exposure levels, give rise to alternatives that complement this approach. In this sense, *in vivo* studies are the gold standard alternative closer to HBM (Figure 3). They give us a more complete vision of most systemic interactions (absorption, distribution, metabolism, and excretion). However, these studies exhibit certain limitations mainly due to animal ethical questions, but also due to physiological and

biochemical species dissimilarities that sometimes make conflictive the extrapolation of the results. Especially for carcinogenesis assays, the application of the 3Rs principle (Replacement, Reduction, and Refinement) is demanded, since these analyses require many animals, high costs, prolonged duration and shows several limitations, which can affect the understanding of the human carcinogenesis. In this context, the development of alternative methods is a priority in the context of regulatory toxicology (Annys et al., 2014).

1.5.1. *Ex vivo* studies

Ex vivo systems are a promising alternative approach to *in vivo* studies since they provide an almost similar *in vivo*-like environment (Figure 3). This method refers to experimentation done in or on tissues obtained from an organism and maintained under optimum conditions mimicking the natural condition. The classical *in vitro* culture system of cell lines is far from recapitulating the heterogeneity of the microenvironment in a disease. Instead, *ex vivo* is developed under controlled conditions and allows evaluations that would not be possible to carry out in humans or live animals, due to the ethical problems they would entail.

In this scenario, this Thesis dissertation proposes an *ex vivo* model using human blood to represent the biological interactions of NMs with human cells. Cells from peripheral blood are easily accessible from donors and they are the first target when talk of the intravenous injection of NMs used for diagnostic or therapeutic purposes. Nevertheless, they can also be affected when the agents arrive by other routes of exposure, for example crossing intestinal or lung barriers (Dusinska et al., 2017). Previous works showed that assays in more complex systems such as whole human blood are more reliable than *in vitro* studies and reflect more accurately the fate of NPs when injected into human blood *in vivo* (Patel et al., 2014). In addition, this *ex vivo model* enables the study of other blood components, such as cytokines, proteins, and other factors, that must be considered when analysing the overall response. However, some challenges to be encompassed are the high inter-individual differences between donors and the short survival time in culture (Mann et al., 2016).

1.5.2. *In vitro* studies

In vitro tests are fast, cost-effective, and enable high-throughput screening (HTS) assays on relevant cells from humans and other mammals (Figure 3). Moreover, when testing NMs, *in vitro* assays can be performed in a more controlled manner taking the NMs' physicochemical characteristics and the cellular uptake into account.

When evaluating the effects of a certain compound through the *in vitro* approach, it should mimic as much as possible what happens *in vivo*. Thereby, an important consideration is that the assessment is carried out on a tissue or cell line representative of the type of exposure target, so the primary route of entry or the assumed target will determine the election. In this Thesis, the effects of different compounds have been evaluated *in vitro* considering their possible potential targets. In the case of cerium dioxide NPs (CeO_2NPs), multi-walled carbon nanotubes (MWCNTs), and titanium dioxide NPs (TiO_2NPs), inhalation represents one of the most likely routes of human exposure (Noel et al., 2015; Schwotzer et al., 2017; Ihrie et al., 2019). Once inhaled, the smallest size NPs will travel along the respiratory tract and penetrate deeply into the alveolar region of the lungs, encountering the alveolar epithelium (Bakand et al., 2012). This epithelium is a primary target for inhaled toxic substances, and hence a focus in inhalation toxicology. In this sense, BEAS-2B is among the most widely used immortalized cell lines for *in vitro* inhalation toxicology. They are generated by the immortalization of human bronchial epithelial cells from a non-cancerous individual (Hiemstra et al., 2018). These cells resemble airway basal epithelial cells and are considered a good bronchial cell line model with similar characteristics and responses to carcinogens as the primary lung cells (Liao et al., 2007; Sargent et al., 2009). They have been used in a variety of studies to evaluate the exposure to different NPs proving that it is a suitable model to be used in our experiments (Eom et al., 2009; Heng et al., 2010; Biola-Clier et al., 2017).

1.5.3. Long-term studies

In addition, to evaluating the correct target, another important consideration is the exposure scenario recreated in the *in vitro* model. Historically, most research is focused on dose/response studies using high concentrations and acute exposures of the tested compounds. But, although short-term studies allow us to obtain results more immediately, they may not reflect concentrations and times expected at more realistic exposure scenarios (Stone et al., 2017). Therefore, emphasis should be directed towards these chronic and low-dose exposures to understand NMs associated risks.

Long-term studies make it possible to evaluate effects such as cell transformation that in short-term studies are very difficult to observe. In this context, several researchers have studied the carcinogenic potential of NMs through *in vitro* long-term exposure models. As a representative example, Wang et al. (2011) demonstrated that p53 overexpression, encouraged by the chronic exposure to carbon nanotubes, induces malignant transformation in BEAS-2B cells. Likewise, in our Group several NPs have been assessed from the carcinogenic point of view through long-term *in vitro* studies. Barguilla

et al. (2020) reported the role of MTH1 in the ZnONPs and CoNPs-induced toxicity and carcinogenicity. These two previous studies confirm the usefulness of long-term studies to unravel the mechanisms involved in the carcinogenic potential of NMs.

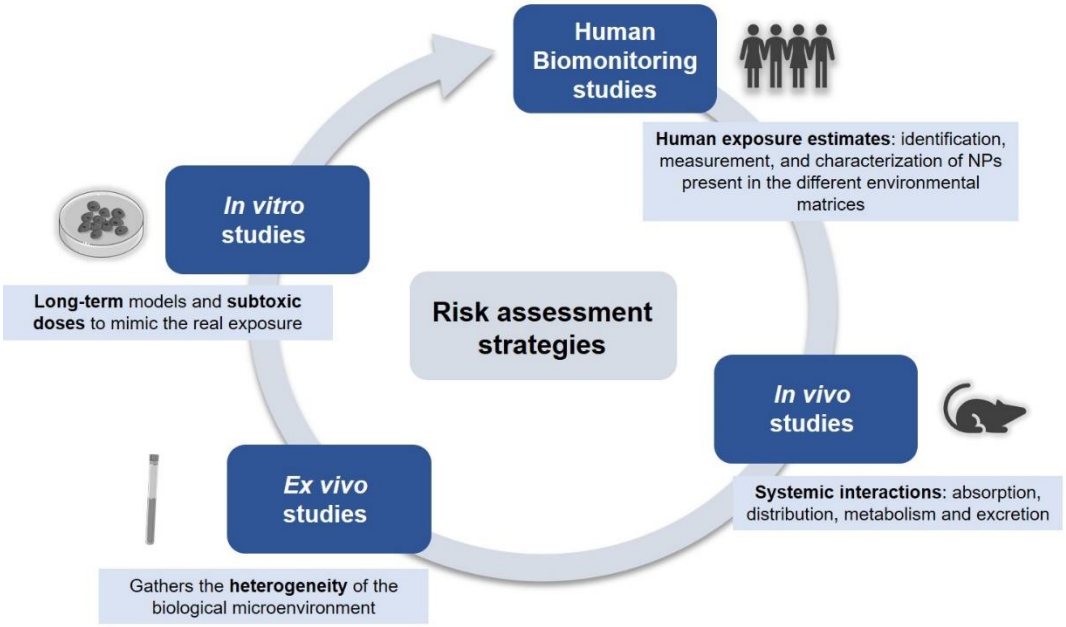

Figure 3. Risk assessment strategies. Different approaches (HBM, *in vivo, ex vivo, and in vitro* studies) to have a complete vision of human NPs' exposure-related effects and their associated molecular mechanisms of action.

1.6. END-POINTS' CLASSIFICATION BASED ON EXPOSURE-RELATED EFFECTS

The exposure risk associated with a certain compound has been traditionally studied with laboratory animals. However, non-animal testing methods are increasingly considered. Among the different *in vitro* techniques, some of them explore different general effects such as genotoxicity or reactive oxygen species (ROS) induction that have been classically studied. Nevertheless, the current situation requires new models aimed to facilitate the parallel process between the NPs innovation industry and the safety assessment. In this context, New Approach Methodologies (NAMs) is an emerging field aiming to improve the understanding of NMs threats and, simultaneously, provide safety assessments for the vast number of NMs both in use and in development (Nymark et al., 2020).

As a part of these NAMs approach, in this Thesis dissertation more novel and specific aspects are evaluated. Specifically, the focus has been directed towards the analysis of the immune system through the evaluation of cytokine expression levels, the effects on the epigenome, through the analysis of a battery of microRNAs, and, finally, a

battery of assays to evaluate the possible long-term carcinogenic effects of NPs have also been addressed.

1.6.1. Effects on DNA: Genotoxicity

Diverse past and ongoing European projects, such as NanoTETS, NanoGENOTOX, NanoReg, HISENTS, and RiskGONE, have made efforts to develop or adapt techniques addressed to measure the genotoxicity of an agent, especially concerning the NMs genotoxic potential (Creton et al., 2012). Among them, the most widely used method to measure DNA damage is the comet assay (Collins et al., 2017). The basic comet assay detects single- and double-stranded DNA breaks in naked supercoiled DNA. Strand breaks cause the supercoiled DNA relaxation, allowing loops of DNA to extend under the electrophoretic field and move towards the anode, resembling a “comet tail” (Figure 4). In addition, a modified version of the classical comet assay uses lesion-specific enzymes, such as formamidopyrimidine DNA glycosylase (Fpg), to detect the presence of oxidative damage in DNA bases. This enzyme is incorporated after the lysis step and removes damaged bases, leaving an apurinic site that is converted into a break by the endonuclease.

Regarding NPs’ genotoxic potential, several *in vitro* studies from our Group have reported NPs genotoxicity. For instance, TiO_2NPs induced significant increases in DNA damage in the human embryonic kidney (HEK293) and mouse embryonic fibroblast (NIH/3T3) cells (Demir et al., 2015). The present dissertation uses the comet assay using cells cultured following a novel *ex vivo* approach.

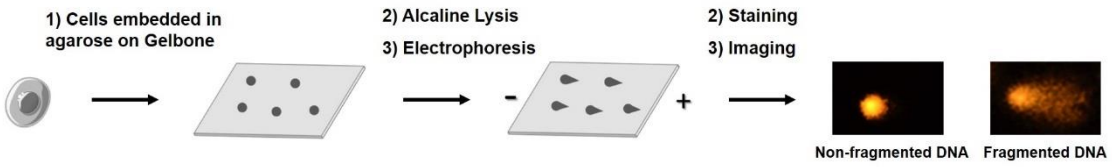

Figure 4. DNA damage detected through the comet assay. Cells are mixed with agarose and dropped on a GelBond film. Then these cells are lysed overnight and placed under the electrophoretic field. Finally, cells are staining and visualized on a fluorescent microscope where cells with non-fragmented DNA and cells with fragmented DNA are distinguished.

1.6.2. Effects on the immune system: cytokine expression changes

Some NPs are intentionally designed to exert an immunomodulatory effect while others, not engineered to such function, end up interacting with the immune system, often triggering an immunotoxic response. When NPs are in contact with blood, they may be

recognized by elements of the immune system. This interaction can be direct, inducing even cellular apoptosis and necrosis, or indirect, affecting immune-specific signaling pathways (Dusinska et al., 2017). All these imbalances can be measured by the expression of surface immune cell markers, cytokines, cell differentiation, and immune activation (Pandey et al., 2018).

Cytokines are released by the immune cells in response to foreign particles considered threats. Macrophages produce most cytokines, which will recruit other cells of the immune system and will exhibit pro- or anti-inflammatory effects (Da Cunha et al., 2019). Thus, the levels of inflammatory cytokines can serve as a biomarker of NP`s immunotoxicity. Several studies have addressed high levels of cytokines after NPs exposure. Thus, Wolf-Grosse et al. (2017) reported high levels of proinflammatory cytokines after exposure to iron oxide NPs. In that study, they used a human whole blood *ex vivo* system to test the potential inflammatory effects derived from NPs exposure. Previous studies have also used this model considering it as an appropriate model to test NPs impact (Orecchioni et al., 2016; Orecchioni et al., 2017; Potter et al., 2018). In this Thesis, the same system was used to characterize the effects of two widely used NMs at the cytokine's level.

