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Mutation Research-Reviews in Mutation Research

journal homepage: www.elsevier.com/locate/mutrev

Insights into the potential carcinogenicity of micro- and nano-plastics

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ARTICLE INFO

Keywords:

Carcinogenesis
Genotoxicity
Microplastic
Nanoplastic
Mammalian models

ABSTRACT

There is a growing concern regarding the potential health effects that continuous exposure to environmental micro- and nano-plastics (MNPLs) may cause on humans. Due to their persistent nature, MNPLs may accumulate in different organs and tissues and may induce in the long term the development of cancer. The present study aimed to review the existing literature on the carcinogenic potential of MNPLs. As studies directly assessing carcinogenicity were expected to be scarce, studies dealing with indirect outcomes associated with the carcinogenic process were considered in the literature search. Of the 126 studies screened, 19 satisfied the inclusion criteria. Besides, 7 additional cross-referenced articles, identified through a careful reading of the previously selected papers, also met the inclusion criteria and, consequently, were included in the review. Most of the selected studies were performed using *in vitro* models whereas about 40% of the studies were done in rodents, although none of them included a 2-year carcinogenicity assay. Most of the reviewed studies pointed out the potential of MNPLs to induce inflammation and genotoxicity, the latter being recognized as a strong predictor of carcinogenicity. These, along with other important findings such as the MNPLs' ability to accumulate into cells and tissues, or their capacity to induce fibrosis, may suggest an association between MNPLs exposures and the carcinogenic potential. Nevertheless, the limited number of available studies precludes reaching clear conclusions. Therefore, this review also provides several recommendations to cover the current knowledge gaps and address the future evaluation of the MNPLs' carcinogenic risk.

1. Introduction

Great interest has been placed on micro- and nanoplastics (MNPLs) as emergent pollutants in the last decade. Although no consensus has been reached regarding the size range for MNPLs [1], plastic particles up to 100 nm are considered nanoplastics (NPLs) while microplastics (MPLs) include particles up to 5 mm [2,3]. Either due to weathering and fragmentation of larger plastics (secondary MNPLs), or mass-production of sized MNPLs for specific purposes (primary MNPLs), the environmental ubiquity of MNPLs poses a health challenge. Their vast distribution throughout the environmental niches (airborne, waterborne, or terrestrial) makes humans susceptible to being unavoidably and continuously exposed *via* different routes such as ingestion, inhalation, or dermal contact [4].

The intrinsic physicochemical characteristics of MNPLs allow them to be uptaken by cells and interact with cellular components. Their reactive surfaces have been reported to cause harmful effects such as cytotoxicity, oxidative stress, and disruption of immune function [5,6]. In addition, due to their persistent nature that limits the clearance from the body, translocation to different tissues and bioaccumulation are also possible. The potential effects on human health of MNPLs have been assessed using different approaches, including a variety of *in vitro* and *in vivo* mammalian models [5,7,8]. Among the outcomes studied, MNPLs' ability to induce oxidative stress, inflammatory responses, genotoxicity, or fitness disturbances are the most highlighted. Hence, the chronic nature of human exposure to MNPLs, along with some of the observed MNPLs-induced effects, raises concern about the potential carcinogenicity of MNPLs.

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<https://doi.org/10.1016/j.mrrev.2023.108453>

Received 16 September 2022; Received in revised form 14 December 2022; Accepted 31 January 2023

Available online 2 February 2023

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Carcinogenesis is a multistage process that consists of three consecutive steps: initiation, promotion, and progression. During the initiation step, a normal cell undergoes a non-reversible genetic change that enables it for autonomous growth. Then, the abnormal clonal expansion of the initiated cell occurs during the promotion stage. Progression is the last stage and consists of the transformation of the neoplastic lesion into an invasive malignant tumor [9]. From a mechanistic point of view, carcinogens can act as initiators, if they are able to produce mutations in a normal cell, or as promoters, if they enhance the carcinogenic process but not necessarily through mutagenic activity [10]. Accordingly, carcinogens are classified into genotoxic carcinogens and non-genotoxic carcinogens [11]. Chemical substances and agents can be genotoxic through a primary mechanism, executed by the compound on the target cell, or by a secondary mechanism involving an inflammatory response that cause downstream secondary effects in the target cell. The primary mechanism can involve a direct interaction of the material with DNA, or an indirect effect mediated by other molecules (e.g., induction of reactive oxygen species (ROS), inhibition of DNA repair mechanisms, etc.) [12,13]. On the other hand, the mechanisms behind non-genotoxic carcinogenicity are diverse or even unknown, such as immune suppression, mitogenic signaling, receptor-mediated endocrine modulation, or epigenetic changes, among others [11,14,15].

Independently of the mechanism of action associated with MNPLs exposure, the assumption of a potential carcinogenic risk triggered by MNPLs is supported by previous experience with other nanomaterials (NMs). As an illustration, carcinogenicity has been demonstrated in mice and rats after the administration of single- and multi-walled carbon nanotubes, respectively [16,17]. Aside from direct carcinogenic effects, NMs have also been described to induce tumor progression by promoting the growth of metastatic tumors or causing DNA damage and elevated mutation rates [18]. These antecedents allow us to roughly draw the carcinogenesis process accomplished by NMs exposure. Nevertheless, it remains an open question as to whether the induced effects are due to the nano size, the chemical composition, or a combination of both.

The hazard evaluation of MNPLs is in its infancy, especially when referring to their carcinogenic risk. Thus, it is anticipated that at present no standardized studies have been carried out to detect the potential carcinogenic risk of MNPLs, which is the central point of this review. However, some publications in the literature have associated MNPLs exposure with different biomarkers linked to the carcinogenesis process such as chronic inflammation, genotoxicity/mutagenicity, and fibrosis. Consequently, such biomarkers can result useful when the potential carcinogenic risk is under consideration [19,20]. We can find examples of these carcinogenesis-related biomarkers for other NMs. In this case, as

illustrated in Fig. 1, when NMs establish contact with epithelial and immune cells (1), increased synthesis of pro-inflammatory cytokines is induced (2) [18]. The inflammatory response's purpose is to remove aggressive agents and repair lesions or damaged tissue, and for that, the recruitment of leukocytes into the injured tissue is accomplished (3) [21]. Under this scenario, reactive oxygen species (ROS) and reactive nitrogen species (RNS) would be produced (4) and would cause DNA damage and damage to proteins and lipids, which would end up in tissue damage (5). Stem cells activated for tissue regeneration may eventually be damaged by ROS and RNS, and gene mutations can be accumulated, generating cancer stem cells (6) [22]. Chronic inflammation may occur if the injury becomes persistent, which would induce the harm and healing of damaged tissue, driving to fibrosis and malignancy (7) [21]. Other signaling pathways promoting invasion and pro-metastatic immune responses would be activated, and signals to prevent autoimmunity and exacerbate immune responses, as well as the epithelial-mesenchymal transition would be stimulated (8) [18,23]. Furthermore, and as above explained, unrepaired/misrepaired DNA damage may raise mutations playing a crucial role in the initiation of carcinogenesis [24]. Therefore, genotoxicity is assumed to be an early predictor of the carcinogenic potential of a compound [25]. The OECD guideline for the testing of chemicals recommends assessing the genotoxic potential due to its veritable close association with cancer development [26]. Considering the role of genotoxicity as a surrogate biomarker of carcinogenicity, special emphasis is placed on the MNPLs' genotoxic potential in this review.

The present review aims to investigate the potential carcinogenic risk of MNPLs exposure based on the existing literature. Due to the lack of studies directly assessing carcinogenicity, studies dealing with indirect outcomes associated with the carcinogenic process have been considered. Additionally, a list of recommendations to cover the current knowledge gaps and address the future evaluation of the MNPLs' carcinogenic risk is proposed.

