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INVESTIGATING THE POTENTIAL ROLE OF MICROPLASTICS AND NANOPLASTICS ON COLORECTAL CANCER DEVELOPMENT: A PROOF-OF-CONCEPT STUDY USING HT-29 CELLS

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“The reward of the young scientist is the emotional thrill of being the first person in the history of the world to see something or to understand something. Nothing can compare with that experience.” – Cecilia Payne-Gaposchkin

ABBREVIATIONS

5-LOX	5-lipoxygenase
CMS	Consensus molecular subtypes
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CO_2	Carbon dioxide
DAMP	Danger-associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DPPC	Dipalmitoyl phosphatidylcholine
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EOCRC	Early onset colorectal cancer
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
HCA	Heterocyclic amine
HCl	Hydrogen chloride
HDI	Human development index
HNPCC	Hereditary nonpolyposis colorectal cancer
HOC	Hydrophobic organic chemical
IBD	Inflammatory bowel disease
IC50	Half-maximal inhibitory concentration
IGF	Insulin-like growth factor
LDS	Lithium dodecyl sulfate
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MMP-2/9	Matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
MP	Microplastic
MPNP	Microplastic and nanoplastic
MSI	Microsatellite instability
MTT	3-[4,5-dimethylthiazol-2-]-2,5-diphenyltetrazolium bromide
NaF	Sodium fluoride
NaVO3	Sodium orthovanadate
NM	Nanomaterial
NOC	N-nitroso compound
NOX	NADPH oxidase
NP	Nanoplastic
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density
PA	Polyamides
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PE	Polyethylene
PES	Polyester

PET	Polyethylene terephthalate
POP	Persistent organic pollutant
PP	Polypropylene
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PUFA	polyunsaturated fatty acid
PVC	Polyvinyl chloride
PVDF	Polyvinylidene difluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDG	Sustainable development goal
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TLR4	Toll-like receptor 4
VEGF	Vascular endothelial growth factor

ABSTRACT

Microplastics and nanoplastics (MPNPs) are emerging contaminants with potential implications for human health, particularly in the context of colorectal cancer (CRC). This study investigates the effects of different sizes and types of MPNPs particles on cell viability and intracellular signaling pathways in HT-29 CRC cells. Cells were exposed to 6 μm and 54 μm polyethylene (PE) microspheres and 50 nm and 100 nm polystyrene (PS) nanospheres, with a subsequent assessment of cell viability and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway activation. Our results demonstrated that 6 μm PE microspheres induced a significant, dose-dependent decrease in cell viability, most notably at 1000 μg/mL. In contrast, 54 μm PE microspheres resulted in only a slight reduction in viability at the same concentration. Additionally, MAPK/ERK pathway activation was markedly increased following exposure to 6 μm PE microspheres compared to 54 μm PE microspheres, particularly at higher doses. Conversely, 50 nm and 100 nm PS nanospheres did not significantly affect cellular viability, although there was a noticeable trend towards increased viability. These findings highlight the importance of particle size in determining the toxicity of microplastics (MPs) and suggest that smaller particles may pose a greater risk to cellular health by both decreasing viability and activating MAPK/ERK signaling pathway. These discoveries suggest the potential contribution of MPNPs to the development of CRC via cytotoxic effects and triggering crucial signaling pathways involved in cell survival and inflammation.

Keywords: colorectal cancer, microplastics, nanoplastics, polyethylene microspheres, polystyrene nanospheres, in vitro models, cell viability, MAPK/ERK pathway.

RESUM

Els microplàstics i nanoplastics (MPNPs) són contaminants emergents amb potencials implicacions per a la salut humana, especialment en el context del càncer colorectal (CCR). En aquest estudi, s'han analitzat els efectes de diferents mides i tipus de partícules de MPNPs en la viabilitat cel·lular i l'activació de vies de senyalització intracel·lulars en cèl·lules d'adenocarcinoma colorectal humà HT-29. Les cèl·lules van ser exposades a microesferes de polietilè (PE) de 6 μm i 54 μm i nanoesferes de poliestirè (PS) de 50 nm i 100 nm, avaluant el seu impacte en la viabilitat cel·lular i l'activació de la via de senyalització MAPK/ERK. Els nostres resultats van demostrar que les microesferes de PE de 6 μm induïen una disminució significativa dosi-dependent de la viabilitat cel·lular, especialment a 1000 μg/mL. En canvi, el tractament amb microesferes de PE de 54 μm va resultar en una lleu reducció de la viabilitat cel·lular a la mateixa concentració. Paral·lelament, es va observar una major activació de la via MAPK/ERK després de l'exposició a les microesferes de PE de 6 μm en comparació amb les de 54 μm, particularment a dosis altes. D'altra banda, l'exposició a nanoesferes de PS de 50 nm i 100 nm no va afectar significativament la viabilitat cel·lular, tot i que s'observà una tendència a l'augment de la viabilitat. Aquests resultats subratllen la importància de la mida de les partícules en la toxicitat dels microplàstics (MPs), proposant que les partícules més petites promouen una major disminució de la viabilitat cel·lular així com un augment de l'activació de la via de senyalització de MAPK/ERK. Aquests descobriments suggereixen la possible contribució dels MPNPs en el desenvolupament del CCR a través d'efectes citotòxics així com l'activació de vies de senyalització crucials per a la supervivència cel·lular.

Paraules clau: càncer colorectal, microplàstics, nanoplàstics, microesferes de polietilè, nanoesferes de poliestirè, models in vitro, viabilitat cel·lular, via de senyalització de MAPK/ERK.

INTEGRATION OF FIELDS

The main scope of this work is **Toxicology**, as it investigates the harmful effects of MPNPs, which are toxic compounds, on the colorectal adenocarcinoma cell line HT-29. By assessing the cytotoxicity of MPNPs on HT-29 cells, potential risks associated with exposure to these environmental contaminants can be elucidated. Moreover, by honing in on their impact on HT-29 cells, this study endeavors to uncover the intricate mechanisms underlying MPNP toxicity and its relevance to carcinogenic processes.

On one hand, this research is integrated into the field of **Cell Biology**, as the experimental component involves conducting cellular assays. The cytotoxic effects were studied using the MTT assay, and molecular alterations were observed by investigating the activation of the MAPK/ERK signaling pathway through Western Blot analysis.

On the other hand, another secondary covered area is **Physiology and Physiopathology**, since this work provides critical insights into how MPNPs influence key processes involved in CRC, such as inflammation and potential carcinogenic pathways. Additionally, physiology, which is the study of normal biological functions, offers a foundational understanding of how MPNPs interact with the human body. Through this lens, we try to elucidate mechanisms by which these particles may contribute to CRC development.

SUSTAINABLE DEVELOPMENT GOALS (SDGs)

MPNPs are tiny particles of plastic that have become pervasive pollutants in various environments, including oceans, freshwater bodies, and even the air. Their widespread presence raises concerns about potential health risks to humans and ecosystems. Research suggests that MPNPs can enter the human body through ingestion, inhalation, and dermal absorption, potentially accumulating and causing adverse health effects such as inflammation, oxidative stress, and cellular damage. Additionally, there are growing concerns about MPNPs acting as carriers for harmful chemicals and pathogens, exacerbating their health impacts. Moreover, they have also been associated with disruptions to the gut microbiome, which is crucial for human health.