1.6.3. Effects on the epigenome: microRNAs

As mentioned above, the classical studies of nanotoxicology were focused on cytotoxic and genotoxic effects, but more recently, attention has been paid to epigenetic changes induced by NPs (Sierra et al., 2016). Epigenetic responses have been suggested as a novel biomarker of exposure or disease risk to NMs. DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) are the most prevalent novel epigenetic end-points in the toxicity testing of NMs. Among them, microRNAs have been the most described ones (Balansky et al., 2013; Sun et al., 2015).

MicroRNAs are single-strand RNAs that are 20-24 nucleotides in length whose main function is posttranscriptional regulation of gene expression (Ha and Kim, 2014). By enacting gene silencing through mRNA degradation or inhibiting mRNA translation into proteins (Figure 5), microRNAs act as key regulators in many biological processes including proliferation, development, differentiation, apoptosis, and metabolism (Wei et al., 2017). Moreover, they also control important processes in cancer metastasis such as epithelial-to-mesenchymal transition process (EMT). In this process, microRNAs modulate different signaling pathways by which epithelial cells undergo conversion to mesenchymal state, acquiring migratory and invasive abilities that enable them to spread to other organs (Legras et al., 2017).

Changes in microRNAs' expression have been related to numerous pathologies. Thus, this differential expression profiling has emerged as a powerful tool to be used as biomarkers in disease diagnosis and progression, as well as prognosis and response to treatment (Lan et al., 2015). However, all these expression changes are difficult to analyze because of their specificity at the level of species, tissues, cells, exposure and times, and to the complex biological environment that involves them. Furthermore, it must be considered that a particular microRNA can control the transcription of many target genes and, therefore, it can present variations in their expression behavior. Thus, it may be found overexpressed in some cancer types, acting as an oncogene, while downregulated in other cancers, acting as a tumor suppressor gene (Babashah et al., 2011).

Numerous nanotoxicological studies have demonstrated the relationship between exposure to NMs and changes in microRNA expression. For instance, the study of Hu et al. (2020) evidenced the biomarker role of miR-155-50p in the polystyrene nanoplastics (PSNPLs) and nano-TiO_2-ARS induced toxicity in a monocytic human cell line. Another similar study identified changes in the microRNAs expression after the treatment with AgNPs, AuNPs, and uperparamagnetic iron oxide NPs in HepG2 cells (Brzóska et al., 2019). Nevertheless, these studies have some limitations, since there is a lack of long-term low-dose studies of NMs exposure to better mimic daily human exposure, together with the need for mechanistic links associating the molecular responses with the functional effects at the cellular level.

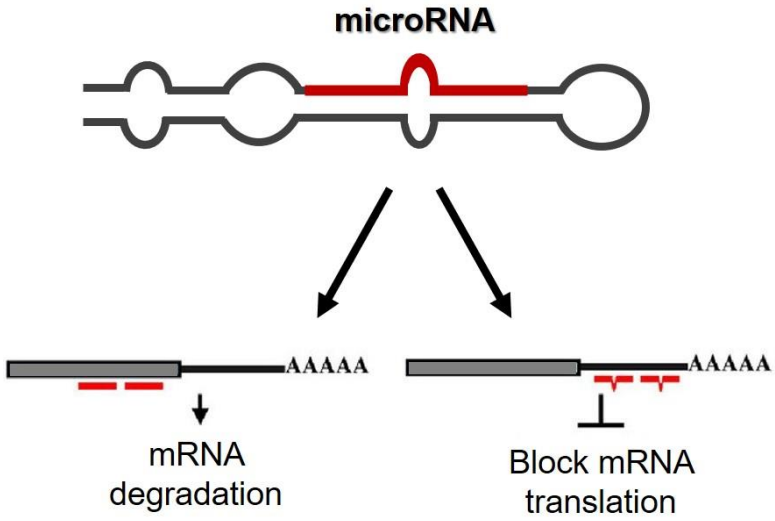

Figure 5. MicroRNAs regulate post-transcriptional gene expression by mRNA degradation or blocking mRNA translation (Adapted from Vella, 2005).

1.6.4. Long-term associated carcinogenic effects

Classical proliferative and morphological changes, genotoxicity markers, and novel makers such as cytokines or microRNAs appear from the earliest stages of exposure and therefore, they can be measured both in the short and long-term scenarios. However, some types of markers are only a consequence of long-term exposure, such as markers of the cell transformation effect. During *in vitro* cell transformation, a series of alterations like those *in vivo* occurs. All these alterations are associated with the neoplasia process, in which cells pass through different stages -early, advanced, or aggressive stages- (Figure 6). Each of them is characterized by a series of cellular phenotypic manifestations, which can be analysed in *in vitro* systems and help us to distinguish between tumour and non-tumour cells.

In this context, cell transformation assays (CTAs) have been proposed as *in vitro* methods for the identification of several hallmarks of cancer, associated with carcinogenic-NPs exposure. These assays can complement *in vitro* genotoxicity tests*,* identifying non-genotoxic carcinogens. However, they are still not accepted for regulatory purposes, since they present certain limitations such as the subjective nature of using morphological criteria for identifying transformed cells, the lack of understanding on either molecular key event occurring during the carcinogenesis process, or the mechanisms of action (MOAs) of the tested compounds (Steinberg et al., 2017; Mascolo et al., 2018). Nevertheless, several works have shown that CTAs are useful tools to predict carcinogens (Creton et al., 2012). Especially, in our Group, a battery of *in vitro* tests to predict the carcinogenic potential of several NPs has been assessed, including the anchorage-independent growth, the invasion potential, and the tumorspheres formation ability.

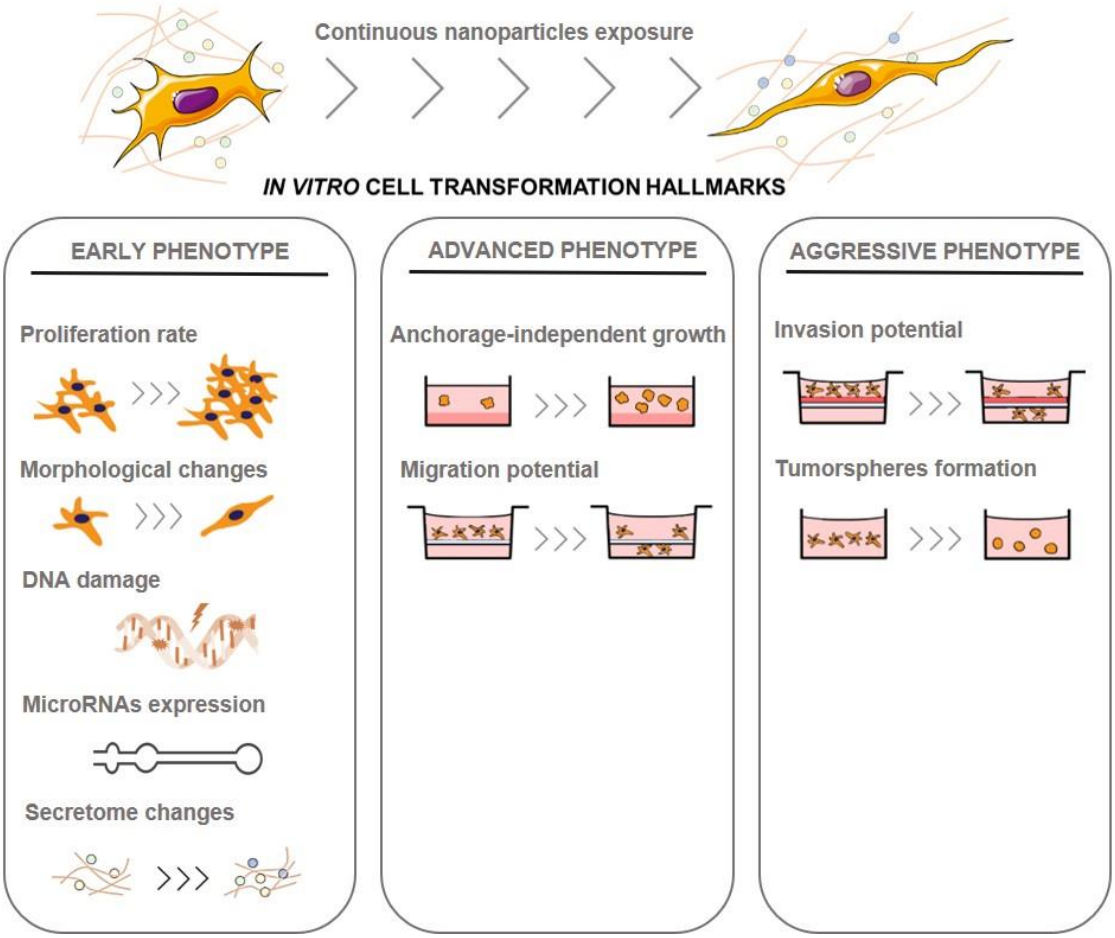

Figure 6. Cell transformation phenotypes -Early, Advanced and Aggressive- can be characterized through different *in vitro* assays. Some manifestations are distinguished from the earliest stages of the tumoral process while others are typical manifestations from more advanced stages (Barguilla, 2021).

1.6.4.1 Anchorage-independent cell growth

Anchorage-independent growth is the ability of transformed cells to grow independently of a solid substrate. When a tumoral process begins, normal cells trigger resistance to a particular type of apoptotic signal (anoikis), starting to grow independently and given rise to future steps in the metastatic process (Paoli et al., 2013). This ability has been considered as a hallmark of carcinogenesis, connected with tumour cell aggressiveness in vivo, and employed as a marker for in vitro transformation -evaluated via the soft-agar assay (Borowicz et al., 2014).

Our group has extensively used the soft-agar assay to evaluate the long-term carcinogenesis induced by NMs (Demir et al., 2015; Vales et al., 2015; Annangi et al., 2016; Vales et al., 2016). However, a variation of this method has been created (Indirect Soft-Agar Assay) to evaluate changes in the secretome functionality (Annangi et al., 2015;

Bach et al., 2016; Vila et al., 2017) (Figure 7). In this system, the conditioned media (CM) of the cells treated with the NM is used to grow a standard cancer cell line (HeLa) in a soft-agar substrate, being this cell line able to grow independently of anchorage and to form colonies in agar. Thus, changes in the size and number of colonies indicate differences in the ability of the CM to impact the neighbors' cells. This technique unravels how secretome impacts cells neighbours, regardless of whether these changes are related to carcinogenic effects.

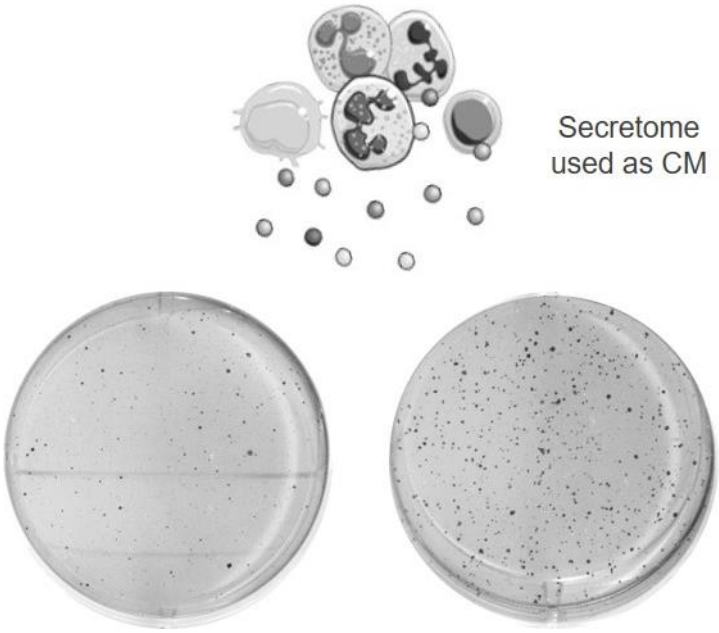

Figure 7. Soft-agar plates showing cell anchorage-independent growth in a cancer cell line after the secretome exposure of non-treated cells (left) and NPs' treated cells (right). CM: Conditioned media.

1.6.4.2. Invasion potential

The EMT is a process extensively studied as an actor of tumour progression and metastasis. During this process epithelial cells start to exhibit features of mesenchymal cells, including migration, invasiveness, stem cell properties, and resistance to apoptosis. The migration and invasion capacities are indicative of an advanced tumoral phenotype, involving the movement of cells on/through a substrate and the proteolysis of the extracellular matrix components (ECM) such as integrins or metalloproteinases, respectively. However, although they are different cellular processes, invasion requires migration, since a cell cannot invade without migration (VanZijl et al., 2011).