2. Search strategy

PubMed database was selected to carry out the bibliographic search. The search string was built up considering indirect outcomes related to carcinogenesis and key characteristics of carcinogens, as those reported by Smith et al. [15,27]. The advanced search builder was used by entering the following keywords included in the title/abstract: (microplastic* OR nanoplastic*) AND (biopersisten* OR tumor* OR carcinogen* OR mesothelioma OR "neoplastic transformation" OR "malignant transformation" OR fibrosis OR "chronic inflammation" OR genotoxic* OR "long term toxicity" OR "long-term toxicity" OR "DNA

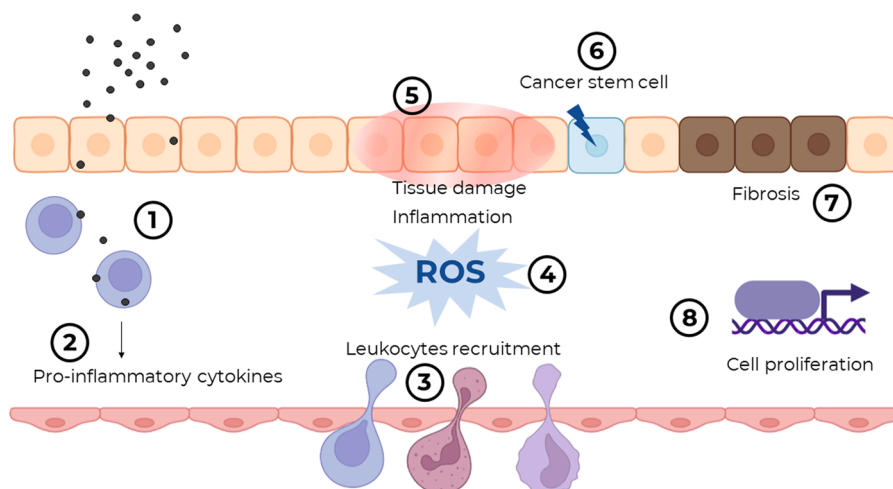


Fig. 1. Schematic representation of the carcinogenesis process triggered by nanomaterials exposure. Numbers indicate the process described in the text.

repair” OR “genomic instability” OR epigenetic* OR immunosuppressive OR immortalization OR “DNA methylation” OR “histone acetylation”). All the articles published until the 21st of January 2022 were selected. A total of 126 publications were obtained and were evaluated for acceptability.

Firstly, the following inclusion and exclusion criteria were applied to select the articles after reading the abstracts:

Inclusion criteria:

- The article contains information on mammalian cells, tissues, or whole organisms’ effects that could be related to the induction of carcinogenesis by MNPLs.

Exclusion criteria:

- The full text was not available in English
- The article was a review, a comment, or a meta-analysis
- The field of the article was out of the scope of this review
- The article was based on environmental and ecotoxicity studies

In case of doubt, articles were in-depth analyzed, which implied reading the full article. After the described process, 19 articles were finally selected.

The search by the above-described terms resulted in a limited number of studies related to human carcinogenesis. Therefore, 7 additional cross-referenced articles meeting the inclusion criteria were identified after a careful reading of the previously selected papers.

3. Results

As described above, 126 articles were retrieved. Two outcomes were discarded since they were a retracted article and the corresponding retraction notice. From the resulting 124 outcomes, and as illustrated in Fig. 2A, review articles (23, including 1 commentary and 2 meta-analysis articles) accounted for 19% of the total results, while the rest of the publications (101) were research articles. From these research articles, 6% of the publications (included in the category “others” in Fig. 2) mainly focused on MNPLs’ environmental sampling and characterization, sorption and desorption behavior of different agents, or separation and degradation methods, among others. Research articles focused on ecotoxicity models accounted for 54% of the total search results, while only 21% of the articles were research studies using *in vitro* or *in vivo* mammalian models. These results highlight the current focus on environmental risks regarding MNPLs’ studies, more than focusing on human health effects. Accordingly, most of the articles were excluded after applying the inclusion/exclusion criteria, and only 19 articles resulting from the systematic search were selected for further discussion. Seven additional cross-referenced articles were included during the review process as they fitted to the inclusion criteria. Thus, 26 articles are discussed in the following sections. As shown in Figs. 2B, 1 of the

discussed articles included both *in vitro* and *in vivo* studies (4%), while 15 articles (58%) were performed using *in vitro* models and 10 papers (38%) included only *in vivo* approaches.

As expected, no studies evaluating the incidence of tumors and cancer in humans exposed to MNPLs, or studies assessing such incidence in rodents, were found in our literature search. Therefore, in this review we have focused on reviewing those studies involving different biomarkers related to the carcinogenicity process. As genotoxicity is a surrogate biomarker of carcinogenicity, we also reviewed whether MNPLs can induce DNA damage. Furthermore, epigenetic alterations also play an important role in carcinogenesis. However, no studies addressing this topic were found.

3.1. *In vitro* studies on carcinogenicity-related effects

A summary of the studies in this section is presented in Table 1.

Forte and co-workers described increasing expression of pro-inflammatory cytokines IL-1 β , IL-6, and IL-8 after 1 h-exposure of human gastric adenocarcinoma epithelial cells (AGS) to polystyrene nanoplastics (PSNPLs, 44 nm, 10 μ g/mL) [28]. Moreover, a slight increase in *c-Myc* oncogene expression was also induced by the PSNPLs. It must be pointed out that *c-Myc* is closely related to the metabolic transformation accomplished by transformed cells (e.g., mitochondrial biogenesis, rRNA and protein biosynthesis, or glycolysis stimulation) [29]. The AGS cells exposed to both 44 and 100 nm PSNPLs (10 μ g/mL) showed increased levels of *TGF-1 β* and decreased levels of *NF-k β 1* mRNA, suggesting an upregulated metabolic transformation not linked to proliferation induction [28]. Similarly, a significant up-regulation in the *IL-6*, *IL-8*, *NF-k β* , and *TNF- α* expression was observed after 8 h of exposure of A549 cells to PSNPLs (25 and 70 nm, 25 μ g/mL and 160 μ g/mL, respectively) [30]. However, the expression of *IL-1 β* was not altered by any of the PSNPLs, whatever their size. The expression of genes involved in cell proliferation (*cyclin D*, *cyclin E*, and *Ki67*) was also upregulated after the exposure.

Shi and co-workers evaluated the expression of *IL-1 β* , *IL-6*, *IL-8*, and *TNF- α* , which are associated with the pro-inflammatory response [31]. In their study, A549 cells were exposed to PSNPLs (~100 nm; 20 and 200 μ g/mL) alone or in combination with 5 μ g/mL of two of the most common plasticizers (dibutyl phthalate, DBP, and di-(2-ethylhexyl) phthalate, DEHP) for 24 h. The expression of the pro-inflammatory cytokines analyzed was significantly increased after both plasticizers’ exposures alone and in combination with the highest concentration of PSNPLs. Interestingly, the combination of low doses of PSNPLs with plasticizers decreased the plasticizers’ pro-inflammatory effects, and low doses of PSNPLs alone did not induce changes in the expression by themselves. Contrary, the highest concentration of PSNPLs increased the inflammatory response compared with the untreated control. The generation of oxidative stress, as well as the levels of the product of lipid peroxidation malondialdehyde (MDA), measured in the cells after the same exposure conditions, followed the same trend as the expression of

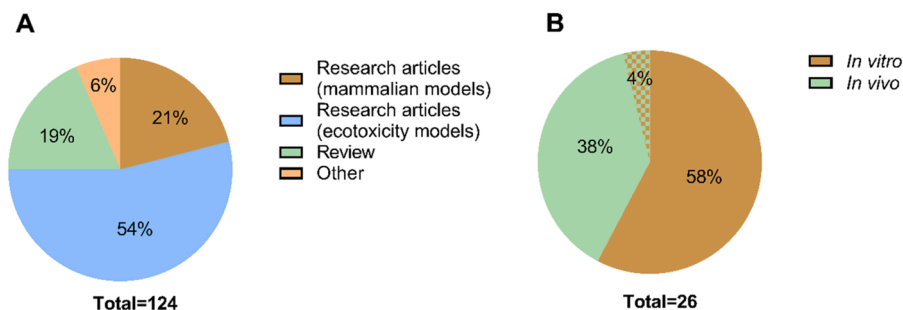


Fig. 2. Overview of the results. Summary of the total search string outcomes (A) and the revised articles included in the review (B). The percentage of articles in each category over the total is indicated inside the different sections represented in the figure. In A, the categories describe the type of article, whereas in B, the categories refer to the use of *in vitro/in vivo* approaches in mammalian models.

Table 1
In vitro studies assessing carcinogenicity-related effects of MNPLs.