Specifically, preliminary evidence suggests a possible association between MPNPs exposure and harmful damage to colorectal cells, potentially predisposing individuals to CRC development. However, further studies are needed to fully understand this relationship and determine underlying mechanisms. Thus, due to the growing interest in the impact of MPNPs on health, the aim of our experimental work is to study the effect of MPNPs on the development of CRC.

Overall, while more research is necessary to fully comprehend the health implications of MPNP exposure, current evidence suggests it poses a potential risk to human health. This underscores the importance of reducing plastic pollution to safeguard human and environmental well-being. Moreover, experts emphasize the need for greater public awareness of the risks of MPNPs exposure and the implementation of measures to reduce their presence in the environment and diet.

Taking all of this into account, the SDG addressed in this work is included in **Goal 3: Good Health and Well-being**, as CRC is a serious disease that affects the health and well-being of individuals worldwide. Additionally, studying the impact of MPNPs on this illness, as well as others, contributes to promoting the health and well-being of people by identifying potential risk factors.

This project also fits within **Goal 9: Industry, Innovation, and Infrastructure**, because research on the field of CRC involves the development of new technologies and methodologies for early diagnosis, treatment, and monitoring of the disease. These technologies and methods allow, among other things, investigating the impact of risk factors, such as MPNPs, on CRC. Additionally, it promotes collaboration between the public and private sectors to improve health infrastructure and continue research.

Another goal that would include this work is **Goal 12: Responsible Consumption and Production**. This is because studying the impact of MPNPs on human health, as well as on ecosystems, can promote more responsible production and consumption practices. This includes reducing the generation of plastic waste.

Lastly, considering MPNPs, the work could also be aligned with **Goal 17: Partnerships for Goals**. The fight against CRC requires collaboration between different institutions, such as government, academia, and the medical and pharmaceutical industry. Through this multidisciplinary partnership, research can progress, and insights into MPNPs and their impact on diseases such as CRC can be gathered from these diverse organizations.

INDEX

1. INTRODUCTION

1.1. COLORECTAL CANCER

CRC is a malignancy that specifically affects the colon or rectum and arises due to abnormal growth of glandular epithelial cells. It manifests in three main forms: sporadic, hereditary, and colitis-associated (1). In patients experiencing symptoms, the illness can manifest as alterations in bowel movements, such as diarrhea or constipation, visible or occult bleeding in the colorectal area, along with discomfort and cramping in the abdomen. Additionally, weight loss, weakness, and fatigue may occur, particularly in individuals with advanced stage tumors (2).

The most efficient strategy for preventing CRC and lowering related mortality rates within the population is by screening average-risk individuals. Consequently, numerous countries have implemented such screening programs, such as the detection of occult blood in stools, tailored to the age and location of the individuals involved (1). Moreover, screening holds relevance in CRC due to its prevalence and the belief that it typically follows a stepwise progression known as the adenoma-carcinoma sequence. Although the precise duration for an early adenoma to evolve into a CRC remains uncertain, research indicates that this process likely spans at least a decade. This extended time frame provides ample opportunity for detection through screening. Consequently, CRC can be prevented by eliminating colorectal adenomas, and the sooner CRC is identified, the lower the likelihood of fatal outcomes for the patient (1,3).

Additionally, as transitioning countries experience a rise in CRC cases, the feasibility of screening in these regions is often limited due to the high costs of colonoscopy and the challenges associated with establishing the infrastructure required for diagnostic and treatment services. However, there is growing evidence suggesting that CRC screening using noninvasive methods, characterized by high specificity, good sensitivity, and ease of use, could prove to be cost-effective in many transitioning regions (4).

1.1.1. EPIDEMIOLOGY

CRC incidence continues to rise steadily. It currently ranks as the third most common cancer type worldwide, trailing only lung and breast cancers. The risk of CRC increases significantly, by over 10-fold, before the age of 50, peaking at the age of 85 and older. Men face about a 50% higher risk compared to women. In addition, CRC ranks as the third most common malignancy in men and the second most common in women (3).

Projections indicate that the global burden of CRC will reach approximately 2.2 million new cases annually by 2030, marking a further 20% increase from the current 1.85 million cases per year (3). Colon cancer incidence rates exhibit an approximately 10-fold difference across different regions worldwide. The highest rates are observed in European regions, Australia/New Zealand, and Northern America. Rectal cancer follows a similar

regional distribution pattern, with high rates observed in Eastern Asia. Conversely, both colon and rectal cancer incidence rates tend to be low in most regions of Africa and in Central Asia (4). The cumulative risk of developing CRC by the age of 74 is 2.27%, with higher rates in countries with very high human development index (HDI) compared to those with low HDI. Moreover, the cumulative risk of dying from CRC by the age of 74 is 0.92%, considerably higher in Europe than in Africa, as well as in countries with very high HDI compared to those with low to medium HDI (3). Despite the overall higher frequency in highly developed nations, recent trends show stabilization or even a decrease in incidence rates (3). This decline is often attributed to population-level shifts towards healthier lifestyles and the implementation of screening programs (4). In contrast, some low- and middle-income countries are experiencing a rise, possibly due to the adoption of more westernized lifestyles (3). Likewise, as countries undergo significant socioeconomic development, there has been a consistent increase in CRC cases, particularly in regions such as Eastern Europe, South-Eastern and South-Central Asia, as well as South America. These changes include increased consumption of animal-source foods and a shift towards a more sedentary lifestyle, characterized by reduced physical activity and a higher prevalence of overweight and obesity (4). Geographically, CRC mortality rates vary, with the condition ranking as the 20th cause of death in the South-East Asia region, 7th in Europe, and 11th in the Americas (3). Even so, in several high-income countries like the United States, Canada, and Australia, there have been numerous recent reports indicating an increase in CRC cases among younger adults—those under 50 years old at the time of diagnosis. The incidence of CRC in this age group is rising by 1-4% annually (4).

Adenocarcinoma stands out as the most common form of CRC, accounting for up to 95% of cases. Its differentiation levels vary, with well-differentiated adenocarcinoma showing over 95% tumor gland formation, while moderately and poorly differentiated adenocarcinomas exhibit gland formation in 50-95% and less than 50% of cases, respectively. Moderately differentiated adenocarcinoma is the most prevalent diagnosis in clinical practice, making up approximately 70% of cases, while poorly and well-differentiated adenocarcinomas represent around 20% and 10% of cases, respectively. Moreover, the left colon is more commonly affected than the right one (5).

Remarkably, approximately 90% of CRC cases are detected at early stages, while the remaining 10% are diagnosed at a metastatic stage (Figure 1). Early detection significantly impacts survival rates, with a 5-year survival rate of around 90% for CRC diagnosed at an early stage, compared to only 13% when the diagnosis is delayed. However, the often-asymptomatic nature of the disease contributes to delayed diagnoses (3).