In vitro these capacities are commonly evaluated through the transwell invasion assay (Figure 8). The principle of this assay is to mimic the metastasis process *in vitro* through the construction of a physical barrier, which tries to simulate all the components

of an ECM. The ECM blocks non-invasive cells from migrating through the porous membrane, while invasive cells can degrade the matrix and move through the ECM layer to the bottom (Kramer et al., 2013; Justus et al., 2014).

Regarding NPs exposure, long-term exposure to NPs can trigger the development of an invasive phenotype. As a representative example, the study of Medina-Reyes and co-workers suggests an increase of the invasion capacity in lung epithelial A549 cells after TiO_2NPs exposure (Medina-Reyes et al., 2015). Similarly, cell lines derived from the primary tumours exposed to MWCNTs reported an elevated invasive capacity (Lu et al., 2019). Thus, there is evidence that the invasion potential can be proposed as an end-point to be evaluated in the carcinogenic risks associated with NMs.

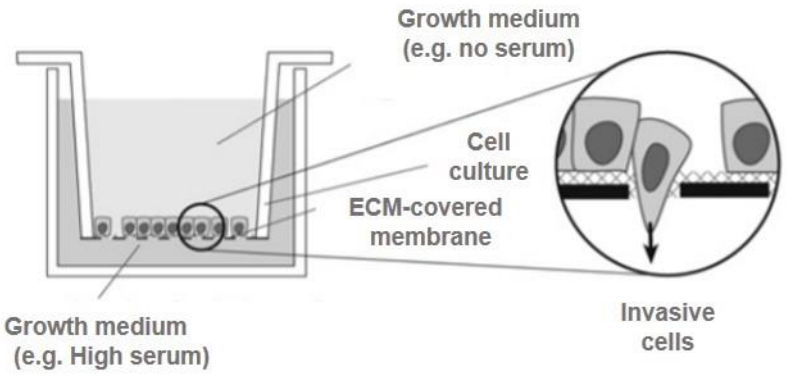

Figure 8. Transwell invasion assay. Cells are cultured on the top of the transwell without serum and the bottom is filled with enriched medium to act as chemoattractant. The invading cells will reach the bottom of the transwell by ECM digestion (Adapted from Kramer et al., 2013).

1.6.4.3. Cell stemness and tumorspheres formation

Cancer stem cells (CSCs) are a subpopulation of cells within tumours with capabilities of self-renewal and asymmetric division (producing a stem cell and a differentiated cell). These cells can form new tumors and support different oncogenic processes such as tumor growth and metastatic potential (Atashzar et al., 2020). Indeed, although they represent only a small proportion of tumor total cells, they usually exhibit resistance to chemotherapy and radiotherapy (Chang et al., 2016). Under specific *in vitro* conditions, CSCs can form tumorspheres. This sphere-forming capability is related to a high invasive tumoral phenotype. Hence, it has been recognized as a potential marker for screening both potential anti- and carcinogenic agents, giving information about the aggressiveness and stemness of a cell population (Lee et al., 2016).

Recently, the induction of cancer stem-like cells has been one of the mechanisms proposed to explain the carcinogenesis produced by NMs exposure. Luanpitpong and co-workers demonstrated CSCs formation as a mechanism underlying single-walled carbon nanotubes (SWCNTs) carcinogenicity, conducted by the overexpression of Cav-1 protein (Luanpitpong et al., 2014). Similarly, acute exposure to carbon nanotubes induces cancer-associated fibroblasts (CAFs), which induce tumour growth of human lung carcinoma cells by CSC induction mechanisms (Luanpitpong et al., 2016). Hence, tumorspheres formation test is a novel assay in the nanotoxicology field, very useful to screening the NMs' carcinogenicity.

1.7. NANOMATERIALS AS MODELS TO EVALUATE NEW END-POINTS

Once it has been described the potential types of exposure, the different experimental models, and the diverse end-points used to assess NMs exposure, several NPs have been used as models to test all the points mentioned above. These NMs are characterized by being widely represented in different environmental matrices, having different routes of exposure, functionalities, and applications.

1.7.1. Polystyrene NPLs

In recent decades, an exponential increase in the demand and production of plastics has occurred (SAPEA, 2019). Unfortunately, most of these plastics are discarded into the environment, causing an environmental problem. Once in the environmental matrices, plastics undergo a constant degradation and fragmentation by several agents or routes, such as solar radiation, mechanical forces, and microbial action inducing their transformation into smaller fragments ranging the micro and nanoscale, known as micro or nano-plastics (MNPLs) (Da Costa et al., 2017).

The MNPLs in the different environmental matrices have triggered an alarm regarding their possible effects on humans, fauna, and various ecosystems. Recent studies estimate that the oceans contain an average of 8.3 million MNPLs per cubic meter of water (Brandon et al., 2020). Also, it starts to appear evidence that they are present in daily consumable food products from marine ecosystems (Toussaint et al., 2019), in bottle mineral water (Ioakeimidis et al., 2016), and as airborne microplastics (Dris et al., 2016). Given its ubiquitous presence, their contact with humans is inevitable, having demonstrated its existence and toxicological consequences in the human body (Figure 9) (Rubio et al., 2020b; Prata et al., 2020).

Among the different types of synthetic plastic polymers, polystyrene (PS) is highly used in different industrial applications such as food packaging, electronics, automotive sector, containers for transportation, and house appliances, among many others (ChemicalSafetyFacts.org, 2020), but also in biomedicine as a part of diagnostic components (tissue culture trays, test tubes, Petri dishes, medical devices) and in pharmacological studies (Loos et al., 2014; Lehner et al., 2019). Despite its widespread use, some studies have shown they potential risk to human health when are in the micro-nano range (Banerjee et al., 2021). Among the different toxicity studies carried out using polystyrene MNPLs some authors report the induction of oxidative stress, genotoxicity, cytotoxicity, necrosis, or inflammation (Deng et al., 2017; Schirinzi et al., 2017). Most of these studies focus on the ingested or inhalation route, but they do not have into account that PS can cross the epithelial barriers (intestinal and airway epithelial barriers) and enter the bloodstream (Domenech et al., 2020). Once there, PSNPLs can interact with different blood cell types and even reach other target organs. Previous studies carried out by our group have demonstrated that PSNPLs induce toxicity, ROS, and genotoxicity in human leucocytic cell lines (Rubio et al., 2020a). However, there is a lack of knowledge about the effects that this interaction could trigger in primary cells of e*x vivo* or *in vivo* models. To fill in this gap, this Thesis dissertation tries to help us to understand potential effects on peripheral blood mononuclear cells (PBMC) after *ex vivo* exposures.

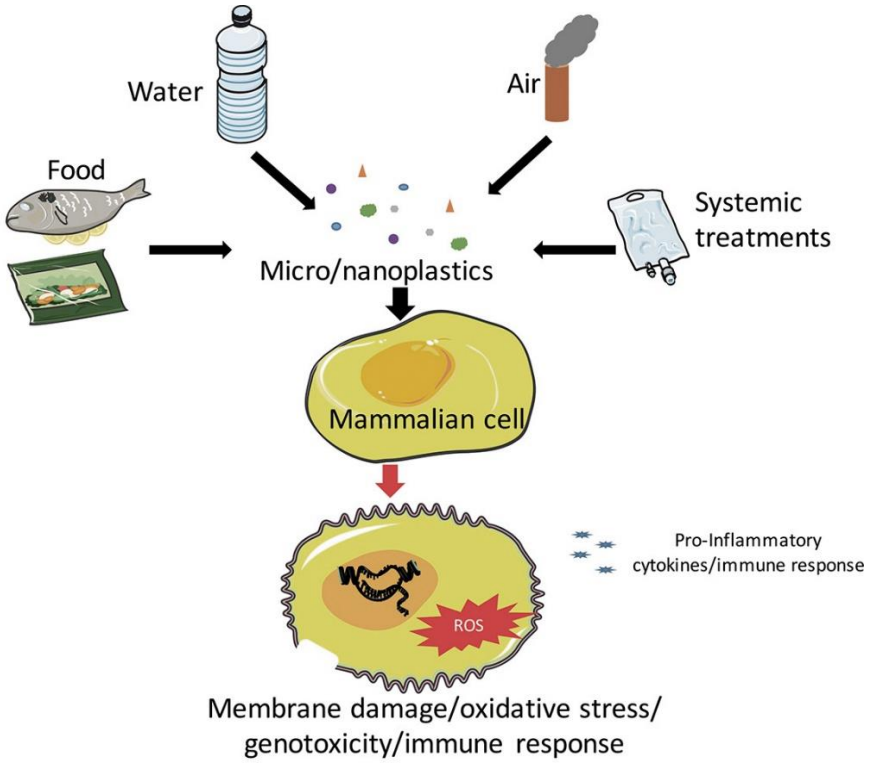

Figure 9. Ubiquitous presence of MNPLs in the environment and their consequences on mammalian model cells (Banerjee et al., 2021).

1.7.2. Graphene-based nanomaterials

Graphene consists of a single- or multi-layered sheet of carbon atoms packed into a two-dimensional (2D) lattice, like a honeycomb. It was synthesized and characterized for the first time in 2004 (Novoselov et al., 2004) and since then the research about their properties and applications has been growing steadily. Depending on the method of synthesis, graphene can exist in various morphologies such as sheets, platelets, ribbons, onions, and quantum dots. The most used are graphene oxide, reduced graphene oxide, quantum dots, graphene nanoribbons, and graphene nanoplatelets.

Graphene-based nanomaterials (GBNMs) have superior mechanical, electrical, and thermal properties making them ideal in multiple biomedical applications, such as anticancer therapy, photothermal therapy, photodynamic therapy, drug or gene delivery, cell imaging, and biosensors tissue engineering (Figure 10) (Chung et al., 2013). Most of these applications require the graphene intravenous injection, which allows graphene to encounter immune cells from the bloodstream, forming a complex environment of blood cells and proteins immediately after injection that stimulates a strong immune response (Zhou et al., 2012; Feito et al., 2014; Mukherjee et al., 2018). In this sense, many studies have focussed their research on the biocompatibility and the toxicity of graphene materials. It has been found that depending on the characteristics of each graphene, such as lateral dimension, oxidation state, or functionalization, the immune response may be altered. Moreover, the attachment of plasma proteins to NPs' surfaces forms a NP protein corona (PC), which composition may determine biodistribution, therapeutic efficacy, and immunotoxicity of the NPs (Orecchioni et al., 2016). For that reason, it is important to take extreme care in the design of each material when used in biomedicine to ensure that there is no toxicity.

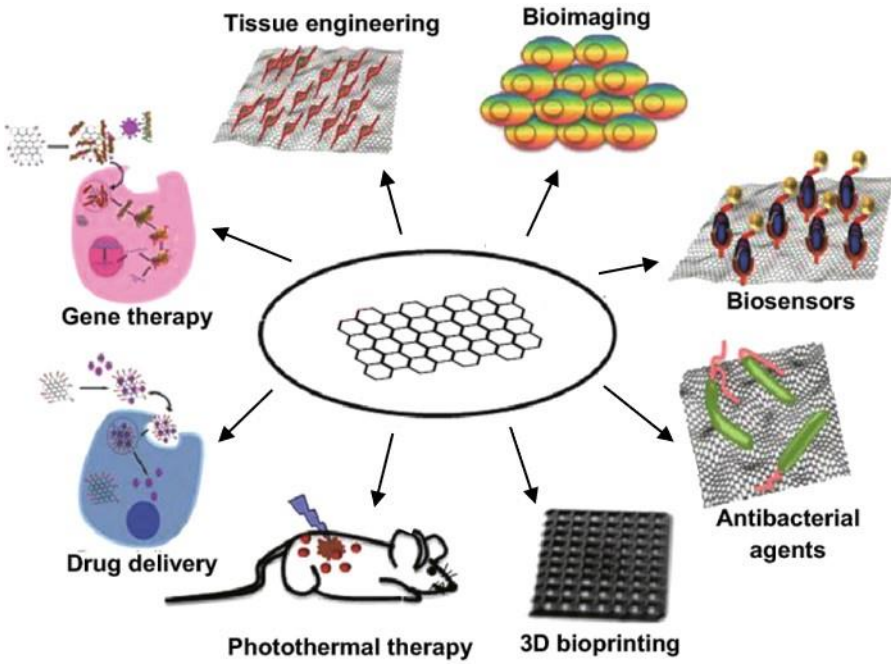

Figure 10. Biomedical applications of graphene-nanomaterials (Adapted from Syama et al., 2019).