Material	Size (μm)	Cell line	Concentration range (time of exposure)	Uptake	Carcinogenicity-related endpoint (method)	Outcome	Ref.
PSNPLs	0.044, 0.1	AGS	10 $\mu\text{g}/\text{mL}$ (1 h)	Proved (10, and 60 min)	Inflammatory response (RT-qPCR)	Increased expression of TGF β 1 (0.044, 0.1 μm), IL-1 β , IL-6, and IL-8 (0.044 μm) and decreased expression of NF- κ B1 (0.044 and 0.1 μm)	Forte et al. [28]
					Proliferation (RT-qPCR)	Increased expression of c-Myc (0.044 μm), decreased expression of p38 (0.1 μm) and decreased expression of Ki67 (0.044 and 0.1 μm)	
PSNPLs	0.025, 0.07	A549	0.025 μm : 25 $\mu\text{g}/\text{mL}$, 0.07 μm : 160 $\mu\text{g}/\text{mL}$ (8 h)	Proved (0.025 μm : 1.14 $\mu\text{g}/\text{mL}$, 0.07 μm : 25 $\mu\text{g}/\text{mL}$; 10 min - 2 h)	Inflammatory response (RT-qPCR)	Increased expression of IL-6, IL-8, NF- κ B, and TNF- α	Xu et al. [30]
					Proliferation (RT-qPCR)	Increased expression of CCND (cyclin D), CCNE (cyclin E), and Ki67	
PSNPLs	-0.1		20, 200 $\mu\text{g}/\text{mL}$ (24 h)	Proved (20 $\mu\text{g}/\text{mL}$)		Increased expression of IL-1 β , IL-6, IL-8, and TNF- α (200 $\mu\text{g}/\text{mL}$)	
PSNPLs/DBP	-	A549	20, 200/5 $\mu\text{g}/\text{mL}$ (24 h)	-	Inflammatory response (RT-qPCR)	Increased expression of IL-1 β , IL-6, IL-8, and TNF- α (200/5 $\mu\text{g}/\text{mL}$)	Shi et al. [31]
PSNPLs/DEHP	-		20, 200/5 $\mu\text{g}/\text{mL}$ (24 h)	-		Increased expression of IL-1 β , IL-6, IL-8, and TNF- α (200/5 $\mu\text{g}/\text{mL}$)	
PSMPLs	1	Liver organoids	0.25, 2.5 and 25 $\mu\text{g}/\text{mL}$ (48 h)	NA	Inflammatory response (ELISA)	Increased expression of IL-5	Cheng et al. [32]
					Inflammatory response/ fibrosis (ELISA)	Increased expression of COL1A	
					Stress-related genes' expression (RT-qPCR)	Altered stress response	
					Epithelial-mesenchymal transition and tumor progression (RT-qPCR)	Increased expression of <i>Snail</i> and <i>Twist</i> genes, and decreased expression of <i>Slug</i> and <i>Zeb1</i> genes	
					microRNA expression	Increased expression on 14 microRNA and decreased expression of 2 microRNA out of 33 microRNA analysed	
PSNPLs	-0.05	Prone-to-transformation progress MEF	25 $\mu\text{g}/\text{mL}$ (6 months)	Proved (24, 48, and 72 h)	Anchorage-independent growth induction (soft-agar assay)	Potential to grow independently of anchorage forming colonies	Barguilla et al. [33]
					Migration induction (direct migration assay)	Acquisition of migration ability	
					Invasion induction (direct invasion assay)	Acquisition of invasion ability	
					Activation of cancer stem cells (tumorsphere formation induction and expression of pluripotent markers by RT-qPCR)	Induction of the dedifferentiation of the cells	
					Anchorage-independent growth induction (soft-agar assay)	Lack of colony formation	
PSNPLs	-0.05	Prone-to-transformation progress MEF	25 $\mu\text{g}/\text{mL}$ (12 weeks)	Proved (25, 100 $\mu\text{g}/\text{mL}$; 24 h)	Migration induction (direct migration assay)	Unable to migrate	Barguilla et al. [34]
					Invasion induction (direct invasion assay)	Unable to invade	
					Activation of cancer stem cells (tumorsphere formation induction)	Lack of cancer stem cells activation	
					Morphological changes (microscopy)	Increased proportion of spindle-like cells	

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Table 1 (continued)

Material	Size (µm)	Cell line	Concentration range (time of exposure)	Uptake	Carcinogenicity-related endpoint (method)	Outcome	Ref.
PSNPLs/ As ^{III}	-		25 µg/mL / 2 µM (12 weeks)	Proved (25 or 100 µg/mL / 2 µM; 24 h)	Anchorage-independent growth induction (soft-agar assay)	Potential to grow independently of anchorage forming colonies	
					Migration induction (direct migration assay)	Acquisition of migration ability	
					Invasion induction (direct invasion assay)	Acquisition of invasion ability	
					Activation of cancer stem cells (tumorsphere formation induction)	Lack of cancer stem cells activation	
PSMPLs	9.5–11.5	AGS, MKN1, MKN45, NCI-N87, and KATO III	8.61 × 10 ⁵ particles/mL (4 weeks)	NA	Morphological changes (microscopy)	Increased proportion of spindle-like cells	Kim et al. [35]
					Proliferation (alamarBlue assay)	Increased proliferation rate (all cell lines)	
					Invasion induction (direct invasion assay)	Increased invasion rate (AGS, MKN1, MKN45, and KATO III)	
					Migration induction (direct migration assay)	Increased migration rate (all cell lines)	
					Cancer stemness (immunocytochemistry and RT-qPCR)	Increased expression of CD44 (all cell lines)	
					Multidrug resistance (alamarBlue assay)	Multidrug resistance induction after PSNPLs treatment (all cell lines)	

the pro-inflammatory cytokines. However, the activity of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) was reduced after the exposure of A549 cells to 200 µg/mL PSNPLs, DBP, DEHP, or their combinations. The low concentration of PSNPLs alone or in combination with plasticizers did not affect the antioxidants' activity. Furthermore, liver organoids developed from human embryonic stem cells H1 exposed to polystyrene microplastics (PSMPLs; 0.25, 2.5, and 25 µg/mL; 1 µm) showed a dose-dependent increase in the IL-6 and collagen type 1a (COL1A1) inflammation biomarkers after 48 h of exposure [32]. In addition to that, other outcomes regarding hepatotoxicity were observed. The decreased activity of GST, GSH, and SOD levels accompanied by the increase in MDA content suggested the antioxidative imbalance, which may arise due to oxidative stress. Based on the increased inflammatory response and oxidative stress triggered by PSMPLs, as well as other outcomes obtained in the study, the authors identified different adverse outcomes pathways (*i.e.*, AOP 38 and AOP 220) that suggested the risk of liver fibrosis and liver cancer, respectively.

In short, the above *in vitro* short-term approaches suggest the ability of MNPLs to induce oxidative stress that can lead to lipid peroxidation, and acute inflammatory responses. Unfortunately, no data addressing the capacity of MNPLs to induce chronic inflammation in *in vitro* systems is available up to date. Additionally, insights on the activation of cell proliferation were also reported in these studies.

Three studies focused on the potential carcinogenic risk of MNPLs *in vitro*, by using cell transformation approaches, have been recently published. Two of these studies used mouse embryonic fibroblasts deficient in repairing oxidized DNA base lesions (MEF Ogg1^{-/-}) in a chronic exposure scenario with exposures lasting for 12 and 24 weeks to relatively low doses of PSNPLs (0.05 µm; 25 µg/mL) [33,34]. The authors reported, after 12 weeks of chronic exposure to the co-treatment with arsenic (2 µM As^{III}), a well-known carcinogen in humans, a synergistic effect increasing a set of cancer hallmarks, such as the number of colonies formed in the anchorage-independent growth assay, and the acquisition of invasion and migration abilities, although PSNPLs alone did not induce these effects [34]. Nevertheless, PSNPLs generated DNA damage by both oxidative and non-oxidative mechanisms after the 12-week chronic exposure. The same authors also demonstrated that a longer period of exposure to the same NPLs (24 weeks) was able to alter early tumoral phenotype biomarkers [33]. Changes in stress-response-related genes were observed with a significant upregulation of *Gstp-1* and a downregulation of *Keap*, *Nrf2*, *Pgp*, *Sod1*, and *Sod2*. In addition, the dysregulation of a battery of microRNAs closely linked to the oncogenic process (miR-21, miR-23a, miR-25, miR-30c, miR-30d, miR-96, miR-135b, miR-148b, miR-155, miR-199b, miR-200a, miR-210, miR-218, miR-502, miR-34a, and miR-203) was observed. Moreover, and as indicators of an advanced tumoral phenotype, there was a significantly elevated number of colonies able to grow in soft-agar and increased cell migration capacity, as well as an aggressive tumoral phenotype, namely, increased invasion capacity, changes in pluripotency markers (upregulated *Klf4*, *Nanog*, *Notch2*, and *Oct3/4* and downregulated *Sox2*) and formation of tumorspheres due to the treatment of the cells with PSNPLs for 24 weeks. On the other hand, Kim and colleagues studied carcinogenicity-related endpoints in AGS, MKN1, MKN45, NCI-N87, and KATO III human gastric cancer cell lines after the exposure to 8.61 × 10⁵ particles/mL of PSMPLs (9.5–11.5 µm) for 4 weeks [35]. As a result of the exposure, all cell lines increased their proliferation and migration rates, and most of them except NCI-N87 cells underwent invasion induction. The upregulation of the CD44 marker was identified in the studied cell lines upon the PSMPLs treatment. CD44 + cells have been described as human gastric cancer stem cells. Also, the authors demonstrated that PSMPLs exposure induced resistance to different anticancer drugs in all the selected cell lines.