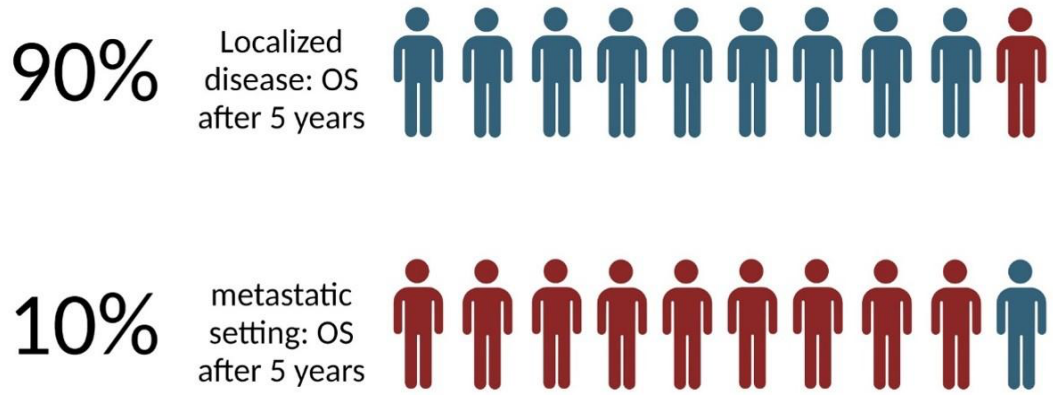

Figure 1. CRC cases diagnosed at early and metastatic stages. This figure shows the distribution of CRC cases according to their detection stages. Early-stage CRC refers to instances where the tumor is localized to its original site, whereas metastatic-stage CRC indicates that the cancer has progressed and spread to distant organs or tissues. Created with Biorender.com.

1.1.2. CARCINOGENESIS

CRC displays genetic diversity and can arise through multiple pathways. For instance, numerous CRC cells showcase several somatic mutations stemming from varied levels of gene expression. Consequently, CRC is thought to possess one of the highest mutational burdens among all malignancies. Depending on the quantity of somatic mutations, CRC is generally classified as hypermutated (exceeding 12 mutations per 10^6 bases) or non-hypermutated (below 8.24 mutations per 10^6 bases) (1).

Like other tumors, CRC is categorized into stages ranging from stage I (carcinoma in situ) to stage IV. Hyperproliferation of colon cancer cells triggers the formation of a benign polyp or adenoma. About 10% of adenomatous polyps may transform into malignant adenocarcinomas, penetrating the muscularis propria (stage I). As the tumor expands, it further infiltrates tissue layers, reaching the serosa (stage II) and visceral peritoneum (stage III). Finally, stage IV is characterized by the spread of cancer to distant organs, such as the liver or lungs. The disease's severity, prognosis and treatment options are determined by its stage (6).

Typically, the progression towards CRC begins with the formation of dysplastic tissue due to non-cancerous growth. After undergoing multiple abnormal DNA alterations, CRC development begins (1). Thus, the development of CRC starts with an aberrant crypt focus, the earliest identifiable neoplastic lesions in the colon carcinogenic model, evolving into neoplastic precursor lesions -commonly known as polyps- and eventual progressing to CRC over an estimated 10–15-year period (7) (Figure 2). Hence, regular screening, detecting, and removing polyps at the early stage is crucial to prevent CRC.

The loss of genomic and epigenomic stability accelerates the accumulation of mutations and epigenetic alterations in oncogenes and tumor suppressor genes. These changes drive

the malignant transformation of colon cells through successive rounds of clonal expansion, favoring cells with the most aggressive and malignant behavior. A widely accepted theory suggests that the cell of origin for most CRC is a stem cell or a cell with stem cell-like properties located at the base of the colon crypts. According to this model, mutations in oncogenes and tumor suppressor genes occur in these cells, leading to the formation of cancer stem cells. These cancer stem cells play a crucial role in initiating and sustaining tumor growth (7).

Globally, there are two major distinct precursor lesion pathways, which are the traditional adenoma-carcinoma pathway and the serrated neoplasia pathway. The traditional adenoma-carcinoma pathway (also referred to as the chromosomal instability sequence), leads to 70-90% of CRCs (7). Thus, chromosomal instability phenotypes typically develop following genomic events initiated by an *APC* mutation, followed by *RAS* activation or the loss of *TP53* function. On the other hand, the serrated neoplasia pathway causes 10-20% of CRCs and is defined by the development from normal cells to hyperplastic polyp, sessile serrated adenomas, and eventually cancer (1,7). Conversely, this pathway is associated with *RAS* and *RAF* mutations, and epigenetic instability, characterized by the CpG island methylation phenotype, which is involved in pathways related to inflammation (7). In fact, chronic inflammation leads to the development of normal cells into indeterminate dysplasia, which then advances to low-grade dysplasia, further progressing to high-grade dysplasia, and ultimately culminating in cancer (1) (Figure 2).

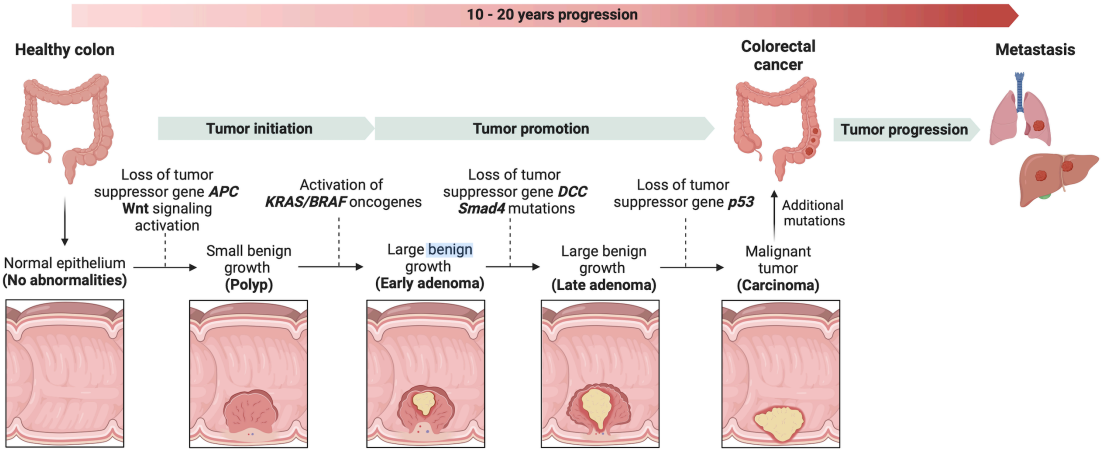

Figure 2. CRC carcinogenesis pathway overview. This figure provides a simplified overview of the process of CRC carcinogenesis, illustrating the key steps leading to the development of cancer. The process begins with initiation, where genetic mutations occur, followed by promotion, characterized by enhanced cell proliferation and survival. Progression then ensues, marked by tumor growth and the acquisition of invasive properties. Created with Biorender.com.