1.7.3. Cerium dioxide nanoparticles (nanoceria)

Cerium is the most abundant of rare-earth metals. Among the different forms, CeO_2NPs, in their nanoscale form, are being used in multiple industrial and commercial applications such as for catalysis, fuel cells, and fuel additives. Unfortunately, this extensive use has facilitated their presence in both aquatic and terrestrial media. Some CeO_2NPs used as a diesel fuel additive can escape in the air emissions and accumulate in soils; others are introduced to landfills as solid wastes of electronics, soils via recycling of sewage sludge, and in the aquatic environment via wastewater discharge from ceramic manufacturing plants (Figure 11) (Dahle et al., 2015). All these accumulations not efficiently removed from the ecosystems, converts nanoceria into a long-term environmental problem since its contact with organisms becomes evident. Nonetheless, conflicting results on the toxicity of CeO_2NPs have been published in the last decade, with some studies reporting CeO_2NPs to be toxic (Mittal et al., 2014; Frieke Kuper et al., 2015), while others found it to have protective effects against oxidative stress (Rubio et al., 2016; Panda et al., 2019; Saifi et al., 2019).

Since CeO_2NPs are present in different environmental matrices, their interaction with other environmental contaminants should not be ignored. However, there is limited data about these potential interactions and, therefore, the assessment of a co-exposure model to have a more comprehensive vision of what happens in real-life exposure scenarios is necessary. In previous studies, our Group confirmed that using early stages

hallmarks of cancer, the transformation potential of cigarette smoke condensate (CSC) was potentiated in combination with nanoceria (Rubio et al., 2018). Accordingly, in this dissertation it is presented further studies with late stages hallmarks of cancer and epigenetics, confirming this interaction.

Figure 11. Environmental fate of CeO_2NPs based industrial and consumer products (Dahle et al., 2015).

1.7.4. Titanium dioxide nanoparticles

Nanoscale titanium dioxide is one of the most manufactured NPs. Its strong catalytic activity makes it suitable for many applications such as white pigment in paints and papers, photo-catalyst in solar cells, optical coating in ceramic, additive in cosmetic and food products and, more recently, in biomedicine (Wolf et al., 2003; Jafari et al., 2020).

This wide field of applications has increased the concern for human exposure and environmental release. In 2010 the International Agency for Research on Cancer (IARC) classified TiO_2NPs as a human carcinogen group 2B (possible human carcinogen) since there was enough evidence that TiO_2NPs may cause lung cancer by inhalation exposure (IARC, 2010). Since then, a vast amount of *in vivo* and *in vitro* studies has been conducted to investigate their human health impact (Shi et al., 2013). The most prevalent hypothesis

explaining the toxicity of TiO_2NPs involves the production of ROS (Chibber et al., 2017). However, a study carried out by our group did not report genotoxic nor reactive oxygen species production, but a potential carcinogenic risk after long-term low-dose exposure to TiO_2NPs in BEAS-2B cells (Vales et al., 2015).

To continue the previous study, this Thesis evaluates TiO_2NPs exposure effects at the level of microRNAs expression. In this sense, only a few studies have evaluated their effects at the epigenetic signature, demonstrating changes in the global DNA methylation and microRNAs in different *in vitro* cellular model systems (Alinovi et al., 2017; Pogribna et al., 2020). Therefore, further studies are needed for a complete assessment of the risks associated with TiO_2NPs exposure.

1.7.5. Multi-walled carbon nanotubes

MWCNTs are widely used in different consumption, medical, and industrial applications due to their excellent chemical, physical, thermal, and electrical properties. These advantages have substantially increased their use and their presence in the environment, entailing a potential health risk for humans. There is little information regarding their toxicological effects, some authors suggested that the diameter (Nagai et al., 2011), surface area (Kim et al., 2011), agglomeration, or functionalization could influence their reactivity. The study of Zhou et al. (2017) compares both pristine and functionalized MWCNTs suggesting that pristine induces more cell death while functionalized is more genotoxic in A549 lung cancer cell line. Moreover, a recently published study reported that continuous exposure (6 weeks) to low-doses of MWCNTs increased the expression of N-cadherin, vimentin, and fibronectin, as well as, a decrease of E-cadherin, which are some of the most important EMT biomarkers (Barthel et al., 2021). In addition, our group has previously demonstrated that sub-chronic MWCNTs exposure induces high levels of intracellular ROS, chromosome damage, changes in cytokine expression, and increases in attachment-independent growth (Vales et al., 2016).

Regarding their potential effects on the epigenome, different studies have reported MWCNTs effects in global DNA methylation. Sierra et al. found an enrichment of the hypomethylated CpG sites in the X-chromosome of BEAS-2B cells chronically exposed to MWCNTs (Sierra et al., 2017). In this context, to add new information to this field, this dissertation presents a microRNAs induction study where the effects are associated with the acquisition of the tumoral phenotype, to establish a potential battery of biomarkers that contribute to monitoring the exposure effects.

2. OBJECTIVES

2. OBJECTIVES

As mentioned in the Introduction section, it exists the necessity to complement the classical models and end-points of hazard assessment with NAMs approaches that help us to better understand the mechanisms of action derived from the exposure to different NMs. In this sense, the objectives of this Thesis are to evaluate the risks derived from the exposure to different NMs throughout novel models and new end-points.

To achieve these goals, we have proposed the following four specific objectives:

1. To set up the whole blood *ex vivo* model to demonstrate its usefulness in NMs testing. This model was applied to study the following PSNPLs effects (chapter 1):
    a. The internalization levels in WBCs.
    b. The levels of genotoxic damage induced in WBCs.
    c. The specific cellular sensitivity of the different WBCs.
    d. The whole blood secretome expression associated to PSNPLs exposure.
2. To adapt the *ex vivo* model to evaluate the secretome effects induced by graphene-based nanomaterials, including graphene oxide, graphene nanoplatelets, and graphene nanoribbons (chapter 2).
3. To use a long-term low-dose exposure approach to evaluate the transforming and epigenetic effects generated after the exposure to nanoceria alone, or in combination with cigarette smoke condensate in human bronchial epithelial BEAS-2B cells (chapter 3).
4. To propose a set of microRNAs as new biomarkers of the long-term NMs' exposure effects. Specifically, this was established after TiO_2NPs and MWCNTs exposures, and validated using cells long-term exposed to CoNPs, MSiNPs, ZnONPs and CeO_2NPs (chapter 4).

3. RESULTS

3. RESULTS

Part of the results derived from this Thesis has been published in different peer-review journals. Specifically, three articles have already been published and the fourth has been submitted for its consideration. Consequently, the three chapters of this section are constituted by the content of the published articles being the fourth one positioned in the Annexes section.

The four articles/manuscripts are ordered according to the objectives of the Thesis as follows:

1. **Chapter 1 (Article 1)**: Genotoxic and immunomodulatory effects in human white blood cells after *ex vivo* exposure to polystyrene nanoplastics.
2. **Chapter 2 (Article 2)**: *Ex vivo* exposure to different types of graphene-based nanomaterials consistently alters human blood secretome.
3. **Chapter 3 (Article 3)**: Nanoceria, alone or in combination with cigarette smoke condensate, induce transforming and epigenetic cancer-like features in vitro.
4. **Chapter 4 (Annex 1)**: MicroRNAs as a new biomarker to detect the effects of long-term exposures to nanomaterials. Studies on TiO_2NPS and MWCNTs

3.1. CHAPTER 1 (Article 1)

Genotoxic and immunomodulatory effects in human white blood cells after *ex vivo* exposure to polystyrene nanoplastics.

Environmental Science: Nano, 7(11): 3431–3446 (2020)

doi: 10.1039/D0EN00748J

Impact Factor: 8.131

3.2. CHAPTER 2 (Article 2)

Ex vivo exposure to different types of graphene-based nanomaterials consistently alters human blood secretome.

Journal of Hazardous Materials, 414: 125471 (2021)
doi: 10.1016/j.jhazmat.2021.125471
Impact factor: 10.588

3.3. CHAPTER 3 (Article 3)

Nanoceria, alone or in combination with cigarette-smoke condensate, induce transforming and epigenetic cancer-like features *in vitro*.

Nanomedicine, 16(4): 293–305 (2021)
doi: 10.2217/nnm-2020-0367
Impact factor: 5.307

3.4. CHAPTER 4 (Annex 1)

microRNAs as a new biomarker to detect the effects of long-term exposures to nanomaterials. Studies on TiO_2NPS and MWCNTs

Submitted manuscript

4. DISCUSSION

4. DISCUSSION

EPs include a wide variety of chemicals that are increasingly detected in different environmental matrices, without clear knowledge of their potential effects on humans. NMs are found within this wide range of EPs (Vasilachi et al., 2021). As numerous studies show, the effects of NMs are not harmless, but rather they represent a problem for both the ecosystems and for human's health (Abedin et al., 2021). Although there is a wide set of useful methods to evaluate the potential health effects of NMs, there is a lack of powerful tools to identify the induction of different mechanisms, such as carcinogenesis. Furthermore, although traditional animal toxicological studies can provide sound data for risk assessment approaches, they have some disadvantages like costs, time-consuming, and ethical considerations related to animal welfare. Therefore, diverse non-animal safety testing methods are increasingly considered (Nymark et al., 2020). The European Chemicals Agency (EACH) favours alternative methods in opposition to conventional *in vivo* testing (Annys et al., 2014). In this context, a variety of "New Approach Methodologies" stems from recent technical and informatics-driven developments and could, potentially, improve the needed hazard assessments of the steadily increasing number of EPs, avoiding the use of animals. NAMs context includes a broad spectrum of biomarkers, using *in silico* approaches, as well as *in chemico* and *in vitro* assays. Altogether, they provide sound information tailored to the exposure assessment, hazard assessment, dose-extrapolation and, finally, risk assessment (Nymark et al., 2020). Within this framework, the focus of this Thesis has been directed towards the hazard assessment of NMs and, more specifically, it pursues to implement new *in vitro* models and biomarkers with the potential of retrieving subacute effects and mechanistic insight. This can provide us with relevant information on the modes-of-action (MOAs) of the compounds under study. Thus, in this Thesis two advanced models have been developed: a whole blood *ex vivo* model, and a long-term low-dose *in vitro* model. Also, novel biomarkers have been established and evaluated, such as changes in the expression of microRNAs, secretome alterations, and oncogenic features driven by NMs exposure.

4.1. ADVANCED MODELS FOR THE HAZARD ASSESSMENT OF NMS

4.1.1. Whole blood ex vivo model

Some authors have pointed out the gap between basic studies in cell culture and pharmacokinetic studies in animals (Clift et al., 2011). To bridge this gap, this Thesis presents a whole blood *ex vivo* model to evaluate the effects of different NMs in the blood system. Importantly, blood is the gateway for all NMs, both for NMs with biomedical applicability that are administered intravenously –or via other routes- into the patient, and

for the rest of NMs that translocate to the blood once they unintentionally enter the human body after crossing the lung and intestinal barriers (De la Harpe et al., 2019). The principle of this model is the measurement of different parameters after *ex vivo* exposure of fresh human blood to different NMs. The suitability of this model to obtain information about the immune reaction of a blood donor has been proven previously (He et al., 2018; Liebers et al., 2018, 2020). However, there are other approaches in which blood cells are first harvested, then cultured, and treated outside the cellular microenvironment (blood) to study the effects of a given exposure. The discussion around which of these two approaches is better when studying different compounds has been the focus of a wide discussion. Most studies highlight the advantages of the *ex vivo* whole blood assay, as it is faster, cheaper, easily reproducible because it does not require complex laboratory equipment, it uses small volumes of human blood, and it maintains better the physiological environment and, therefore, better mimics the *in vivo* situation (Silberer et al., 2008). Moreover, several studies have compared results from isolated cells *vs* whole blood cultures showing that results from isolated cells present some inconsistencies and low reproducibility (Silva et al., 2013). Specifically, they reported that the isolation procedures (e.g., Ficoll or Lymphoprep-related density gradient centrifugation techniques) negatively influenced the results by increasing the levels of apoptosis in PBMC cultures pointing out that interaction between all cellular blood components is important to maintain cell viability (Hodge et al., 2000). Additionally, in the *ex vivo* system NMs are embedded in a complex system of inorganic (salts) and organic substances (amino acids, fatty acids, and proteins) that can be adsorbed on the NPs' surface forming a protein corona that is known to influence cellular uptake (Lee et al., 2021). Several studies have demonstrated the coronas' formation and have remarked that this corona influences trafficking and biological effects, as well as organ distribution and clearance rate of NMs (Monopoli et al., 2011, 2012; Yan et al., 2013; Neagu et al., 2017). Taking as an example one of the NMs used in this Thesis, recent studies have reported an increase in the genotoxic effects of PSNPLs due to the characteristics of the protein corona formation. The coronated-NPLs caused higher genotoxic effects in human lymphocytes at 5 µg/mL concentration than virgin-NPLs (Gopinath et al., 2019). Therefore, it is certainly beneficial to analyse the potentially hazardous effects of NMs in a system closely related to the *in vivo* situation. Lastly, this model can also help us to identify those NMs able to induce other subacute alterations at the molecular level, such as in the secretome. This is constituted by several bioactive molecules (including proteins, cytokines, chemokines, growth factors, but also nucleic acid) released by cells and involved in cell-to-cell communication and crosstalk. Therefore, from our perspective, the whole blood *ex vivo* system developed in this Thesis is also a

promising experimental model for the *in vitro* identification of immunotoxic and immunomodulatory NMs-related effects (Baumann et al., 2013; Galbiati et al., 2018).