Table 2
In vivo studies assessing carcinogenicity-related effects of MNPLs.

Material	Size (µm)	Experimental model (strain; sex)	Doses (route of administration)	Treatment schedule	Presence in target tissue	Carcinogenicity-related endpoint (method)	Outcome	Ref.
PSMPLs	1–5	House Dust Mite-induced allergic asthmatic and healthy mice (BALB/c; females)	300 µg/mouse (intranasal)	Every 3 days for 24 days	Proved (lung)	Inflammatory response (multiplex ELISA)	Increased expression of IgG1 and TNF-α (healthy mice)	Lu et al. [36]
						Inflammatory response (histopathological analysis)	Increased eosinophils and lymphocytes infiltration (healthy mice)	
PSMPLs	0.5	Mice (ICR)	5, 25, and 50 µg/mouse (oral)	Daily for 2 weeks	NA	Inflammatory response (western blot)	Upregulation of the ASC-inflammasome, and NF-κβ pathways	Choi et al. [37]
						Inflammatory response (RT-qPCR)	Increased expression of NF-κβ (25 and 50 µg), IL-6, TNF-α, IL-1β, TGF-β, and IL-10	
						Inflammatory response (immunology multiplex assay)	Increased expression of IL-2 and IL-6 (6 µg), IP-10 and RANTES (60 µg), IL-5 and IL-9 (600 µg), G-CSF (60 and 600 µg), and IL-1α	
PE	10–150	Mice (C57BL/6; males)	6, 60, and 600 µg/mouse (oral in food)	Daily for 5 weeks	NA	Inflammatory response (flow cytometry)	Decreased percentage of Th17 and Treg cells (60 and 600 µg)	Li et al. [38]
						Inflammatory response (histopathological analysis)	Increased lymphocytes and plasma cells infiltration (600 µg)	
						Inflammatory response (immunofluorescence staining)	Increased expression of TLR4, AP-1, and IRF5 (600 µg)	
PSMPLs	10	Mice (C57BL/6; males mated BALB/c; females)	250 µg/mouse (intraperitoneal)	Twice on 5.5 and 7.5 days of gestation	NA	Immune balance/Immuno-suppression (flow cytometry)	Decreased CD45 ⁺ leukocytes, and CD49b ⁺ NK cells in CD45 ⁺ leukocytes, Increased M2-subtype macrophages polarization	Hu et al. [39]
						Inflammatory response (RT-qPCR)	Increased IL4, decreased TNF-α, increased tendency IL6, decreased marginal IL2 and IFN-γ	
						Inflammatory response (biochemical assay)	Increased activity of AST and ALT (high-fat diet fed mice, 1 µg)	
						Inflammatory response/fibrosis (histopathological analysis)	Increased macrophage infiltration and increased collagen deposition (high-fat diet fed and normal mice, 1 µg)	
PSNPLs	0.042	High-fat diet fed and normal mice (C57BL/6; females)	1 and 5 µg/mouse (i. v.)	Every 3 days for 15 days	NA	Inflammatory response (RT-qPCR)	Increased expression of IL-1β (high-fat diet mice, 1 and 5 µg), IL-12, IL-2, and IFN-γ (high-fat diet fed mice, 1 µg)	Li et al. [40]
						Fibrosis (RT-qPCR)	Increased expression of α-SMA and Col1a (high-fat diet fed mice, 1 µg)	
						Fibrosis (histopathological analysis)	Increased collagen (50 mg/L) and fibronectin (5 and 50 mg/L) deposition	
PSMPLs	0.5	Rats (Wistar; males)	0.5, 5 and 50 mg/L of drinking water/rat (oral in drinking water)	Daily in drinking water for 90 days	Proved (cardiomyocytes, 5 and 50 mg/L)	Inflammatory response/fibrosis (western blot)	Increased expression of Wnt, TGF-β, p-β-catenin, α-SMA, Collagen I and fibronectin (5 and 50 mg/L) and β-catenin and Collagen III (50 mg/L)	Li et al. [41]

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Table 2 (continued)

Material	Size (µm)	Experimental model (strain; sex)	Doses (route of administration)	Treatment schedule	Presence in target tissue	Carcinogenicity-related endpoint (method)	Outcome	Ref.
PSMPLs	0.5	Rats (Wistar; females)	0.015, 0.15 and 1.5 mg/day/rat (oral in drinking water)	Daily in drinking water for 90 days	Proved (ovary, 0.15 mg/d)	Fibrosis (histopathological analysis) Inflammatory response/fibrosis (western blot)	Increased collagen and fibronectin deposition (1.5 mg/d) Increased expression of Wnt and TGF-β (0.15 and 1.5 mg/d) and β-catenin, p-β-catenin, α-SMA, Collagen I, fibronectin and Collagen III (1.5 mg/d)	An et al. [42]
PSMPLs	5, 20	Mice (ICR; males)	0.01, 0.1, and 0.5 mg/day/mouse (oral gavage)	Daily for 1, 2, 4, 7, 14, 21, and 28 days	Proved (liver, kidney, gut, 0.1 mg/d)	Inflammatory response (histopathological analysis)	Inflammation indicators observed (0.5 mg/d, 28 days)	Deng et al. [43]
PSMPLs	9.5–11.5	Mice (BALB/c nude; males) Mice (BALB/c nude PSMPLs-exposed cell xenografted; males)	1.72 × 10 ⁴ particles/mL/mouse (oral) 8.61 × 10 ⁵ particles/mL 4-weeks-exposed NCI-N87 (subcutaneously)	Daily for 4 weeks Once	Proved (stomach) -	Gene expression (RNA-seq) Tumor growth	Altered expression of 194 genes associated to digestive system diseases and cancer Accelerated tumor growth	Kim et al. [35]
PLGA	1–2	Mice (NOD; female)	100 µg/mouse (subcutaneously)	Once a week for 5 weeks	-	Immune tolerance (flow cytometry) Immune tolerance (ELISA)	Augmentation of Treg and TGF-β1 release Increased expression of IL-10 and TGF-β1, INF-γ and IL-17A	Liu et al. [44]