Furthermore, it has been demonstrated that molecular features of right-sided (proximal) colon cancers are different when compared with left-sided (distal) colon cancers and rectal cancers. Apart from molecular differences, embryological, biological, and anatomical differences exist between left-sided and right-sided CRC (8).

Remarkably, in 2014, CRC was classified into four molecular subtypes based on gene expression profiles, known as consensus molecular subtypes (CMS 1-4). Each CMS is characterized by unique genes or pathways: microsatellite instability immune (CMS1), canonical (CMS2), metabolic (CMS3), and mesenchymal (CMS4). Right-sided CRCs are more often MSI-immune and metabolic tumors. Although tumor sidedness and mutations in *RAS* or *RAF* genes are currently factors that help determine which patients will benefit from different systemic treatments, the CMS classification is being explored in clinical trials as a prognostic or predictive marker (9).

1.1.3. RISK FACTORS

The etiology of colorectal neoplasms has not been fully understood and the immediate causes are still unknown. However, many years of research have allowed us to determine many risk factors. The occurrence of CRC is associated with non-modifiable risk factors, including age and hereditary factors, as well as modifiable factors related to the environment and lifestyle (7) (Figure 3).

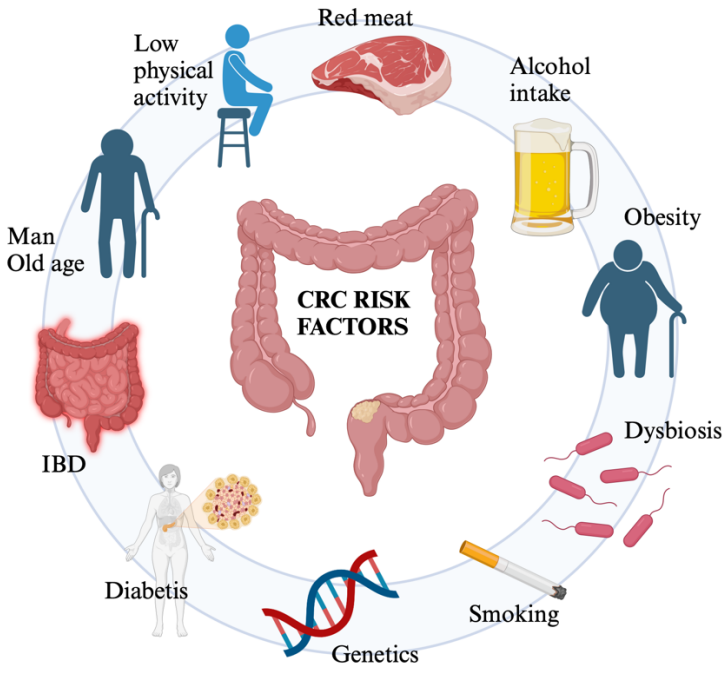

Figure 3. Key risk factors for CRC development. Figure depicting a simplified overview of the main risk factors for CRC development, including genetic predisposition, diets high in red and processed meats, physical inactivity, obesity, smoking, and heavy alcohol consumption. Created with BioRender.com.

On the one hand, paying attention to non-modifiable risk factors, we should focus especially on family history, age, sex, and ethnicity. Regarding family history, it seems to have a part in approximately 10-20% of all patients with CRC, with varying risk depending on number and degree of affected relatives and age of CRC diagnosis (7). Based on twin and family studies, estimates for heritability of CRC range from 12-35%. Although several genome-wide association studies of CRC have successfully identified

cancer susceptibility genes that are associated with CRC risk, most factors causing heritability are still elusive and subject to further study (10). A retrospective analysis conducted in the United States investigating risk factors associated with early-onset CRC discovered that individuals aged 18-49 years with a family history of CRC face a heightened risk of developing CRC compared to those without such familial history. Moreover, in contrast to patients with late-onset CRC (aged ≥50 years), those with early-onset CRC were more likely to have a family history of CRC (11).
The two most common forms of hereditary CRC are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (11). Both conditions stem from specific inherited mutations that heighten the susceptibility to CRC among relatives. Thus, FAP is an uncommon genetic disorder triggered by a mutation in the *APC* gene, which typically functions to suppress tumor growth in the intestinal tract. Individuals with FAP develop hundreds to thousands of small adenomatous polyps along the lining of their large intestine and rectum, often appearing during adolescence. These polyps continue to proliferate throughout the colon and can eventually progress into cancerous growths (1). In fact, the risk of CRC development in individuals with untreated FAP is nearly 100% (11). On the other hand, in HNPCC, such as Lynch syndrome and familial CRC, the risk of developing CRC in these patients is 70-80% (11, 12).

Concerning age, it is considered the main cause. Although cancer occurs also in young people, the chance of developing it increases after the age of 50, since 9 out of 10 people who develop cancer are over 50 years of age. The peak incidence occurs after the age of 70 (11). The average age at diagnosis for men with colon cancer is around 68 years, while for women, it's typically around 72 years. However, both men and women are diagnosed with rectal cancer at an average age of 63 years. Additionally, when considering gender, CRC is more prevalent in men than in women (1).
Based on the CDC report, in 2020, black men and women exhibited the highest incidence and mortality rates among races, followed by white men and women, Asian or Pacific Islanders, and American Indians or Alaska Natives. Additionally, individuals of non-Hispanic origin demonstrated higher incidence and mortality rates compared to Hispanic counterparts (13).

On the other hand, we have modifiable factors related to the environment and lifestyle, which can be modified or altered to reduce the risk for CRC. Low physical activity is one of the most important behavioral factors in the development of CRC, alongside obesity (7). Thus, regarding obesity, adipocytes present within the tumor microenvironment serve as a source of energy that fuels the proliferation of cancer cells. Adipose tissue contributes to the initiation and progression of CRC by boosting the secretion of various factors, including adipokines, proinflammatory cytokines, insulin, or insulin-like growth factor (IGF), while decreasing the secretion of adiponectin. These molecules activate signaling pathways that support tumor cell proliferation and metastasis, thereby playing a significant role in oncogenesis. Furthermore, obesity contributes to intestinal dysbiosis and elevated levels of total bile acids. Numerous studies suggest that these bile acids can induce colorectal oncogenesis by damaging epithelial cells and triggering inflammatory

processes (14). Similarly, a diet rich in fat, particularly animal fats, meals prepared at high temperatures, consumption of red meat, high-calorie intake, and low consumption of fruits and vegetables are significant risk factors (4). The exact mechanism linking excessive consumption of red and processed meats to an increased risk of CRC remains unclear. However, several potential underlying mechanisms have been proposed. These include the formation of carcinogenic substances during the cooking process, which can lead to greater exposure to compounds such as N-nitroso compounds (NOCs), heterocyclic amines (HCAs), and polycyclic aromatic hydrocarbons (PAHs). Heme iron molecules present in red meat can also generate carcinogens and act as DNA mutagens themselves. Additionally, other factors such as polyunsaturated fatty acids (PUFAs), bile acids, non-human sialic acid, and infectious pathogens may contribute to the risk of CRC (15).