4.1.2. In vitro long-term model

As explained in the introduction (1.5.2 and 1.5.3 sections), another strategic model use cell types able to support long-term/low-dose exposures of a given compound to study the effects and mechanisms operating under prolonged exposures. In this sense, most of the literature dealing with hazard evaluation of environmental pollutants contemplates acute exposure treatments only which are, in many cases, far from reflecting the real environmental situation. Another important aspect that reinforces the need for long-term studies is the high bio-persistence observed for many pollutants of human concern. Focusing on the NMs evaluated in this Thesis under a long-term regime of exposure (CeO_2NPs, TiO_2NPs, and MWCNTs), several studies demonstrated their bio-persistence in different tissues. For example, TiO_2NPs have been described to bio-persist in the liver, lungs, and spleen of rats intravenously treated with 1 ng/kg one year after the administration (Disdier et al., 2015). As well, MWCNTs were still present in the lungs 6 months after intratracheal instillation in rats (Elgrabli et al., 2008). In addition, a study using nanoceria intravenously infused into rats reported 90 days bio-persistence in organs of the mononuclear phagocyte system, which includes lungs, being this persistence associated with the observed adverse effects (Yokel et al., 2012).

Additionally, long-term studies enable the evaluation of potential carcinogens and their underlying mechanisms of action. In this sense, our Group has extensive experience: Vales et al. (2015, 2016) described cell transformation induced by TiO_2NPs and MWCNTs mediated by a non-genotoxic mechanism, and induction of chromosome instability, respectively. In both studies, BEAS-2B cells were exposed for up to 4 weeks to 1, 10, and 20 µg/mL of the corresponding NM. Similarly, Vila et al. (2017) demonstrated the carcinogenic potential of AgNPs after 6 weeks long-term *in vitro* exposures to 0.5 and 1 µg/mL in Caco-2 cells. In such a study, a wide set of oncogenic biomarkers were included to evaluate the NMs-induced cancer-like phenotype, such as anchorage-independent cell growth, migration capacity, and overexpression of matrix metalloproteinases. Also, the potential carcinogenicity of CoNPs was demonstrated after 12 weeks of exposure to sub-toxic doses (0.05 and 0.1 µg/mL) in mouse embryonic fibroblast (MEF) wild-type and MEF *Ogg1* knockout. Here, the cell transformation was observed due to an increase in matrix metalloproteinases and anchorage-independent cell growth capacity, two important transformation features. Interestingly, the cell line most transformed was that more sensitive to the oxidative damage (MEF *Ogg1*$^{-/-}$), and thus, the oxidative damage observed after CoNPs exposure could be the cause of the malignant features observed (Annangi et

al., 2015). Likewise, these same cellular types reported negative results after long-term exposure to ZnONPs. Thus, they were unable to induce genotoxicity or cell transformation after 12 weeks of exposure to subtoxic doses while, at short-term (24 h), ROS, genotoxicity, and oxidative DNA damage were observed. Moreover, to test the biocompatibility of mesoporous silica nanoparticles (MSiNPs), used as nanocarriers for biomedical applications, long-term studies have been performed in our Group. Although not published yet, BEAS-2B cells were exposed to 10 µg/mL of three different types of MSiNPs for 8 weeks, showing an increased expression in some biomarkers of transformation, such as migration, invasion, and anchorage-independent cell growth ability in two of the three tested NPs. The exposure dynamics of all these studies was similar. In them, the cell culture medium was renewed with the NMs' concentration every 2-3 days, and passages were performed every 2-3 days according to the growth rate of the experimental cell-line. Furthermore, in parallel to the exposure time, passaged-matched controls were carried out to identify if the observed results were due to the damage that the cells may suffer during the passages, and not to the treatment itself. Further, other groups have also studied the *in* vitro long-term carcinogenic effects of different NMs. For example, the incubation with ZnONPs under long-term treatments (16 and 24 weeks) using sub-toxic doses, induced cell malignant progression in MEF wild-type cells (Wang et al., 2019).

The selection of the cell type used with the long-term model is another important factor to be considered. Besides being a relevant cell target, other questions arise for the selection criteria, such as the potential of the cell type to show positive traits for the different evaluated biomarkers or its performance in the different employed *in vitro* assays/methodologies. In this regard, the human bronchial BEAS-2B cells have been extensively used to investigate the cell transformation process of different carcinogens (Park et al., 2015; Zhong et al., 2018). Many of the studies quoted in the previous paragraph used BEAS-2B cells for testing different biomarkers of the transformation process, demonstrating the suitability of these cells in long-term studies. Furthermore, this cell-line has also been previously used to study the epigenetic changes generated after NMs' exposures. Sierra et al. (2017) used the BEAS-2B cell-line to confirm the global DNA methylation changes induced by MWCNTs. In such study, long-term exposure to sub-toxic doses of TiO_2NPs and MWCNTs reported only minor effects on genome-wide DNA methylation, and consistent DNA hypomethylation, respectively. Additionally, several studies have described microRNA expression alterations after the exposure to some chemicals, including NMs, using BEAS-2B as a model cell-line. As an example, miR-200b was found upregulated in BEAS-2B cells exposed to cigarette smoke. Its overexpression

promoted the migration of BEAS-2B cells by targeting *ETS1*, a target gene related to ETM (Wang et al., 2021). Similarly, Gao et al. (2016) demonstrated that miR-34a (tumour suppressor gene) overexpression enhanced TiO_2NPs-induced autophagy and cell death by targeting *Bcl-2*, a known oncogene. Therefore, all these studies indicate that BEAS-2B are good model to study the transforming potential and the epigenetic cancer-related alterations triggered by NMs' exposure. Thus, the third and fourth studies of this Thesis have used this cell-line to evaluate some biomarkers of the transformation process, as well as a battery of microRNAs related to this process. Moreover, since inhalation (together with ingestion) is one of the most likely routes of human unintentional exposure to environmental pollutants, this is another strength making these cells a good model to handle with.

4.2. NEW BIOMARKERS FOR THE IDENTIFICATION OF SUB-ACUTE EFFECTS AND MODES-OF-ACTION OF NMS

As indicated previously, one important point in hazard assessment is the understanding of the NMs' mode-of-action. To this end, several novel biomarkers have been developed and examined in this Thesis. These biomarkers include a panel of microRNAs related to the cancer process and an array of cytokines related to the cells' secretory phenotype. On the other hand, several oncogenic biomarkers have also been evaluated by using the indirect soft-agar assay, and the invasion and tumorspheres assays to test potential NMs carcinogenicity.

NMs' exposure has been reported to trigger epigenetic changes as shown by extensive reports on altered DNA methylation patterns induced by gold nanoparticles (AuNPs), silver nanoparticles (AgNPs), MWCNTs, and TiO_2NPs, among others (Gonzalez-Palomo et al., 2021; Sierra et al., 2017; Tabish et al., 2017). Regarding miRNAs, they are central players in gene expression regulation, controlling diverse processes such as cell proliferation, EMT, tumour invasion pathways, angiogenesis, apoptosis, or metastasis (Hammouz et al., 2021). All this involves them in the pathogenesis of numerous diseases, including cancer. In this sense, many studies in cancer point out alterations in the epigenetic signature, revealing a microRNA expression profiling different between healthy *vs* cancer patients (Fehlmann et al., 2020). Likewise, there is a growing literature that associates microRNAs' alterations with the induction of tumoral phenotype upon NMs' exposure (Li et al., 2020; Zhao et al., 2016). Therefore, a panel of microRNAs related to the carcinogenic processes has been designed and applied in this Thesis.

The blood secretome has been implicated in the pathology of several diseases related to the inflammatory process, including cancer (Da Cunha et al., 2019). A recent study analysed the blood secretome from healthy and cancer patients revealing a specific panel of cytokines in each one of the different phases of the carcinogenic process (Coluccio et al., 2020). In turn, it has also been shown that exposure to certain pollutants, including NMs, can trigger an inflammatory response (Pandey et al., 2018). In this sense, although some NMs are designed to be immunomodulatory (*e.g.,* vaccine adjuvants, anti-inflammatory, immunosuppressive drugs), others are not intended for that function but also stimulate an inflammatory or an immune response, promoting the release of numerous cytokines that play essential roles in these responses. This cytokine secretion has a substantial influence on many cellular processes, such as intercellular communication, cell differentiation, cell migration, cell proliferation, cell-death programs, etc., and therefore, it is expected that secretome alterations may influence the cells' state (Kany et al., 2019). Hence, this Thesis pursues to understand the NMs' impact at the level of the secretome through the analysis of a large panel of cytokines that influence different biological processes.

Long-term studies enable us to study cell-acquired transforming features derived from the NMs' exposure. In this Thesis several carcinogenic features such as the stem-like properties, the anchorage-independent growth, and the invasion abilities have been analysed as they are considered important indicators of carcinogenesis. Their evaluation is useful to study some key stages of *in vivo* multistep carcinogenesis and to detect possible non-genotoxic NMs with carcinogenic potential. However, the main concern regarding these studies is the lack of understanding of the molecular mechanisms underlying transformation (Creton et al., 2012). Thus, from our perspective, a good approach would be to complement these tests with the ones previously discussed to explain the NMs' carcinogenic mechanisms. For example, an explanation that could be behind the increase in cancer stem cells could be the dysregulation of certain microRNAs that have been related to stem-like properties (Pan et al., 2021).

4.3. USABILITY OF THE DIFFERENT MODELS AND BIOMARKERS IN THE EVALUATION OF THE HAZARDOUS EFFECTS INDUCED BY NMS EXPOSURE

Following the previous description of NAM's concept and the general strategy followed in this Thesis, the next sections of this Discussion are dedicated to the four different chapters included in this Thesis. Thus, the previously presented NAMs will be discussed in the context where they have been developed and/or applied.

4.3.1. Genotoxic and immunomodulatory effects detected in human white blood cells after ex vivo exposure to polystyrene nanoplastics

Plastic production is growing year by year and, consequently, environmental plastic wastes are steadily growing. Plastic wastes are submitted to a continuous degradation by different physicochemical mechanisms generating by-products with a wide range of sizes. Those with sizes comprises in the micro- and nano-range are defined as micro and nanoplastics, respectively. Regarding their small sizes, micro and nanoplastics constitute an entity nominated micro-nanoplastics (MNPLs). These MNPLs constitute a new widespread type of emergent pollutants found in many environmental compartments such as water, air, and soils (Mofijur et al., 2021). Thus, its interaction with humans -and other organisms- is inevitable and evidence of their potential risk for human health is recently emerging (Yee et al., 2021). As for other pollutants, it is considered that there are three main routes for MNPLs to end up into the human body i.e., inhalation, ingestion, and dermal absorption, being ingestion possibly the most significant one (Li et al., 2015; Santillo et al., 2017; Lehner et al., 2019; Wang et al., 2020; Domenech and Marcos, 2021). Once they reach the human body, particles <110 μm can translocate through the respiratory and gastrointestinal body barriers, reaching the blood circulation and, for particles <20 μm, they can be distributed and accumulated in secondary organs (Wright et al., 2017). In this direction, studies carried out by our group showed that MNPLs can translocate across an *in vitro* 2D human intestinal barrier simulated through two co-culture models: differentiated Caco-2/HT29 intestinal cells and Caco-2/HT29+Raji-B cells (Domenech et al., 2020). Again, this highlights the fact that PSNPLs have the potential to enter the circulatory system and interact with all blood components.