3.2. In vivo studies on carcinogenicity-related effects

The *in vivo* inflammatory responses caused by MNPLs exposure have been studied in mice and rats (Table 2). Lu and co-workers treated intranasally healthy, and House Dust Mite (HDM)-induced allergic asthmatic female BALB/c mice, either with PSMPLs (1–5 µm; 300 µg in 20 µL of saline per mouse) or saline solution (control), every 3 days up to 24 days [36]. Plastic particles' accumulation was detected in lung sections of the MPLs-exposed groups, co-localizing with macrophages. In addition to MPLs-loaded macrophages, strong recruitment of inflammatory cells (eosinophils, lymphocytes, neutrophils, plasmocytes, and macrophages) into the airspace of lungs of HDM-induced allergic asthmatic mice, either treated or not with MPLs, was reported. Besides, the presence of eosinophils in the bronchoalveolar lavage fluid (BALF) of healthy mice exposed to MPLs was higher than in healthy mice exposed to saline. Thus, MPLs exposure triggered the recruitment of inflammatory cells into the lungs of healthy mice and aggravated the inflammation response in allergic mice. Further analyses were performed with BALF and plasma to determine IL-4, IL-5, IL-10, IL-13, IL-17A, IL-33, IFN-γ, TNF-α cytokines, and immunoglobulins E (IgE) and IgG1. TNF-α levels were increased after the exposure of healthy mice to MPLs, compared with the healthy control group. IgE and IgG1 were significantly overexpressed in both MPLs-exposed and unexposed asthmatic mice compared with the healthy control group, but with no differences between asthmatic groups. Importantly, healthy mice exposed to MPLs showed increased IgG1 levels compared to the untreated control. In general, the exposure to PSMPLs did not alter the cytokines and immunoglobulins' expression in asthmatic mice, but it induces pro-inflammatory effects in healthy mice. The transcriptomic analysis conducted in this study revealed that MPLs administration altered processes related to immune activation, cell cycle control, and signaling pathways such as the MAPK cascade. Likewise, Choi and colleagues detected an upregulation of the ASC-inflammasome pathway (NLRP3, ASC, and cleaved Cas-1/Cas-1) in the mid colon of ICR mice after the oral administration with PSMPLs (0.5 µm; 10, 50, and 100 µg/mL;

0.5 mL/day) daily for 2 weeks [37]. Moreover, the authors associated the response with the activation of the NF-κβ, which is linked with the expression of pro-inflammatory genes. To prove that, the expression of *NF-κβ*, *TNF-α*, *IL-6*, *IL-1*, *IL-10*, and *TGF-β1* was determined. The results significantly showed dose-dependent increased levels of mRNA for all the cytokines in PSMPLs treated mice groups, compared to the untreated control group. Similarly, Li et al. studied the levels of various cytokines (IL-1α, G-CSF, IL-2, IL-5, IL-6, IL-9, IP-10, and RANTES) in serum of C57BL/6 mice administered with polyethylene microplastics (PEMPLs, 10–150 µm; 6, 60, and 600 µg per mouse) in food every day for 5 weeks [38]. *IL-1α* expression was significantly increased in PEMPLs-fed mice groups. However, the expression of the other analyzed cytokines did not change or even decreased after the PEMPLs exposure. Other inflammation markers, such as the expression of *TLR4*, *AP-1*, and *IRF5*, or the infiltration of lymphocytes and plasma cells in the duodenum of mice administered with 600 µg PEMPLs, proved the induction of intestine inflammation.

Besides that, the maternal-fetal immune balance effects of PSMPLs were evaluated in C57BL/6-mated BALB/c pregnant mice after intraperitoneal administration (PSMPLs of 10 µm, 250 µg/200 µL of saline) on days 5.5 and 7.5 of gestation periods [39]. It resulted in significantly lower levels of CD45⁺ leukocytes derived from the peripheral blood, spleen, as well as from placenta of the PSMPLs' exposed mice. In addition, there was a decrease in CD49b⁺ natural killer (NK) cells in CD45⁺ leukocytes from placenta; nevertheless, no changes in the population of NK cells in peripheral blood and spleen of the exposed pregnant mice were observed. Furthermore, PSMPLs' exposure of mice significantly skewed the macrophages' polarization towards the M2-subtype dominance (CD206⁺) with immunosuppressive capacities over M1-subtype (CD86⁺) in the peripheral blood, spleen, and placenta tissues. Similarly, several pro/anti-inflammatory cytokines secretion like *IL-2*, *IL-4*, *IL-6*, *IL-10*, *TNF-α*, and *IFN-γ*, evaluated at mRNA level from the exposed mice placenta, were affected. Notably, there were increases in the levels of *IL-4*, and decreases in *TNF-α*. Moreover, an increasing tendency of *IL-6* and a marginal decrease of *IL-2* and *IFN-γ* were also observed due to

PSMPLs' exposure. The authors suggested this as a shift towards the immunosuppressive state.

Hepatic inflammation was observed in normal and high-fat diet (HFD) fed C57BL/6 mice administered *via* tail vein with PSNPLs (41.54 nm; 10 and 50 µg/mL in 100 µL of PBS per mouse) every 3 days for 15 days [40]. The expression levels of *TNF-α*, *IL-1β*, *IL-12*, *IL-2*, and *IFN-γ* revealed higher transcription of the inflammatory genes in mice treated with PSNPLs in HFD mice groups. Although the mRNA levels of the mentioned cytokines were higher in HFD mice, compared with normal mice in every treatment condition, higher concentrations of PSNPLs did not induce stronger effects. Intriguingly, massive collagen deposition was found in liver tissue of HFD mice administered with 10 µg/mL PSNPLs. The increased expression of *α-smooth muscle actin* (*α-SMA*) and *COL1a*, which play important roles in fibrogenesis, confirmed the notable potential of PSNPLs to induce fibrosis in the HDF group of mice. These results are in concordance with the also reported increased levels of two markers of hepatic cell injury in HFD mice treated with 10 µg/mL PSNPLs, such as aminotransferases ALT and AST. These biomarkers have been linked with chronic hepatitis (inflammation of the liver tissue), liver cirrhosis (fibrosis), and hepatocellular carcinoma. In another study [41], the authors described that the expression of *Wnt*, *TGF-β*, *p-β-catenin*, *α-SMA*, *Collagen I*, and *fibronectin* raised significantly in the heart tissue of male Wistar rats after the daily administration of 5 and 50 mg/L of PSMPLs (0.5 µm) for 90 days in drinking water, whereas the highest dose also induced an increase expression of *β-catenin* and *Collagen III*. These effects were accompanied by a significant increase in collagen deposition and hyperplasia (50 mg/L PSMPLs), as well as fibronectin deposition (5 and 50 mg/L PSMPLs) in the heart of treated rats. An accumulation of PSMPLs was also found inside cardiomyocytes after 90 days of exposure. Equivalent results were observed in the ovaries of female Wistar rats exposed to PSMPLs (0.5 µm; 0.015, 0.15, and 1.5 mg/day/rat in drinking water) for 90 days following the same exposure procedure. Furthermore, while *p-β-catenin*, *β-catenin*, *α-SMA*, *Collagen I*, *Collagen III*, and *fibronectin* expression increased only after the highest dose treatment, *Wnt* and *TGF-β* expression raised after the administration of 0.15 and 1.5 mg/day PSMPLs in female rats [42]. In addition, some authors have associated the induction of fibrosis by PSMPLs with cell apoptosis caused by oxidative stress, which in its turn, activates the expression of the reported cytokines [41,42].

It is important to point out here the study of Deng and co-workers where administered PSMPLs (5 and 20 µm; 0.1 mg/day/mouse) were found to accumulate in the liver, kidney, and gut of ICR mice after 28 days of oral gavage exposure [43]. The retention of PSMPLs was determined one week after the termination of the exposure and both sizes of PSMPLs were still observable in liver, kidney, and gut tissues. Moreover, liver tissue showed inflammation signals after a 4-weeks of exposure to 0.5 mg/day of both sizes of PSMPLs. Oxidative stress-related biomarkers were also evaluated after the exposure to PSMPLs (5 and 20 µm; 0.01, 0.1, and 0.5 mg/day) and increased activity of GSH-Px and SOD were shown in mice after the termination of the exposure, while CAT activity decreased in the treated mice.

Liu and co-workers evaluated the impact of poly[lactic-co-(glycolic acid)] (~1–2 µm, PLGA) microparticles on the immune functions of non-obese diabetic female mice [44]. Twenty weeks after the treatment (100 µg, once a week for 5 weeks), PLGA MPLs stimulated the splenic Treg augmentation and TGF-β1 release. Nevertheless, the effect was no longer observed at week 30. To confirm the stimulation of immune tolerance by PLGA, multiplex serum cytokine ELISA was performed. The substantial increase of IL-10 and TGF-β1 levels supported the tolerance-inducing property of these MPLs. In addition, a slight increase in serum INF-γ and IL-17A, indicated the stimulation of antigen presentation.