In this context, type II diabetes has also been identified as a risk factor in CRC. Hyperinsulinemia affects the colon, potentially establishing a link to CRC through various mechanisms. IGFs, crucial for cell growth, are often overexpressed in cancer cells, promoting cell cycle progression and inhibiting apoptosis. Insulin exacerbates cancer risk by reducing IGF-binding proteins, increasing free IGF levels. This activates several pivotal signaling pathways involved in the development and proliferation of cancer cells, such as the Ras-Raf-MEK-MAPK pathway (also known as the MAPK/ERK pathway) and the PI3K-AKT-mTOR pathway. Moreover, enhanced insulin signaling heightens metabolic activity, leading to increased oxidative stress and DNA damage (16).

Smoking is another modifiable risk factor, since the risk of CRC tends to rise with the number of cigarettes smoked. Nicotine, a carcinogen present in cigarettes, can enhance cell proliferation by modifying receptor expression and phosphorylation patterns within various mitogenic pathways. Exposure to nicotine results in heightened phosphorylation of the EGFR and increased expression of 5-lipoxygenase (5-LOX) in colon cancer. Additionally, nicotine promotes the growth of CRC cells by upregulating acetylcholine and noradrenaline receptors. Furthermore, nicotine stimulates the process of angiogenesis and neovascularization in colon cancer by elevating levels of vascular endothelial growth factor (VEGF), 5-LOX, cyclooxygenase-2 (COX-2), and matrix metalloproteinase-2/9 (MMP-2/9) (17).

Moreover, numerous research investigations indicate a direct link between drinking alcohol and the likelihood of developing CRC. Combining data from various prospective studies and meta-analyses, have revealed a slight but noticeable connection between consuming large amounts of alcohol (more than 50 grams per day) and increased mortality rates related to CRC. Interestingly, this correlation appears to be more pronounced among Asian individuals compared to those of white ethnicity, potentially influenced by genetic makeup and dietary habits (18). In addition, there is a time-dependent relationship between the duration of alcohol consumption and CRC. Thus, the longer the period of alcohol consumption, the higher the risk of having CRC. Alcohol metabolism involves the breakdown of ethanol into various metabolites, which can induce

carcinogenic effects in the colon. The production of these ethanol metabolites can be influenced by the colon microbiota, which is now recognized as another significant factor in colon cancer development. Processes such as the formation of DNA adducts, oxidative stress, lipid peroxidation, epigenetic alterations, dysfunction of the epithelial barrier, and modulation of the immune system are all associated with the production of acetaldehyde and other alcohol metabolites (1).

Actually, the overall incidence of CRC was significantly higher among those with low education attainment or living in low socioeconomic districts. Much of the socioeconomic difference risk can be attributed to the higher incidence of adverse health behaviors in low-status populations and lower education levels. Hence, recent epidemiological evidence suggests that psychosocial factors may be considered risk factors for certain types of cancer, including CRC (19).

Another important risk factor is chronic inflammatory processes. Indeed, the risk of developing CRC increases twenty times in ulcerative colitis and three times in Crohn's disease patients (3). Chronic colitis resulting from inflammatory bowel disease (IBD) is linked to a heightened risk of CRC, with the risk escalating with prolonged duration of IBD. However, IBD accounts for only around 1% of CRC cases in Western populations (20). While the association between Crohn's disease and CRC risk is not as clearly established as with ulcerative colitis, several studies suggest an increased incidence of CRC and cancer-related mortality among patients with Crohn's disease. Interestingly, a study on early-onset CRC discovered that patients with a history of IBD, such as Crohn's disease, had a higher incidence of metastatic disease, poorer histological differentiation, and increased rates of lymphovascular and perineural invasion compared to those with sporadic cancer. Furthermore, survival rates were lower among these patients (14). In addition, some drugs, such as aspirin or non-steroidal anti-inflammatory drugs (NSAID), menopausal hormone therapy drugs and statins, may be related with the onset of CRC (7).

Remarkably, recent studies have highlighted the crucial role of gut microbiota and dysbiosis in CRC. Dysbiosis, characterized by imbalances in gut microbial composition, is increasingly recognized as a contributing factor in CRC carcinogenesis (21). The gut microbiota consists of a diverse community of microorganisms that interact closely with cells in the intestine of the host, influencing both immunity and the metabolome within the gastrointestinal tract. Thus, some studies have shown that the intestinal microbiota in CRC patients differs from that of healthy individuals, exhibiting a global compositional change and dysbiosis (22, 23). For example, in animal models, certain bacteria such as *Fusobacterium nucleatum*, *Escherichia coli*, and *Bacteroides fragilis* have been implicated in CRC development. These microorganisms can induce a pro-inflammatory microenvironment by releasing various pro-inflammatory chemokines and activating NF-kB and STAT3 signaling pathways, thus contributing to the progression of CRC (23-27) (Figure 4).

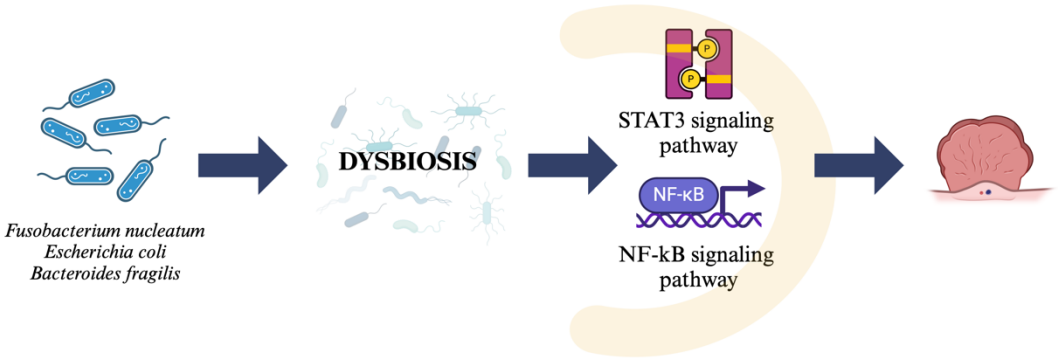

Figure 4. Dysbiosis involving *F. nucleatum*, *E. coli*, and *B. fragilis* can precipitate CRC. The figure outlines how specific bacterial imbalances contribute to a pro-inflammatory environment by activating the STAT3 and NF-kB pathways, thereby promoting the development of CRC. Created with Biorender.com.

However, human studies have yielded fewer clear results, partly due to challenges in distinguishing microorganisms with a primary influence from those secondary to cancer. Methodological differences, such as variations in sample cohorts, diverse disease stages, and disparities between the right and left colon, contribute to this ambiguity. Moreover, the observed diversity in cancer-related microbiota may also stem from differences in the immune-inflammatory response associated with various cancer profiles (24). Therefore, understanding the implications of the gut microbiota in CRC can lead to the development of new strategies for detecting or reducing the progression of CRC precursors, ultimately contributing to improvements in detection and treatment approaches. Various potential clinical applications related to the intestinal microbiota and CRC have been evaluated, such as using the microbiota as a prognostic and/or predictive biomarker, as a modulating treatment for CRC, or as a preventive measure (22).