In this study, we have used commercial polystyrene nanoparticles (PSNPLs) as a representative MNPL. Results indicate that our particles did not seem to exert cytotoxicity on WBCs at any tested dose. A similar study showed no significant cytotoxicity of PSNPLs on PBCs at concentrations up to 500 μg/mL (Hwang et al., 2020), although other studies detected a decrease in the cell viability after exposure to 500 μg/mL of PSNPs (Kik et al., 2021). This last study compared PSNPLs of different sizes (29, 44, and 72 nm) showing the most significant decrease in the number of cells with the smallest NPLs exposure. However, it is important to emphasize that while most reported studies are being conducted using this type of intentionally manufactured particles, it is critical to include new models and materials (polyethylene, polyesters, or polyurethanes), as well as samples gathered from environmental sources to characterize the effects of more realistic samples. Likewise, MNPLs coexist in the environment with other contaminants and, therefore, the evaluation of their potential interactions is also relevant. Based on De Souza

et al. (2018), the combination of several pollutants could reinforce their individual biological effects. A recent study carried out by our Group has demonstrated that the physical interaction between silver and PSNPLs takes place and that the formed complexes can modulate the uptake of silver nanoparticles and slightly modify some toxic effects of silver compounds (Domenech et al., 2021). This initial evidence makes it urgent to decipher the potential role of MNPLs as carriers of other environmental pollutants sharing similar niches.

Another important appreciation is that the concentrations used in most of the studies with MNPLs are just an approximation to the real exposure concentrations. This is due to the lack of methods allowing to quantify the amount of NPLs present in the different environmental and biological matrices. Furthermore, the ubiquity of these MNPLs makes difficult to estimate their impact on human health, due to the lack of real exposure data levels (O'Neill et al., 2021). Recently, Ragusa et al. (2021) showed the presence of microplastic fragments (ranging from 5 to 10 μm in size) in four of six placentas explored in humans. However, there are only a few studies as the previous one, that have directly assessed human exposure, since most studies mainly made estimations from external sources of exposure and are based in the presence of microplastics but not in nanoplastics, what are supposed to be more dangerous from the health point of view. For instance, the annual dietary exposure for European shellfish consumers is estimated to reach 11,000 MPLs particles (Van Cauwenberghe et al., 2015), although other estimates indicate that this dietary exposure could be higher (Oliveri Conti et al., 2020). Usually, the amount of MPLs is calculated as particles per m^2 or per kg but it is too difficult to translate these values to μg/mL of MNPLs (Cox et al., 2019). Thus, in our study, we have chosen a wide range of subtoxic concentrations (0-100 μg/mL) based on the cell viability results and, also, based on the *in vitro* concentrations used by us with other similar NMs.

In our model, we have demonstrated a significant uptake of PSNPLs by certain WBCs. Although the uptake route has not been determined, several studies pointed out that cells exploit different uptake mechanisms depending on the particle size, the NPs' functionalization, and the cell type. Fiorentino et al. (2015) concluded that PSNPLs can rapidly cross the cell membrane through passive non-endocytic mechanisms, whereas Dos Santos et al. (2011) showed active energy dependent pathways when PSNPLs uptake takes place. Moreover, Lunov et al., (2011) showed that macrophages were able to internalize more carboxy-PSNPs than THP-1 cells, while the last ones took up more aminated-PSNPLs. Additionally, differential phagocytic activity of PS microplastics was observed in PBMCs. Thus, neutrophils and macrophages internalized the particles, but not lymphocytes (Hwang et al., 2020). This differential uptake between cell types has also

been demonstrated in our study. While lymphocytes showed low levels of internalization, PMNs and monocytes display high rates of uptake, being monocytes those with the highest rates. In a context in which there is a “cellular competition” for the uptake, these results are expected, since monocytes are intended for phagocytosis. Furthermore, it is important to consider the microenvironment in which the cells are immersed, since similar studies have shown some uptake of these NPs in lymphoblastic (TK6) and Raji-B (B-lymphocytes) cell lines, but these results were found in individualized cell cultures, where there is no "cell competence" for the internalization. Furthermore, as found in our study, the referred study also showed a greater uptake by THP-1 (monocytes) cells, although due to the saturation, they only observed this trend at the lowest concentrations, because at the highest concentrations there were no differences between cell types (Rubio et al., 2020a). All this highlights the importance of studying cell responses within a realistic microenvironment. Due to their relevance, effects on DNA must be considered among the harmful effects of any environmental pollutant. Studies on the genotoxic effects of MNPLs using different cellular and animal models are starting to appear but reporting contradictory results. Negative results were reported after the exposure to PSNPLs. For instance, our group did not observe any significant genotoxic effects in human intestinal Caco-2 cells exposed to 1, 25, 50, and 100 µg/mL of PSNPLs. Similarly, negative results were found in two *in vitro* biological barriers, GI and placental barrier, simulated using two different co-culture models. These barriers were exposed to different concentrations (0.01 to 50 µg/mL) of 50 nm and 0.5 µm COOH-modified PSNPLs and the genotoxicity was assessed by using the reporter *p53* gene and the micronucleus assays (Hesler et al., 2019). However, Poma et al. (2019) reported that PSNPs induced increased levels of DNA damage in human fibroblast hs27 cells. Very recently, Cobanoglu et al. (2021) identified MPLs’ genotoxicity in human peripheral blood lymphocytes after exposure to five concentrations (25, 50, 100, 250, and 500 µg/mL) of PSNPLs by using the cytokinesis-block micronucleus assay. Swiss mice exposed to PSNPLs and ZnONPs, alone or in combination, showed DNA damage in erythrocytes as measured by the comet assay. Interestingly, these genotoxic effects were associated with the production of free radicals after exposure (Estrela et al., 2021). In this context, our research showed a different cell sensitivity to genotoxic damage induction, depending on the cell type. Thus, after 24 h of incubation with 50 and 100 µg/mL of PSNPLs, lymphocytes did not show any DNA damage, while monocytes presented significant levels of damage at the highest dose, and PMNs suffered high levels of damage at both tested doses and, therefore, they were considered the most sensitive cells to the genotoxic damaging effects of PSNPLs. This differential sensitivity to damage points out the importance of studying the different cell

populations separately since most of the works found in the literature study collectively all the blood cell types (Cobanoglu et al., 2021; Sicinska et al., 2021).

Interestingly, in our study, we have detected a masking effect produced by high differences in the levels of DNA damage between immune cell subtypes that are, moreover, not equally represented in the sample. When we measure genotoxicity in the total cell population it is important to consider the percentage of each cell sub-population present in the sample. Nucleated cells in whole blood mainly consist of neutrophils (60–75%) followed by lymphocytes (20–30%), whereas PBMCs isolated by Ficoll-Paque consist of 95–98% of lymphocytes (Collins et al., 2008). Therefore, it is important to consider that lymphocytes represent the absolute majority of PBMCs and that this could be hiding the effects produced on other cells more sensitive to damage, but less representative, when we analyse PBMCs collectively. Additionally, and contrary to the assumptions made by the ComNet project that pointed to increased damage by isolating lymphocytes from whole blood (Collins et al., 2014), the study of Bausinger et al. (2016) showed that this isolation may reduce mutagen induced effects. The reason for this reduction was not completely clear, they hypothesized that it may be due to the repair activity of the cells during the time needed for lymphocyte isolation, or due to the different cell populations studied or an impact of the isolation process itself. From our results, we have been able to verify that PSNPLs produced DNA damage in some sub-populations, despite WBCs were previously isolated. Furthermore, we claim that the cell population under study influences the genotoxicity results and, for further studies, the repair systems or antioxidant system will have to be considered when DNA damage is evaluated.

Another interesting point observed in our results is that NPLs uptake and genotoxicity are not necessarily correlated. Here, while monocytes presented the highest uptake, they were not the cells more sensitive to genotoxic damage. Instead, although PMNs did not internalize as many PSNPLs as monocytes, they were the cells most sensitive to DNA damage. Similarly, to our findings, Paget et al. (2015) analysed two model cell lines, THP-1 and Calu-3, exposed to PSNPLs with different functionalized surfaces (non-functionalized, carboxylated, and aminated), and demonstrated NPLs' surface characteristics influences on the genotoxic and cytotoxic effects and, these observed effects were not correlated with NPLs internalization. Therefore, these results pointed out that cell genotoxicity is not necessarily correlated with NPLs uptake, but they may depend on the intrinsic characteristics of each cell. Thus, areas of interest for future studies include deciphering which specific mechanisms of each cell type are related to sensitivity to MNPLs toxicity.

Regarding the evaluation of the effects triggered by PSNPLS on the blood secretome at the global qualitative level firstly, we performed a variation of the classical soft-agar assay, namely indirect soft-agar assay (Borowicz et al., 2014). This classical technique is included in the battery of assays evaluating cell transformation, as it measures the ability of cells to grow independently from anchorage to a substrate. Nevertheless, with the modified assay we aim to evaluate whether exposed blood cells can secrete factors that induce some functional effects on the growth of a model cell line. Thus, this would indicate PSNPLs' ability to modify the blood microenvironment. HeLa cells, which can spontaneously form colonies in agar, were exposed to the serum of the blood samples exposed to 10 and 25 µg/mL of PSNPLs (conditioned media). The results showed that at low exposure levels there is a great inhibitory effect on the growth of colonies, but at the highest concentration, the number of colonies increases significantly, showing a different growth-induction pattern according to the exposure level. Interestingly, in this study, we have not applied the highest concentrations of PSNPLs as those used with the genotoxicity test, since we have already seen effects at the lowest concentration. That means that the effects on the secretome appear when there is still no genotoxicity induction. In line with this, it gives the impression that the activation mechanisms of the secretome differ from those of the genotoxic damage. Thus, the obtained results evidence that PSNPLs exposure induces changes in the secretome composition, able to alter the cell growth of other cell-lines, even at low concentrations. These results demonstrate the importance of using the indirect soft-agar assay in the battery of assays used for the illustration of the secretome changes.

Giving strength to the previously founded secretome alterations, we use an array of 105 cytokines in our *ex vivo* whole blood model. In the current investigation, instead of selecting candidate cytokines, we investigated a wide panel of cytokines to have a more complete view of the immunomodulatory effects of PSNPLs exposure. Several studies have already reported MNPLs-induced inflammatory processes. Hwang et al., (2019) found that polypropylene MPLs stimulated the immune system increasing the levels of cytokines and histamines in PBMCs, Raw 264.7, and HMC-1 cells. Later, the same group demonstrated that PSNPLs smaller than 1 µm increased the secretion of TNF-α, while PSNPLs smaller than 10 µm produced changes in the secretion of IL-6 both at the concentration of 500 µg/mL (Hwang et al., 2020). According to that, our results showed secretome alterations after the exposure to 10 and 25 µg/mL of PSNPLs. From our panel, most of the cytokines were under-expressed at the lowest concentration while most of them were overexpressed at 25 µg/mL. In addition, CSF3, IL1β, IL31, MPO, TGFα, and

TNFα were overexpressed in both concentrations, meaning that cells can already enhance the expression of some cytokines independently of the exposure levels.

To analyse the involvement of the overexpressed cytokines, they were grouped into four different categories: immune response, inflammatory response, stress response, and proliferation response. Some cytokines were category-specific, for example, IL-31 and TGFα participate only in the inflammatory response and in the cell proliferation response, respectively. Instead, other cytokines have a role in all shown categories, such as CXCL10, IL1α/β, IL3, IL15, IL34, and TNFα. It is interesting to highlight TNFα, as well as IL1α and β as the most described inflammatory cytokines in the tumour microenvironment (Balkwill et al., 2012). TNF-α is a pro-inflammatory cytokine produced by macrophages or lymphocytes, among other cells, after the stimulation with various agents. Thus, the upregulation of this cytokine is a potential indicator of an immune response and inflammation (Bradley et al., 2008). Previous studies demonstrate that TNF-α is up-regulated after exposure to PSNPLs in A549 human lung epithelial cells (Xu et al., 2019). Moreover, CXCL10 is secreted by immune cells in response to an inflammatory stimulus, being deregulated in multiple inflammatory diseases (Zhao et al., 2017). IL-1β was secreted by human macrophages in response to amino-functionalized PSNPLs of ~100 nm in diameter (Lunov et al., 2011). Additionally, CCL3 is produced by macrophages and activates the inflammatory and immune response. Moreover, it has been identified in several types of leukemia (Baba et al., 2014). It is worth highlighting the role of TGFα as a part of the proliferation response. Exposures to other NMs, as SiNPs, induced the release of TFGα by human mononuclear cells (THP-1) promoting the proliferation and EMT of BEAS 2B cells (Li et al., 2019).