Only one study focusing on *in vivo* tumorigenesis capacity of PSMPLs (9.5–11.5 µm) was found [35]. Mice xenografts (BALB/c nude mice injected with NCI-N87 cells previously exposed or not to 8.61×10^5 PS

particles/mL for 4 weeks) were used to demonstrate an induction in tumor growth in the PSMPLs-exposed NCI-N87 mouse model compared to the control. Additionally, differential expression of 194 genes related to digestive system disease and cancer, and a decrease in the survival rate were reported in the PSMPLs-exposed mice (1.72×10^4 particles/mL per mouse, orally administered daily for 4 weeks). The authors also demonstrated the particles' accumulation in the stomach of the PSMPLs-exposed BALB/c nude mice.

3.3. *In vitro* genotoxicity studies

Until now, several *in vitro* studies have reported genotoxic effects of MNPLs exposure using several genotoxicity endpoints, relevant cell lines, different sizes and concentrations of MNPLs, and different exposure times (Table 3). The DNA damage (either produced by oxidative causes or not) measured by the comet assay after the treatment of human hematopoietic cell lines with PSNPLs was cell-line specific, and time- and concentration-dependent [45]. It was found that Raji-B cells treated with PSNPLs (50 nm, 5–50 µg/mL) showed a pronounced dose-dependent increase in DNA damage after 24 h and 48 h of exposure, although the oxidative DNA damage (ODD) was observed only at the highest concentration (50 µg/mL) and after exposures lasting for 24 h. Only ODD was detected in a dose- and time-dependent manner in TK6 cells. Contrary, THP1 cells treated with PSNPLs did not show DNA damage, independently of concentrations and time intervals. The differential cell sensitivity to genotoxic agents could be related to the differential uptake of PSNPLs and the potential generation of ROS. Similarly, a recent study also assessed the cellular-specific DNA damage due to *ex vivo* exposure to different concentrations of PSNPLs (0.05 µM) in total whole blood cells (WBC) and sorted cell types from WBC such as lymphocytes, monocytes, and polymorphonuclear cells (PMNs) obtained from different donors by using the comet assay [46]. PSNPLs were able to cause a significant increase in the percentage of DNA in the tail of monocytes (100 µg/mL) as well as in PMNs (50 and 100 µg/mL), but not in lymphocytes and in the total WBC population after 24 h of exposure. The differential cell sensitivity to PSNPLs did not reveal a correlation with cellular internalization as monocytes showed greater internalization of particles than PMNs, but less genotoxic damage.

DNA damage associated with exposure to different concentrations (1–100 µg/mL) of PSNPLs (mean size ~50 nm) in the *in vitro* human intestinal barrier models (biculture of differentiated Caco-2/HT29 intestinal cells and triculture containing Caco-2/HT29/Raji-B cells) was not significantly increased when analyzed by using the comet assay after 24 h of exposure. The lack of DNA damaging effects could be attributed to the insignificant generation of ROS, though the data demonstrated clear uptake of PSNPLs [47]. The only *in vitro* study presenting results on MNPLs' accumulation after a long-term exposure was performed using undifferentiated Caco-2 cells exposed to pristine PSNPLs (50 nm; $0.26 \mu\text{g}/\text{cm}^2$) for 24, 48, 72, and 96 h, and weekly thereafter up to week 8. Internalization reached a plateau after 2-weeks of exposure, and PSNPLs remained accumulated inside the cells at a similar level during the consecutive weeks [48]. However, undifferentiated Caco-2 cells treated with PSNPLs (50–100 nm; 10 and 100 µg/mL) for 8 weeks did not elicit DNA damage using the comet assay. Nevertheless, there was a dose-related increase in the expression of a few oxidative stress-related genes (*HO1* and *SOD2*).

PSNPLs acting as carriers of silver metal were able to modulate the induced genotoxic and ODD effects of AgNO₃ or silver nanoparticles (AgNPs) in Caco-2 cells [49]. Results of the comet assay after 24 h of exposure showed that the co-treatment of the cells with the highest doses of PSNPLs (100 µg/mL) and AgNPs (5 µg/mL) significantly increased the non-oxidative DNA damage but not ODD. On the other hand, the adsorption of plasma proteins onto PSNPLs (~100 nm) resulted in increased DNA damaging effects observed in human blood lymphocytes by the comet assay, but not with PSNPLs alone; suggesting that interactions of plasma proteins with nanoplastics could turn them

Table 3
In vitro studies assessing genotoxicity of MNPLs.

Material	Size (µm)	Cell line	Concentration range (time of exposure)	Uptake	Endpoint (method)	Outcome	Ref.
PSNPLs	0.05	Raji-B	5, 10, 25, and 50 µg/mL (3, 24, 48 h)	Proved (1, 5, 10, 25, 50 µg/mL; 24 and 48 h)	DNA damage (alkaline and Fpg-comet)	Non-oxidative DNA damage induction (25 and 50 µg/mL, 24 and 48 h) and oxidative DNA damage (50 µg/mL, 24 h)	Rubio et al. [45]
		Oxidative stress (DCFH-DA assay)			Increased intracellular ROS levels (50 µg/mL, 3 h)		
		DNA damage (alkaline and Fpg-comet)			Oxidative DNA damage (5, 10, 25 and 50 µg/mL, 24 h and 10 and 25 µg/mL, 48 h)		
		Oxidative stress (DCFH-DA assay)			Increased intracellular ROS levels (5, 10, 25 µg/mL, 24 h and 50 µg/mL, 3 and 24 h)		
PSNPLs	0.05	THP-1	50 and 100 µg/mL (24 h)	No significant uptake Proved (100 µg/mL) Proved (100 µg/mL)	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Ballesteros et al. [46]
		Oxidative stress (DCFH-DA assay)			Lack of oxidative stress induction		
		Lymphocytes Monocytes PMNCs			Lack of DNA damage induction DNA damage (100 µg/mL) DNA damage (50 and 100 µg/mL)		
PSNPLs	0.05	Caco-2/HT29	1, 25, 50 and 100 µg/mL (24 h)	Proved	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Domenech et al. [47]
		Oxidative stress (DCFH-DA and DHE assays)			Lack of oxidative stress induction		
PSNPLs	0.05	Caco-2/HT29/Raji-B	10 and 100 µg/mL (24 h)	Proved	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Domenech et al. [49]
		Oxidative stress (DCFH-DA and DHE assays)			Lack of oxidative stress induction		
PSNPLs	0.05	Caco-2	10 and 100/0.1, 0.5, 1, and 5 µg/mL (24 h)	Proved (10 and 100/0.5 and 5 µg/mL)	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Domenech et al. [48]
					Oxidative stress (DHE assay)	Lack of oxidative stress induction	
PSNPLs/AgNPs	0.05/0.005	Caco-2	10 and 100/0.1, 0.5, 1, and 5 µg/mL (24 h)	Proved (10 and 100/0.5 and 5 µg/mL)	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Domenech et al. [49]
					Oxidative stress (DHE assay)	Increased intracellular ROS levels (5 µg/mL AgNPs and 100/5 µg/mL PSNPLs/AgNPs)	
PSNPLs/AgNO ₃	-	Caco-2	0.26 and 6.5 µg/cm ² (24 h) and 0.0006, 0.26, 1.3, and 6.5 µg/cm ² (8 weeks)	Proved (0.26 µg/cm ² ; 24, 48, 96 h and weekly up to week 8 and 6.5 µg/cm ² ; 24 h)	DNA damage (alkaline and Fpg-comet)	PSNPLs-dependent DNA damage induction and PSNPLs-dependent oxidative DNA damage induction (0.5 µg/mL AgNO ₃)	Domenech et al. [48]
					Oxidative stress (DHE assay)	Increased intracellular ROS levels	
PSNPLs	0.05	Caco-2	0.26 and 6.5 µg/cm ² (24 h) and 0.0006, 0.26, 1.3, and 6.5 µg/cm ² (8 weeks)	Proved (0.26 µg/cm ² ; 24, 48, 96 h and weekly up to week 8 and 6.5 µg/cm ² ; 24 h)	DNA damage (alkaline and Fpg-comet)	Lack of DNA damage induction	Domenech et al. [48]
					Oxidative stress (DCFH-DA assay)	Lack of oxidative stress induction	

(continued on next page)

Table 3 (continued)