It is important to note that several environmental factors, such as MPNPs, can cause dysbiosis. As will be reviewed in detail in the following sections, MPs are ingested by humans starting at a young age and in growing amounts. As they travel through the digestive system, they engage with the body's natural processes, especially in the colon and rectum, potentially affecting the protective mucus layer in the colon. Consequently, they could change how colon cells are exposed to harmful substances from the gut microbiota, potentially impacting the risk of CRC (28).

1.2. MICROPLASTICS AND NANOPLASTICS

Plastics are synthetic organic polymers characterized by a diverse array of chemical compositions and densities. Among the numerous types are polystyrene (PS), polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyurethane (PU), polyester (PES), polyamides (PA), and so on. These materials find utility across a wide spectrum of industries including building and construction, healthcare, sports and entertainment, electronics, agriculture, packaging, aeronautics, and more. Additionally, plastics are often enhanced with various additives such as fillers, stabilizers, pigments, foaming agents, lubricants, flame retardants, and

plasticizers, among others. These additives serve to tailor the properties of plastics to suit specific applications, making them versatile materials for diverse purposes (29). Indeed, plastic products have become integral to human life due to their affordability, lightweight nature, durability, and resistance to corrosion. Moreover, they exhibit excellent thermal and electrical insulation properties. Thus, these characteristics make plastics highly versatile and desirable materials for a wide range of applications, contributing to their widespread use in various industries and everyday consumer products (30).

Since the 1950s, the production of plastic has increased dramatically, with approximately 359 million tons of virgin plastic being manufactured annually. While plastics undoubtedly enhance human life, this surge in plastic usage has inadvertently led to the emergence of a contaminant posing a serious threat to our environment (31). Plastics primarily originate from fossil hydrocarbons, and the raw materials used for synthesis are predominantly non-biodegradable. As a result, plastic waste accumulates within natural ecosystems, posing environmental risks (32). Thus, with the intensification of industrialization, the world has entered an era dominated by plastics. PE, PP, PVC, PS, PU and PET now comprise approximately 80% of plastic demand (33).

The production and widespread use of plastics have led to the generation of significant amounts of plastic waste, resulting in the emergence of MPNPs as contaminants of global concern. MPs are plastic particles with diameters less than 5 mm, while nanoplastics (NPs) are even smaller, ranging from 1 μm to 100 nm. These particles are challenging to remove from the environment once released, and they have been detected in various ecosystems worldwide, including terrestrial, aquatic, and atmospheric environments (34, 33). Likewise, the transfer of MPNPs through the aquatic food chain has led to their inevitable exposure in the human body. The sources of MP pollution can be divided into two subcategories: primary MPs, which are manufactured within the MPs size range (e.g., pellets), and secondary MPs, originating from larger plastics. The full extent of all secondary MP sources is still unclear, but common sources include weathering-induced fragmentation and tire abrasion (35).

MPNPs represent an emerging pollutant, and their presence in the environment and human exposure routes have sparked widespread concern. They can be ingested, inhaled, or come into contact with the skin, and they have been found in various sources such as air, water, soil, seafood, and packaged foods (32). Consequently, there is a widespread concern that MPNPs might not only pose environmental threats but also have adverse effects on human health. In fact, recent research has documented the presence of MPNPs in human tissues and fluids, raising concerns about their potential health impacts. MPNPs have been associated with liver and gastrointestinal issues, inflammation, and genotoxicity. However, the full extent of their toxicity and health risks remains unclear, and further research is needed to understand their effects on human health (33).

The potential impact of MPNPs on various organisms depends on several factors, including particle size, quantity, plastic type, and how and where they accumulate within

organisms. Given the diversity of plastics found in natural environments, it is premature to definitively assess their toxicity to humans. Furthermore, human exposure to MPNPs may occur over prolonged periods, potentially leading to their accumulation in organisms and resulting in local and systemic detrimental effects (33). Additionally, some experimental studies have shown that MPNPs can induce cytotoxicity in human cell lines and increase the frequency of genetic abnormalities such as micronucleation, nucleoplasm bridge formation, and nuclear bud formation, which can lead to genotoxicity (30).

Numerous studies have established methods for detecting MPs. However, despite extensive efforts to accurately quantify NPs in the environment, their detection remains challenging. As a result, achieving sensitive and specific detection is limited, thus hindering a comprehensive understanding of their environmental and health implications (36).

The toxicity of MPNPs is primarily attributed to their small size and large surface area, which facilitate interactions with biological systems. However, other factors such as surface charge, presence of functional groups, exposure duration, concentration, particle shape, and type of polymer also play significant roles in determining their toxicity. Thus, the small size of NPs promotes their internalization and accumulation within cells, making them more readily internalized compared to MPs. Additionally, their small size is associated with heightened surface energies and interactions, further influencing their biological effects (29).

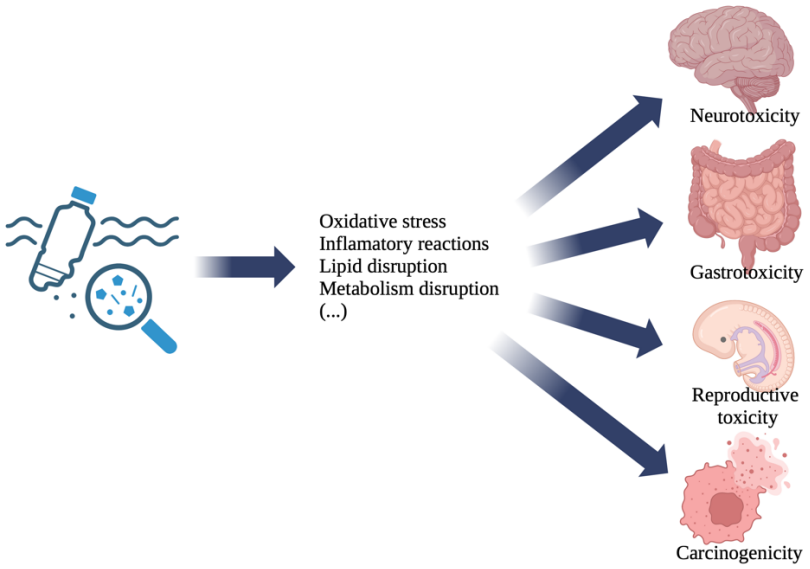

Figure 5. Toxicity of microplastics on various organs. This diagram illustrates how MPNPs induce toxicity across different organs through mechanisms such as oxidative stress and inflammation. Created with Biorender.com.

Furthermore, the detrimental impacts of MPNPs mainly arise from potential toxic processes like triggering oxidative stress, provoking inflammatory reactions, disrupting lipid or energy metabolism, or influencing the expression of related biological factors.