4.3.2. Ex vivo exposure to different types of graphene-based nanomaterials consistently alters human blood secretome

GBNMs are widely used in the biomedical field for diagnosis, prevention, and treatment of different disease. For instance, Kaleekkal (2021) developed hemo-compatible polyetherimide membranes with functionalized graphene oxide (f-GO) to be used in haemodialysis treatments. Another recent study displayed a structure formed by oxidized graphene nanoribbons (OGNRs), decorated with folic acid, and loaded with tamoxifen citrate to fight against breast cancer cells (Abu Lila et al., 2021). Moreover, Adhikari et al. (2021) made an electrochemical immunosensor for haptoglobin detection compose of polysaccharide chitosan and graphene nanoplatelets (GNPs/Chi), since elevated levels of that protein in blood have been related to several disorders (cancer, diabetes mellitus, etc). As it can be appreciated, most NMs used in these therapeutical approaches (including GBNMs) enter in the systemic circulation, when they are administered

intravenously, and come into direct contact with blood cells and plasma proteins, among other components. This would affect their structures and functions leading, in many cases, to toxicities or other potential harmful interactions. Thus, the assessment of the GBNMs' induced immunotoxicity is crucial for the safe use of medical diagnostic nanoproducts and, therefore, appropriate experiments are required.

In the second chapter of this Thesis, we have used the same whole blood *ex vivo* model that we presented in the first chapter to help to determine the hemocompatibility of three different types of GBNMs: graphene oxide (GO), graphene nanoplatelets (GNPs), and graphene nanoribbons (GNRs). Previously, this model was used in other studies with GBNMs, such as the one carried out by Orecchioni et al. (2017), where they showed that the presence of amino groups on GO reduced the perturbations caused by GO on immune cell metabolism, increasing their biocompatibility. Similarly, Orecchioni et al. (2020) also presented a novel approach (cytometry by time-of-flight) to show the immune interactions of functionalized-GO with different populations of immune cells, as a guide for future immune assessments. Thus, our approach is an already validated tool to evaluate different effects derived from the exposure to GBNMs. Furthermore, this model, besides demonstrating an immune response towards the compound, also allows knowing which nanomaterial is more biocompatible among those proposed.

The effects on the immune system triggered by GBNMs have been extensively explored with contradictory results. Some *in vivo* studies report strong immune responses to these compounds: pristine nGO and nGO-PEGs (graphene oxide nanosheets polyethylene glycol-functionalized), stimulating powerful cytokine responses in peritoneal macrophages from exposed C57BL/6 mice. Although the nGO-PEGs were not internalized inside the cells, both forms increase the secretion of IL6, MCP1, IFNγ, TNFα, and IL-12 (Luo et al., 2017). Similarly, an immunological response, with elevated chemokine secretion and enhanced expression of cytoskeletal-related genes, was found in whole blood samples collected from mice intratracheally instilled with GNPs (Park et al., 2017). Also, an *in vitro* study showed that the levels of three cytokines (TNF-α, IL-1β, and IL-6) were increased in primary dendritic cells treated with 100 μg/mL of GO. This exposure also induced apoptosis in T-lymphocytes in a dose-dependent manner, as measured by the Annexin V+/PI+ assay, as well as black dense aggregates within the macrophages (Zhi et al., 2013). Likewise, *in vitro* immunotoxicity of GO was reported in dendritic cells after exposure to mono-GO and multi-GO layered materials. Multi-GO caused stronger toxicity than mono-GO, but both triggered the production of ROS and the release of TNFα (Yang et al., 2020). However, contrary to the results previously indicated, an *in vitro* study

made by our group indicated a low immune response against GO and GNPs exposure, in an *in vitro* model of the intestinal barrier (Domenech et al., 2020).

To further explore the previous contradictory results, GBNMs effects have been analysed at the level of the human blood secretome. The first remarkable observation is that all types of exposures have a noticeable impact on the blood secretome. On the one hand, as we previously explained in the first chapter, the indirect soft-agar assay was conducted to unravel the functional impact of the secretome of the samples treated with GBNMs, over the growth of another cell line. The results revealed a decrease in the number of colonies in all the exposures, being the GO exposure the most pronounced one, followed by GNPs, GNRs exposures being the less inhibited. To have more detailed information on these secretome changes, the effects in a wide panel of cytokines were evaluated. The cytokines' array revealed 20 common cytokines deregulated in the three types of exposures. In general, our study coincides with other studies where it was observed a pro-inflammatory effect of GBNMs (Löfdahl et al., 2020). Among the different pro-inflammatory cytokines deregulated we can point out IL1 (α and β), CCL3, TGFA, MCP-3, TNFA, IL8, MCP1, MCSF, ITAC, ENA78, CCL20, and CXCL1. Some of these cytokines (CXCL1, CCL20, TNFA, and CCL3) are among those most upregulated in our study. Moreover, the upregulation of these cytokines is a potential indicator of an immune and inflammatory response. CCL3 is a chemotactic chemokine secreted by macrophages, participating in the inflammatory response. Lategan et al. (2018) performed a proteome profiling analysis in whole blood cultures exposed to 5 μg/mL GO and founded that IL-8, CCL3 (MIPα), MCP-1, and IL-1ra were upregulated. All these proteins are proinflammatory except for IL-1ra, acting as an anti-inflammatory cytokine blocking the effects of IL-1β by binding to its receptor. IL1β is released primarily by monocytes and macrophages as well as by non-immune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation (Zhang et al., 2007). This cytokine was founded overexpressed in a dose-dependence manner in spleens from zebrafish treated with different concentrations of GO. Additionally, and according to our results, the same study showed an increase in the expression of TNFA and IL6, which were overexpressed in the GO and GNPs exposures, respectively (Chen et al., 2016). Regarding the IL6 cytokine, it plays role in chronic inflammation, which is closely related to chronic inflammatory diseases (rheumatoid arthritis, Crohn' disease, psoriasis), autoimmune disorders, and cancer (Gabay et al., 2006; Hirano et al., 2021). Apart from IL6, another important cytokine that is also over-expressed in GO and GNPs exposures is G-CSF. This cytokine regulates neutrophil development and function, necessary for the innate immune system to fight against bacterial pathogens. Along with other cytokines (IL-1α, IL-6, IL-10, TNF-α, MCP-

1, MIP-1α, MIP-1β, and RANTES), G-CSF was found to be significantly overexpressed in primary murine macrophages and immortalized macrophages after the exposure to subtoxic concentrations of pristine graphene (Schaaij-Visser et al., 2013).

Specific graphene cytokines were also observed. Thus, IL-10 only appeared overexpressed in GNPs exposure. This is an anti-inflammatory cytokine that inhibits the activity of Th1 cells, NK cells, and macrophages during infection. Moreover, is likely to have an important role in limiting the immune response to NMs that may otherwise cause injury and inflammation (Pondman et al., 2016). In this exposure type, other anti-inflammatory cytokines were also observed such as ST2 and TFF3. It seems that TFF3 promoted a protective effect against inflammatory states, like in colitis disease, reducing the levels of TNF-α expression in the colonic endothelium (Teng et al., 2009). On the other hand, the lungs of C57BL/6 mice intravenously injected with graphene nanosheets triggered an increased expression of IL-33 and ST2. That study evidence that ST2 displays an important role in the immunotoxicity induce by GBNs (Brown et al., 2013).

Regarding GNRs exposure, it is important to highlight the overexpression of MMP9. The increased expression of MMPs is observed associated with the onset of cancer. Specifically, they have been related to cell invasion and metastasis (Kessenbrock et al., 2010). Moreover, MMPs have been found up-regulated in almost every type of human cancer, and their expression is often associated with poor survival (Di Nezza et al., 2002). Therefore, attention must be paid to the overexpression of MMP9 after GNRs exposure.

Since the three compounds tested did not present noticeable immune differences among them, we cannot decide which presents more biocompatibility. Nevertheless, a significant number of *in vivo* studies corroborate the increased expression of many of the altered cytokines observed in our study (Schinwald et al., 2012; Brown et al., 2013; Luo et al., 2017) and, therefore, reaffirm the suitability of our *ex vivo* study as an alternative tool to detect harmful effects associated to GBNMs. Normally a balance between pro and anti-inflammatory cytokines is maintained in the body. However, when immune cells encounter an antigen, this balance is shifted and macrophages and monocytes are activated to release different cytokines (Barton et al., 2008). In our study, many of these cytokines have a pro-inflammatory effect and are also released by monocytes. Thus, although this approach was not followed up in this study, as we previously indicated in the first chapter, it will be interesting to study the WBCs populations and especially, the effects on monocytes, since they seem to be the most stimulated ones.

4.3.3. Nanoceria, alone or in combination with cigarette-smoke condensate, induce transforming and epigenetic cancer-like features in vitro

Nanoceria has been extensively studied for biomedical purposes (Xu and Qu, 2014). For example, it has been engineered as a promising therapeutic agent to treat bone cancer (Alpaslan et al., 2015), or as biosensor for detecting lactate in human blood samples to clinical diagnosis of several diseases such as hypoxia, acute heart disorders, lactic acidosis, muscle fatigue, and meningitis (Nesakumar et al., 2013). All these studies point out the powerful antioxidant properties of nanoceria, which arise from the ability of cerium to release or acquire oxygen depending on the environmental conditions. However, there exist several discrepancies regarding their health implications, since some studies reported their ability to cause oxidative stress and DNA damage. For instance, BEAS-2B cells exposed to different sizes and concentrations of nanoceria showed ROS increase, GSH (glutathione) decrease, and the induction of oxidative stress-related genes linked to the apoptotic process (Park et al., 2008). Likewise, this disruption of the redox balance was shown to be one of the most important factors for cancer initiation and progression in human cells (Aggarwal et al., 2019). In this sense, our third study demonstrates the transforming abilities of nanoceria after a long-term exposure scenario and, although we have not studied the induction mechanisms of this transformation, one hypothesis could be related to the redox potential of nanoceria.

Only two previous studies have used *in vitro* long-term methodologies for the evaluation of carcinogenic effects of nanoceria. The first one consists of sub-chronic exposure to 0.18 μg/cm^2 or 0.06 μg/cm^2 of nanoceria concentrations in human primary small airway epithelial cells (pSAECs) for ten weeks (Stueckle et al., 2017). The results showed no significant signs of cell transformation. Similarly, a study carried out by our group at low-dose and long-term exposures showed that nanoceria did not elicit any carcinogenic feature in BEAS-2B cells, when different features associated with early stages of the carcinogenic process were evaluated (Rubio et al., 2017). In our study, where BEAS-2B cells were exposed to 2.5 μg/mL of CeO_2NPs for six weeks, cells showed an acquired invasive potential and an enhanced tumorspheres phenotype, two features associated with the late stages of the carcinogenic process. Moreover, the internalization of nanoceria was confirmed by TEM, showing that CeO_2NPs are mostly internalized in vacuoles. Likewise, other short-term studies confirm the uptake by clathrin-mediated and caveolae-mediated endocytic pathways, and its co-localization in the mitochondria, lysosomes, and endoplasmic reticulum, as well as in the cytoplasm and the nucleus (Singh et al., 2010; Vassie et al., 2017).

Normally, in our daily life, we are not exposed to a single pollutant but a combination of several. These mixtures may interact with each other, having different effects (additive, synergistic, or antagonistic effects) compared to single exposures. Hence, when the carcinogenicity of a compound is evaluated, we can find that although alone it is not carcinogenic, its combination with other compounds can give rise to mechanisms that induce a tumour phenotype (Goodson et al., 2015). Regarding nanoceria, co-exposures with other environmental agents have already been analysed. Thus, Snow et al., (2014) discover that fuel diesel in combination with nanoceria induced more adverse pulmonary effects than the exposure to both compounds separately. The authors combined both compounds since nanoceria is frequently commercialized as a diesel additive for increasing fuel combustion efficiency while decreasing soot emissions. Therefore, these results reinforce our proposal that studying co-exposures novel and unexpected biological effects can be revealed.