Material	Size (µm)	Cell line	Concentration range (time of exposure)	Uptake	Endpoint (method)	Outcome	Ref.
PSNPLs, plasma coronated-PSNPLs, scrub isolated-PSNPLs	0.1	Human lymphocytes	1, 2.5, 5, 7.5 and 10 µg/mL (24 h)	NA	DNA damage (alkaline comet)	DNA damage induction (plasma coronated-PSNPLs, 5 µg/mL)	Gopinath et al. [50]
PSNPLs	~0.05	Prone-to-transformation progress MEF	25 µg/mL (12 weeks)	Proved (25, 100 µg/mL; 24 h)	DNA damage (alkaline and Fpg-comet)	DNA damage induction	Barguilla et al. [34]
PSNPLs/As ^{III}	-		25 µg/mL / 2 µM (12 weeks)	Proved (25 or 100 µg/mL / 2 µM; 24 h)		DNA damage induction	
COOH-PSNPLs, COOH-PSMPLs	0.05, 0.5	HepG2CDKN1A-DsRed biosensor cells	0.01, 0.1, 1, 5, 10, 25, and 50 µg/mL (48 h)	Proved (in intestinal and placental barrier co-culture models, 100 µg/mL, 24 h)	DNA damage (p53 reporter gene assay)	Lack of p53 expression	Hesler et al. [51]
		CHO-k1	0.1, 1, 5, 10, 25, and 50 µg/mL (24 h)			DNA damage (CBMN assay)	
PSNPLs	0.1	Hs27	5, 25, and 75 µg/mL (48 h)	NA	DNA damage (CBMN assay)	Increased levels of MN (25 and 75 µg/mL) and nuclear buds (75 µg/mL)	Poma et al. [52]
			5, 25, and 75 µg/mL (15, 30, 45, 60 min, and 24 h)			Oxidative stress (ROS assay kit)	
PEMPLs	10–45	Human blood lymphocytes	25, 50, 100, 250, and 500 µg/mL (48 h)	NA	DNA damage (CBMN assay)	Increased levels of MN (250 and 500 µg/mL), nucleoplasmic bridges, nuclear buds (100, 250, and 500 µg/mL) and chromosome instability (50, 100, 250, and 500 µg/mL)	Cobanoglu et al. [53]

Table 4
In vivo studies assessing genotoxicity of MNPLs.

Material	Size (µm)	Experimental model (strain; sex)	Doses (route of administration)	Treatment schedule	Presence in target tissue	Endpoint (method)	Outcome	Ref.
PSNPLs	0.023	Mice (Swiss; males)	14.6 ng/kg b. w. (i. p.)	Daily for 3 days	Proved (brain)	DNA damage (alkaline comet assay)	DNA damage induction	Estrela et al. [54]
						Oxidative stress (ELISA)	Increased TBARS concentrations and nitrite levels	
PSNPLs/ ZnONPs	0.023/ 0.069		14.6/14.6 ng/kg b. w. (i.p.)			DNA damage (alkaline comet assay)	DNA damage induction	
						Oxidative stress (ELISA)	Lack of oxidative stress induction	

into bio-incompatible, modulating the subsequent biological response [50].

In another study, PSNPLs alone (0.05 µm, 25 µg/mL), as well as co-exposure of PSNPLs with sodium arsenite (As^{III}) (25 µg/mL / 2 µM), were evaluated for genotoxic effects in mouse embryonic fibroblasts prone to oxidative damage (*Ogg1*^{-/-}) using the comet assay [34]. The data suggested a significant increase in DNA damage produced both by oxidative and non-oxidative mechanisms after 12 weeks of chronic treatment with PSNPLs alone. Furthermore, cells co-exposed to PSNPLs and As^{III} showed even higher and significant DNA damage, as compared to PSNPLs alone at 12 weeks post-treatment.

Neither PSMPLs (500 nm, 0.1–100 µg/mL) nor PSNPLs (50 nm, 0.1–100 µg/mL) could induce micronuclei formation in CHO-K1 cells when exposed for 24 h, or activation of p53 in HepG2CDKN1A-DsRed biosensor cells, upon treatment with PSMPLs or PSNPLs (0.01–50 µg/mL, 48 h) when assessed by the cytokinesis-block micronucleus (CBMN) assay and the p53 reporter gene assay, respectively [51]. Contrary, the treatment of the human skin fibroblast cell line Hs27 with PSNPLs

(100 nm, 5–75 µg/mL, 48 h) resulted in a dose-dependent increase at 25 and 75 µg/mL in the micronuclei (MN) frequency and increased nuclear buds' formation at the highest PSNPLs dose, as evaluated by the CBMN assay [52]. In addition, PSNPLs were able to induce ROS in the cells exposed to 5 µg/mL after 15, 30, and 60 min, and to 25 µg/mL after 30 and 60 min. On the other hand, the PEMPLs' (10–45 µm, 25–500 µg/mL) genotoxic potential was also assessed in human lymphocytes after 48 h of exposure using the CBMN assay [53]. The total number of MN, as well as the number of nucleoplasmic bridges and nuclear buds, significantly increased after the treatment (250 and 500 µg/mL, and 100–500 µg/mL, respectively). Moreover, the authors reported a significant positive correlation between the analyzed endpoints.

3.4. *In vivo* genotoxicity studies

In the literature search, we found only one *in vivo* study reporting the genotoxicity effects of MNPLs (Table 4). In such study, PSNPLs alone

(23.03 nm) or in combination with zinc oxide nanoparticles (ZnONPs; 68.96 nm) were able to induce DNA damage, assessed by the comet assay, in peripheral blood cells from male Swiss mice intraperitoneally administered for 3 consecutive days with 14.6 ng/kg b.w. of PSNPLs, ZnONPs, or equitable doses of both materials [54]. The increase in DNA damage, measured as the tail length, the percentage of DNA in the tail, and the Olive tail moment, showed a statistically significant tendency, ZnONPs > ZnONPs + PSNPLs > PSNPLs. The authors hypothesized that the effect could be due to the observed increased levels of nitric oxide and thiobarbituric acid reactive species, as indicators of an oxidative stress response. MNPLs bioaccumulation was also assessed, and the results showed a significant accumulation of PSNPLs, and ZnONPs, as well as the combination of both, in the mice's brain. Nevertheless, the detection of both nanoparticles was lower when co-administered, which is explained by the authors considering the bigger size of the aggregates formed when both types of particles interact.

4. Discussion

The ever-increasing usage of polymer plastics in day-to-day life is a major cause of concern due to their potential implications for human health. The ongoing research efforts suggest that exposure of humans to MNPLs *via* inhalation and ingestion is inevitable. Nevertheless, there exists a clear knowledge gap and a lack of understanding *vis-à-vis* whether they pose a serious health risk to humans [55]. The carcinogenic potential due to continuous exposure to MNPLs has barely been assessed until now, which highlights the pressing need to address the carcinogenic risk assessment in humans on an urgent basis through concerted research efforts.

As an important point to highlight, the present review confirms the lack of studies performed with rodent carcinogenicity assays to assess MNPLs exposure, probably due to the complexity of such studies. For this reason, indirect approaches to evaluating DNA damage, generation of ROS, chromosomal aberrations, and inflammatory responses in mammalian models have been used in the present review to get some insights into the potential carcinogenic risk of MNPLs. Most of the studies pointed out the potential of MNPLs to induce biological responses that are considered either molecular initiating events (MIE) or key events (KE) in the induction of human cancer. For instance, an increased production of inflammatory cytokines, accomplished by increased formation of ROS that could result in higher rates of DNA damage and mutations have been described as KE in the development pathways of lung (*e.g.*, AOPs 303, 416, 417, and 451) and breast (*e.g.*, AOPs 439) cancer. Increased production of reactive oxygen and nitrogen species can be MIEs leading to mutations (*e.g.*, AOP 296) and induction of breast cancer (*e.g.*, AOP 294) or, if becoming chronic, of gastric cancer (*e.g.*, AOP 298) [56]. Although some of these KE, like inflammation, do not always lead to tumor formation, they can be considered as early indicators of an increased cancer risk. Furthermore, inflammation and ROS induction are among the pivotal events that should be addressed when developing an integrated approach to the testing and assessment (IATA) of non-genotoxic carcinogens [14]. Besides, the MNPLs' ability to accumulate into cells and tissues may favour the interaction of the material with the target cells, which could initiate a chain of events involving the induction of primary or secondary genotoxicity and resulting *e.g.*, in lung cancer (AOP 451; [57]). Although no described for MNPLs, physical interference of the internalized MNPLs with the mitotic apparatus may lead to losses of chromosomes during the mitosis, as it has reported *e.g.*, with carbon nanotubes [58]. Furthermore, the capacity of MNPLs to induce fibrosis, which have been associated with the induction of malignant mesothelioma [59], may also suggest an association between MNPLs exposure and their tumorigenic potential.