Likewise, toxic effects induced by MPNPs have been observed in various cell types, including macrophages, erythrocytes, brain cells, epithelial cells lining different organs, and lung cells. However, the precise effects of MPNPs on human health remain uncertain, despite existing studies indicating that their toxic effects primarily involve chemical and microbial toxicity (32). Overall, MPNPs can exert their effects through various mechanisms, resulting in outcomes such as neurotoxicity, gastrointestinal alterations, reproductive toxicity, and carcinogenicity (29) (Figure 5).

1.3. THE IMPACT OF MICROPLASTICS AND NANOPLASTICS ON THE DEVELOPMENT OF COLORECTAL CANCER

The incidence of CRC among individuals younger than 50 years old, termed early onset colorectal cancer (EOCRC), is increasing worldwide. According to current literature, EOCRC predominantly affects the left colon and rectum, which show higher occurrences of mucinous or signet ring cells histology (37). Consequently, the epidemiological trends of EOCRC indicate a likely environmental influence. This uptick in EOCRC aligns with the period when we would expect to observe the effects of a rapid rise in MPs in the environment (38). Indeed, MPNPs have been detected in diverse amounts, shapes, and dimensions within the human colon, placenta, feces, and gastrointestinal tract (39). The consequences of prolonged exposure to MPs seem to vary greatly and are contingent upon the type and morphology of the particles. Moreover, the effects of a mixture of MPs, as encountered in real-life situations, are likely different from those of individual components. This complexity further complicates the understanding of this issue (40).

MPNPs could get exposed to humans via different routes, although the gastrointestinal tract serves as the main gateway for daily exposure (41, 42). The internalization of MPs through epithelial tissue could potentially expose these compounds to various cell types, including dendritic cells, macrophages/monocytes, and/or T cells (41). However, the rates and pace of uptake significantly rely on the mechanism involved. Various routes have been identified, with dependencies on particle characteristics and cell types highlighted previously. Current studies have demonstrated that smaller particles (<0.5 μm) are often taken up via receptor-mediated processes like clathrin or caveolin-mediated endocytosis, whereas larger particles (>0.5 μm) are typically internalized through phagocytosis or micropinocytosis (42).

Several in vivo studies have demonstrated that interaction with MPNPs results in disturbances in oxidative and inflammatory equilibrium within the intestine, as well as interference with the intestinal wall's permeability. Additional significant outcomes of exposure to MPNPs comprise of dysbiosis and toxicity to immune cells. Moreover, MPNPs can contain additives, adsorb pollutants, and potentially promote the proliferation of bacterial pathogens on their surfaces. As a result, they may carry intestinal toxins and pathogens, thereby potentially leading to additional adverse consequences (40) (Figure 6).

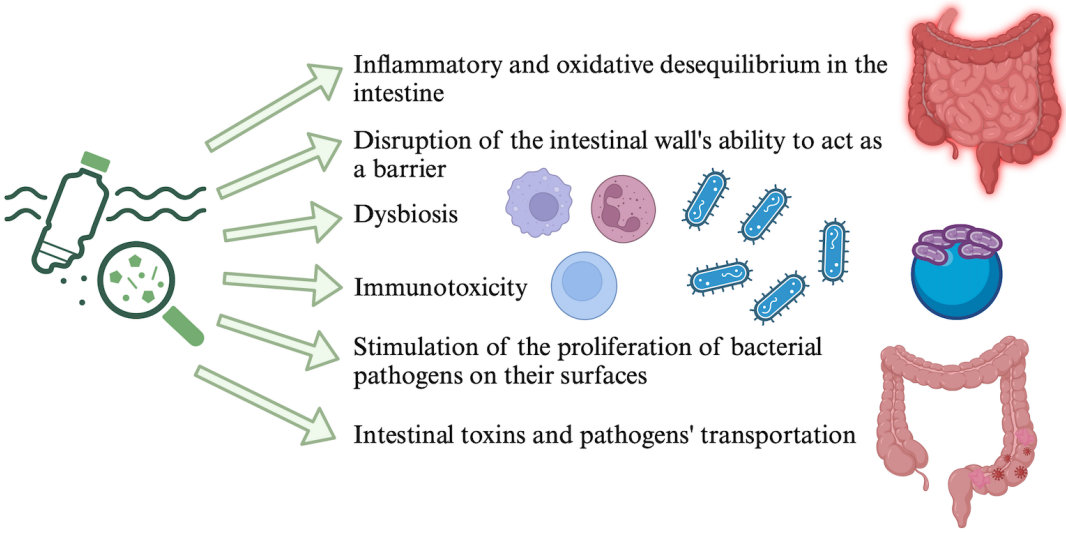

Figure 6. In vivo effects following MPNPs exposure. This image illustrates several significant effects observed after exposure to MPNPs, highlighting the urgent need for further research into the environmental and health implications of MPNP pollution. Created with BioRender.com.

Extensive research has demonstrated that microorganisms can indeed colonize MPs. The hydrophobic surface of MPNPs provides an ideal environment for microbial colonization and the formation of biofilms (43). Furthermore, there is evidence suggesting that interactions between MPs, microorganisms, and gut microbiota could have implications for health. In this context, a study conducted on mice exposed orally to 1000 µg/L of PS MPs of varying sizes (0.5 and 50 µm) investigated the toxic impacts of PS. The findings suggested that PS could disrupt the balance of gut microbiota, primarily evidenced by alterations in composition and diversity. Furthermore, both sizes of PS resulted in decreased hepatic triglyceride and total cholesterol levels, suggesting a potential induction of hepatic lipid disorders in mice (44).

As previously stated, MPs can serve as carriers for environmentally acquired surface-associated toxic chemicals, such as persistent organic pollutants (POPs), PAHs, and hydrophobic organic chemicals (HOCs). These exposures mediated by MPs carry inherent health risks, including the direct delivery of MP-associated toxic chemicals to the underlying epithelium (28). Furthermore, MPs have the propensity to accumulate in food chains, particularly in marine organisms, eventually reaching human food sources. As a result, drinking water and food represent the two primary sources of human exposure to MPs, thereby affecting the gastrointestinal tract (45).

The notion that sustained exposure to MPs likely poses harm to humans has expanded to encompass the consideration of how plastics may induce carcinogenesis in humans (28). The inherent physicochemical properties of MPNPs enable them to be taken up by cells and interact with cellular components. Their reactive surfaces have been documented to induce detrimental effects such as cytotoxicity, oxidative stress, and disruption of immune function (46). Moreover, owing to their persistent nature, MPNPs have the potential to accumulate in various organs and tissues over time, potentially leading to the

long-term development of cancer (39). This is attributed to the chemical composition of MP surfaces, which allows them to adsorb hydrophobic compounds, some of which are carcinogenic, and bind through electrostatic interactions with charged molecules, ions, and toxic metals (45). Therefore, the chronic nature of human exposure to MPNPs, coupled with some of the observed effects induced by MPNPs, underscores concern regarding the potential carcinogenicity of these compounds (46). Furthermore, various in vivo studies have demonstrated that the adverse effects caused by MPNPs depend on particle size, polymer type, shape, charge, concentration, and routes of exposure. These effects caused local inflammation, oxidative stress, changes in microbiota composition and metabolic disruption, leading to gastrointestinal toxicity, hepatotoxicity, reproduction disorders, and neurotoxic effects in different animal species. However, the current understanding of the effects of prolonged exposure to MPNPs, as experienced in real-life scenarios, remains limited, and comprehending the mechanisms of MPNPs is a complex endeavor (47).