Nanoceria is present in the environment due to their use as a fuel additive (Cassee et al., 2011), or in industrial activities (Li et al., 2017). In this scenario, there are many probabilities of coexistence with tobacco components which are well-studied carcinogens mainly targeting the lungs, as nanoceria (Saha et al., 2007). Thus, in the study of Rubio et al. (2017), the interaction of nanoceria with CSC was confirmed, demonstrating that co-exposure enhances the carcinogenic potential of CSC. Smoke tobacco is a mixture of chemical compounds having different MOAs to induce carcinogenesis (Nagah and Amer, 2021). Among them, ROS production and oxidative stress-induction are the most studied mechanisms of cell transformation (Valavanidis et al., 2009). The purpose of this study was to demonstrate whether CeO_2NPs have a protective role in the production of ROS generated by CSC. As we have previously commented, nanoceria has antioxidant properties and, therefore, it could decrease the induction of the tumoral phenotype produced by the CSC. Surprisingly, what it was found was quite the opposite, since CeO_2NPs enhanced the tumoral effects of CSC. In this sense, the results of the third chapter of this Thesis confirmed the previous observations, highlighting the importance of co-exposures study.

Among the different effects evaluated in the co-exposure nanoceria/CSC stand invasion. The invasiveness properties are characteristic of the late stages of cell transformation. Our results showed invasive properties of cells exposed to both compounds by separate, as well as in co-exposures, although this feature was more increased in the co-exposure conditions. Even though we have not found information regarding the invasive properties of nanoceria, rather the opposite (Alili et al., 2011). Contrarily, CSC has been shown to increase cell invasion in several published studies

(Nagaraj et al., 2007; Di Cello et al., 2013). Thus, these findings demonstrate that when both compounds are applied together, they have a positive interaction increasing their carcinogenicity.

Further, the capacity of nanoceria, CSC, and the co-exposure to induce the growth of cancer stem cells (CSCs) was evaluated. The induction of CSCs from normal differentiated cells is gaining importance as an oncogenesis driving mechanism that contributes to tumour aggressiveness (Chengizkhan et al., 2020). Waterpipe tobacco smoke exposure in normal and lung cancer cell-lines triggered changes in the cell morphology moving to a more elongated mesenchymal shape and inducing increased stemness biomarkers (Zaarour et al., 2021). Similarly, Sun et al., (2020) demonstrated that cigarette smoke was able to induce an increase in the tumorspheres formation and upregulation of stemness markers in bladder cell-lines. Contrary to what was expected, our results did not show any increase in the tumorspheres formation after CSC exposure, but the nanoceria and the co-exposures increased the proportion of cells exhibiting stem-like features. Probably, the induction mechanisms of a tumoral phenotype are not the same between nanoceria and CSC.

Alterations at the epigenetic level may be another of the mechanisms explaining the tumor phenotype observed after different exposures. Thus, in the context of this Thesis, a battery of microRNAs related to the carcinogenic process was used. Among the microRNAs contained by this battery, those most overexpressed after the co-exposure were miR-21, miR-23a, miR-96, and miR-505. MiR-21 and miR-23a are two common oncogenes that have appeared dysregulated in many cancers, including lung cancer, and they have been proposed as early biomarkers for the detection of non-small cell lung cancer (Hetta et al., 2019). Moreover, many of the microRNAs founded deregulated can functionally influence some of the phenotypical cancer features evaluated in the present study, such as the invasiveness and stem-like cell features. MiR-96 has been overexpressed in all the exposures. This is a well-known oncogene that appeared highly upregulated in different kinds of cancers, such as breast cancer or lung cancer (Fei et al., 2018; Hong et al., 2016). These studies point out that miR-96 promotes the invasion and metastasis processes by targeting different genes. Regarding miR-210 expression, it is mainly overexpressed in the nanoceria and the co-exposure treatments. This microRNA appeared highly expressed under hypoxic conditions in cancer-derived exosomes enhancing the stemness of normal cells (Kling et al., 2020). In correlation to our study, the high overexpression of this microRNA in these two exposures could be one of the differential mechanisms that justify the significant presence of tumorspheres in these two treatments, compared to the CSC exposure.

4.3.4. MicroRNAs as a new biomarker to detect the effects of long-term exposures to nanomaterials. Studies on TiO_2NPS and MWCNTs

As previously described, NMs have properties that make them more biologically reactive, and, therefore, an accurate assessment of their associated exposure risks is demanded. Among the different models used in hazard assessment, most of the studies focus on short-term and acute doses conditions. However, the most realistic exposure conditions occur over long periods at low concentrations. Accordingly, *in vitro* exposure conditions should be designed to mimic as much as possible such exposure scenarios. In fact, *in vitro* long-term models offer multiple advantages as referred to in a previous section (4.1.2) and highlighted by different authors (Comfort et al., 2014; Huang et al., 2021).

Long-term exposure models offer the possibility of studying the phenotypic and molecular changes occurring when a tumour phenotype develops. Within these models, *in vitro* CTAs are useful to identify potential carcinogens (not necessarily genotoxic) since they mimic different stages of the *in vivo* neoplastic process (Creton et al., 2012). Thus, the two explored NMs used in the 4th chapter, TiO_2NPs and MWCNTs, induced significant increases in some cell-transforming biomarkers after an *in vitro* long-term exposure to subtoxic concentrations, as indicated by the previous studies carried out in our Group (Vales et al., 2015, 2016). In these studies, TiO_2NPs did not induce genotoxic effects, as measured by the comet and the micronucleus assays, but increased the number of colonies growing in soft-agar medium. In the case of MWCNTs, they induced an increase in the attachment-independent growth, as well as in the levels of DNA damage (observed in the micronucleus assay), and the levels of ROS. It must be highlighted that anchorage-independent cell growth is considered one of the most relevant cancer hallmarks biomarker (Vanparys et al., 2012), indicating the potential carcinogenic risks associated with both NPs, although in the case of TiO_2NPs this would be mediated by a non-genotoxic mechanism.

However, those CTAs used to evaluate transforming features (like the soft-agar assay) do not provide information on the MOAs and, therefore, additional tests are needed to further explore the different triggering mechanisms of the induced tumour process (Hwang et al., 2020). Among the diverse MOAs involved in the tumoral process stand the epigenetic alterations that include DNA methylation, histone modification, non-coding RNA expression, and chromatin remodeling. From previous studies, we know that long-term exposure to MWCNTs can induce global DNA methylation changes in BEAS-2B cells (Sierra et al., 2017). To keep the focus on epigenetic mechanisms, changes in the microRNA's expression of a microRNA's battery related to the acquisition of the tumoral phenotype were evaluated aiming to propose new biomarkers of the carcinogenic

transformation process associated with NMs exposure. MicroRNAs dysregulation is characteristic from many human cancers (Karkhane et al., 2020). It causes aberrant expression of their target genes and, consequently, disrupts several pathways related to cancer, including cell growth, apoptosis, metabolism, and invasion (Zhu et al., 2021).

MicroRNA deregulation has been reported as a novel biomarker of environmental agents' exposure (Sisto et al., 2019). Moreover, such altered expression can be used as a biomarker of the adverse effects induced by these agents, including cancer. In our model, exposures to TiO_2NPs and MWCNTs revealed a significantly altered expression in our battery of microRNAs. From the exposure to TiO_2NPs, 29% appeared deregulated at both weeks (3 and 6) and most of them presented significant overexpression except for miR-541, which is a well-known tumour suppressor miR whose overexpression inhibits lung cancer cells proliferation and invasion by directly targeting HMGA2, an acknowledged oncogene (Xu et al., 2018). The exposure to MWCNTs revealed a higher percentage of inhibited microRNAs at both times, with the inhibited miR-541 appearing again. Interestingly, at both weeks, miR-21 presented a high expression. This overexpression is very significant because it represents an intensively studied oncomiR, which deregulation has been reported in many cancers (Feng et al., 2016).

From all the previous changes observed we selected a set of microRNAs (miR-23a, miR-25, miR-96, miR-210, and miR-502) whose expression was elevated in most of the exposures. This set was tested in BEAS-2B and MEF cell lines exposed to different NMs, including ZnONPs, CoNPs, MSiNPs and CeO_2NPs, to validate them as potential biomarkers of NM's effects. All of them were oncomiRs and participate in important pathways related to signal transduction, adherent junctions, and cell cycle arrest, typically deregulated in cancer cells. Interestingly, all microRNAs appeared significantly overexpressed in all the exposures, but cobalt exposure had the biggest impact, and it correlates with its biological significance, since, as we reported in the 4.1.2. Discussion section, their transforming properties were confirmed by Annangi et al., 2015. Likewise, microRNAs expression after ZnONPs exposure, although significant, it was not as substantial as CoNPs exposure, correlating with the previous results, also reported in the mentioned discussion section, where there was a lack of genotoxic and carcinogenesis effects after the long-term exposure to ZnONPs (Annangi et al., 2016). Thus, from our results, we propose this set as potential biomarkers representative of the epigenetic signature induced by NMs' exposure. Also, several previous reviews proposed microRNAs as sensitive indicators of environmental pollutants exposure, including NPs' exposure. Some of the microRNAs collected in this battery appear in these reviews, such as miR-23a, which appeared overexpressed in *in vivo* studies from rats' livers exposed to

perfluorooctane sulfonate, or miR-25 which was overexpressed in the sputum of humans exposed to ozone polluted air (Vrijens et al., 2015; Kotsyfakis et al., 2019; Cheng et al., 2020).

Finally, although we do not know whether microRNAs are the keys to start a tumoral process or the markers of an advanced cancer process, they hold a great potential to be used as biomarkers in the field of environmental risk assessment. Moreover, they can help to elucidate the MOAs of different environmental pollutants. Therefore, further studies need to be accomplished to elucidate the relationship that exists between microRNAs altered expression and their effects that contribute to disease development.

5. CONCLUSIONS

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According to the objectives of this Thesis, to propose new approaches and end-points for hazard assessment, we have reached the following conclusions:

1. The whole blood *ex vivo* exposure approach has shown to be useful to detect different harmful effects induced by PSNPLs and GBNMs exposures.

Regarding PSNPLs effects:

- the different cell sub-types of WBCs present significant differences in the uptake levels. Monocytes showed the highest particle internalization levels, intermediate uptake levels were observed in PMN cells, and practically no internalization was observed in lymphocytes.
- PSNPLs can negatively affect the different cell sub-types of WBCs, showing a clear different cell sensitivity regarding DNA damage induction. Monocytes and PMN cells showed significant levels of DNA damage, while lymphocytes did not present such damage, as measured with the comet assay.
- Significant secretome alterations were detected according to the PSNPLs exposure. Such changes were mainly related with the inflammatory and the immune response.

Regarding GBNMs effects:

- A high immunological response was detected after the exposure to the three GBNMs tested (graphene oxide, graphene nanoribbons, and graphene nanoplatelests) using a panel of 105 cytokines.
- Our results also indicate that the GBN-induced altered secretome can modify the natural anchorage-independent growth capacity of HeLa cells, used as a model.

2. Long-term low-dose exposure to nanoceria induced cell transformation in Beas-2B cells. Interestingly nanoceria showed a positive interaction with CSC, enhancing their carcinogenic potential.

3. Nanoceria, CSC, and the co-exposure intensify the expression of a wide set of microRNAs, producing relevant changes in the microRNAs signature of the exposed cells. These changes were more evident in the nanoceria-CSC co-exposure.

4. Long-term TiO_2NPs and MWCNTs exposure induced significant changes in the expression of different microRNAs directly associated with the transformation process.

Among them, five microRNAs are proposed as representative of the epigenetic alterations induced by NMs, once confirmed with cells long-term exposed to CoNPs, ZnONPs, CeO_2NPs, and MSiNPs.

6. ANNEXES

6. ANNEXES

6.1. ANNEX 1: chapter 4

MicroRNAs as a new biomarker to detect the effects of long-term exposures to nanomaterials. Studies on TiO_2NPS and MWCNTs

Submitted paper

7. REFERENCES

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