Another important question to consider is the fact that MNPLs contain not only polymers but potentially harmful chemicals that have been deliberately added during plastic goods production to get the desired characteristics of the final product. Such additives include

plasticizers, stabilizers, pigments, flame retardants, antioxidants, and antimicrobial agents, to name a few [60]. Of special concern are endocrine-disrupting compounds (EDC) -like phthalates-based plasticizers, bisphenols, alkylphenols, and brominated flame retardants-that could elevate the risk of cancer [61,62]. This poses a new challenge for MNPLs' cancer risk assessment, as the impacts resulting from the mobilization of these additives from the plastic matrix need to be considered. As an example, to illustrate this fact, the compound di-n-butyl phthalate was observed to be released into water from polyvinyl chloride (PVC) tubes [63]. Also, the desorption of a well-known EDC (DEHP) from PS foam coolers was demonstrated after an *in vitro* simulated human digestion of the PS mimicking gut conditions [64]. Nevertheless, it must be highlighted the lack of studies regarding the ability of MNPLs to release these additives into biological matrices after their uptake.

Despite its relevance regarding the carcinogenic risk, plastic additives have been left aside from the scope of the present review due to the large number of potentially harmful additives that can be found in plastics and the already existing extensive reviews of the literature regarding this topic. To give instance, the genotoxic potential of bisphenol A (BPA) was the aim of a recent review [65]. The summary of the reported data indicates that BPA could induce oxidative stress in a wide range of organisms through increased intracellular ROS production, or by altering the activity of antioxidant enzymes. The intracellular oxidation effects could lead to DNA damage induction, mainly by oxidatively damaging DNA bases, or by inducing single- and double-strand DNA breaks. Similarly, some phthalates have been shown to induce carcinogenicity in rodent livers, as well as their potential to cause genotoxicity [66]. In fact, different phthalates can act as genotoxic agents in a wide range of assays and animal models from bacteria to mammals, inducing both point and chromosome mutations. Considering the ways of actions of these plastic additives, the challenge is to identify if the plasticizers incorporated into the MNPLs have the potential to diffuse or leach from the polymer matrix and exert their harmful effects.

Most of the discussed studies pointed towards the micro and nano sizes of plastics as an important physical determinant for the observed biological effects. Nevertheless, the shape, chemical composition, surface charge, or functionalization of MNPLs may also determine the biological outcome since they play a major role in conferring toxicity, genotoxicity, and carcinogenic properties [67,68]. Thus, we consider that the focus on size, as the sole determining feature of MNPLs' harmful potential, turns a blind eye to the other determinants. Then, the role of these physicochemical properties in the risk assessment along with the size should be taken under advisement. On the other hand, most of the studies employed commercially synthesized spherical PS particles. In fact, only three of the discussed studies [38,44,53] focused on other types of environmentally representative MNPLs (PE and PLGA) with a myriad of physicochemical properties. Considering that polypropylene, PE, PVC, and polyethylene terephthalate are the most demanded polymer types in Europe [69], other types of environmentally relevant MNPLs, apart from PS, should be considered for the carcinogenesis risk assessment. Taking these considerations into account, it should be remembered that secondary MNPLs resulting from the degradation of large plastic goods are constituted by a mix of different sizes, shapes, and chemical nature. In this context, it should be indicated the lack of studies using synthetic fibers (despite their high environmental presence) to determine their carcinogenic risk at the nano/micro range. At this time, and despite the work carried out with other fiber-like nano-sized materials, especially carbon nanotubes and carbon nanofibers, limited evidence of carcinogenicity has been reached for most of them [58,70].

Few *in vivo* studies appeared in our literature search due to the lack of studies specifically addressing genotoxic or tumorigenic effects after exposure to MNPLs. However, the effects of MNPLs in rodent models have been recently reviewed [71]. Regarding MNPLs cancer risk, accumulation of plastic particles and inflammation at the accumulation site are the most studied outcomes. Non-fluorescent MNPLs have been

localized in primary absorption sites (gut and liver, considering the main exposure route) leading to increased inflammation. As well as in the *in vitro* scenario, oxidative stress is considered the underlying toxicity mechanism of MNPLs *in vivo* which would induce inflammation. Inflammation in mice intestines caused by MNPLs has been reported to alter the microbiome [72], and these can contribute to metabolic disorders including colorectal cancer [73,74]. Like that described *in vitro* by Cheng et al. [32], liver inflammation in rodents has been shown to induce hepatotoxicity. Among the effects, the downregulation of PPAR γ would lead to fibrosis [71]. Accumulation of MNPLs in reproductive organs has also been reported in rodents. Accumulation in testes generated oxidative stress, which in turn activated the p38 MAPK pathway inducing an increase in the levels of the pro-inflammatory cytokines. Likewise, An et al. [42] reported the oxidative-stress-mediated activation of the Wnt/ β -catenin pathway leading to fibrosis in rats' ovaries after the MNPLs administration.

Another point to be highlighted is the dearth of research directly dealing with the cellular transformation ability of MNPLs. No studies performed with validated guidelines (*i.e.*, the Bhas-42 cell transformation assay) were found in our search. Furthermore, only two studies [33,34], evaluated the transforming capacity of MNPLs using *in vitro* long-term approaches and a wide battery of different hallmarks of cancer. The selection of bioassays for the evaluation of carcinogenesis is of paramount importance. Based on the previous know-how on NMs, the recommended *in vitro* approaches are the anchorage-independent cell growth assessment (using the soft-agar colony-forming assay), and assays measuring invasion and migration abilities, as well as those approaches determining the morphological changes in cells exposed to MNPLs. In addition, to mimic chronic exposures, longer duration treatments, and lower concentrations than those usually applied in short-term systems might be considered.

Special focus must be placed on the dose/concentration of MNPLs used when performing *in vivo* and *in vitro* experiments. This is not devoid of challenges, as the estimation of environmental concentrations of MNPLs has several limitations, such as sampling and identification in real environmental settings, which remains a subject of debate to date. Moreover, the effective dose in many studies dealing with MNPLs is uncertain. The buoyant nature of some plastic particles challenges their study when using *in vitro* models [75]. On the other hand, the lack of harmonization in the MNPLs' administration procedure in *in vivo* approaches hampers the understanding. Two of the *in vivo* discussed studies reported that MNPLs were administered in drinking water or food although no specification on the consumption per animal was given [41,42]. Similarly, Kim and colleagues reported the concentration of particles administered per mouse without mentioning the volume administered [35]. A comprehensive and detailed description of these procedures is fundamental for comparing the results between studies. It also must be taken into consideration that human exposure to MNPLs occurs mainly through ingestion and inhalation [4]. Thus, the selection of the cell types, or the target tissues/organs when using *in vitro* or *in vivo* models, respectively, should be in concordance with the potential routes of exposure to MNPLs, namely, gastrointestinal tract, and pulmonary systems.

5. Conclusions

There is a growing concern in different walks of life regarding the potential of MNPLs as agents increasing the carcinogenic risk in humans. Due to the lack of studies directly evaluating carcinogenic effects, the only way to get some indicative data is the use of surrogate biomarkers. Thus, several *in vitro* and *in vivo* studies demonstrated the potential of MNPLs to cause DNA damage, generation of ROS, and inflammatory response suggesting their human health risk. However, there are very limited studies focusing on their carcinogenic risk as a target. This warrants an urgent need for scientific efforts for a thorough exposure risk evaluation of MNPLs for cancer-causing in humans. Such efforts are

necessary to provide the necessary evidence-based knowledge for informed decision-making by the regulatory bodies and policymakers.

Funding

This work was supported by the European Union's Horizon 2020 research and innovation programme [grant number 965196] (PlasticHeal).

CRediT authorship contribution statement

Conceptualization, Methodology: R. M., A. H., and J. C.; Formal analysis and Writing – original draft: J. D., and B. A.; Writing – review & editing: J. D., B. A., R. M., A. H., and J. C.; Supervision: R. M., A. H., and J. C.; Funding acquisition: J. C., R. M., and A. H.

Declaration of Competing Interest

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

Data Availability

No data was used for the research described in the article.

Acknowledgment

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 965196.

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