In the context of CRC, there are various potential mechanisms through which MPs could disrupt the colonic mucus layer, diminishing its protective effect and consequently increasing the likelihood of CRC (28). In this scenario, Herrala et al. demonstrated that a 48-hour exposure to PE particles measuring 5-60 μm, alongside its extracts at doses varying from 0.25 to 1.0 mg/mL, decreased cell viability, and triggered increased oxidative stress responses in Caco-2 and HT-29 human colorectal adenocarcinoma cell lines (45).

Carcinogenesis can be genotoxic or non-genotoxic (28). Chemical substances and agents can exert genotoxic effects through primary or secondary mechanisms. In primary mechanisms, the compound directly interacts with the target cell, while in secondary mechanisms, an inflammatory response triggers downstream effects in the target cell. Primary mechanisms may involve direct interactions with DNA or indirect effects mediated by other molecules, such as the induction of reactive oxygen species (ROS) or inhibition of DNA repair mechanisms. Indeed, genotoxicity is widely regarded as an early predictor of the carcinogenic potential of a compound (46) (Figure 7). DNA damage can be a significant and potentially harmful outcome of long-term exposure to MPNPs. Studies have demonstrated that MPNPs can induce DNA fragmentation, reduce transcriptional gene expression, and correlate with the expression of genes involved in apoptosis. These effects underscore the potential genotoxicity of MPNPs and raise concerns about their impact on cellular health and integrity (48).

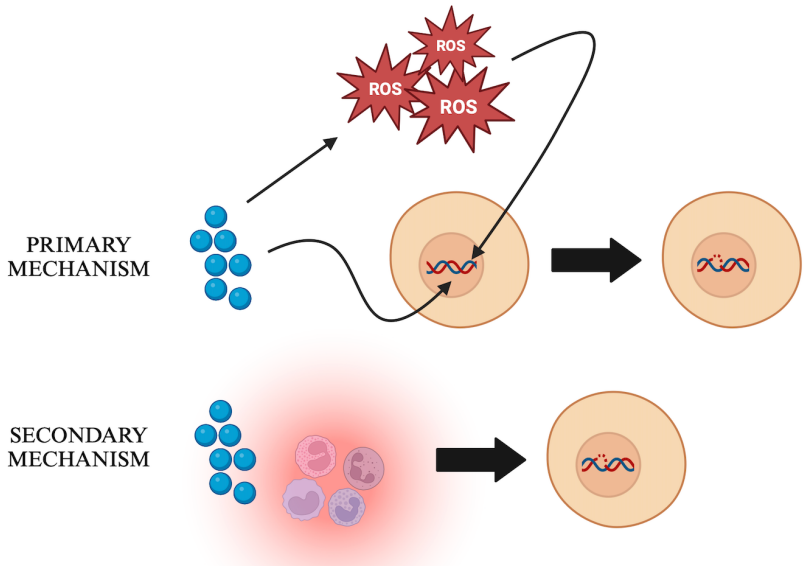

Figure 7. Genotoxic mechanisms induced by MPNPs. This image depicts the genotoxic effects induced by MPNPs. Exposure to MPNPs has been demonstrated to cause DNA damage, oxidative stress, and disruption in various cellular processes, potentially leading to mutations and carcinogenesis. These genotoxic mechanisms, whether primary or secondary, underscore the critical necessity for comprehensive research into the long-term health risks associated with MPNP exposure. Created with BioRender.com.

Non-genotoxic carcinogenesis can occur because of errors during cell division (28). In this context, MPs may induce inflammation through diverse mechanisms, including immune suppression, mitogenic signaling, receptor-mediated endocrine modulation, or epigenetic changes, among others (46). A study utilizing murine macrophages illustrated that these cells were capable of phagocytizing 10-micron sized PS MPs, and this ingestion could trigger an immunometabolically active state in macrophages. The phagocytosis of MPs by macrophages prompted a metabolic alteration towards glycolysis and a decrease in mitochondrial respiration, linked with an augmentation of cell surface markers CD80 and CD86, as well as cytokine gene expression associated with glycolysis. The gastrointestinal consequences of this metabolic transition within the framework of an immune response remain unclear, but there is an appreciated health risk (49).

When nanomaterials (NMs) like MPNPs interact with epithelial and immune cells, they trigger an increase in the production of pro-inflammatory cytokines. This inflammatory response aims to eliminate harmful agents and repair damaged tissue, often involving the recruitment of white blood cells to the affected area. In this process, ROS, and reactive nitrogen species (RNS) are generated, leading to DNA damage, as well as damage to proteins and lipids, resulting in tissue injury. Stem cells activated for tissue repair may also be harmed by ROS and RNS, potentially accumulating genetic mutations and giving rise to cancer stem cells. If the injury persists, chronic inflammation can develop, perpetuating tissue damage and healing processes, ultimately leading to fibrosis and malignancy (50). In fact, the generation of ROS by various types of MPs has been demonstrated through both in vivo and in vitro studies. A study was conducted to assess

the impacts of two distinct sizes (30.5 and 6.2 μm) of PE MPs on diverse human cell lines representing various tissues or cell types. Six cell lines were cultured with differing concentrations of PE, and assessments were made for cell viability, ROS levels, nitric oxide (NO) production, and cytokine expression. Findings revealed that PE generally did not significantly reduce cell viability except at the highest concentration (1000 μg/mL), which exhibited a minor decrease in intestinal epithelial Caco-2 and lung epithelial A549 cells. Furthermore, both sizes of PE triggered elevated NO levels in all cell lines, and an increase in ROS generation was observed in THP-1, Jurkat, and U937 immune cell lines. These results underscore the fact that MPs exert disparate effects on various cell types (41). It is well recognized that inflammation in the intestine is increasingly recognized as a pivotal factor in the initiation and progression of CRC (51). In particular, the activation of the NF-κB (p50/p65) transcription factor, a central regulator of inflammation and immune response, is commonly upregulated in CRC cells, playing a prominent role in promoting cell proliferation and metastasis, angiogenesis, therapy resistance, evasion of apoptosis, and the establishment of a pro-tumorigenic microenvironment (52, 53). Interestingly, a recent study showed that exposure to NPs can activate inflammatory responses in HT-29 cells by promoting the nuclear translocation of p50, an NF-κB subunit, which correlates with increased expression of Toll-like receptor 4 (TLR4) (54). Accordingly, Bahadur et al. demonstrated that polytetrafluoroethylene (PTFE)-MPs exposure promotes the activation the MAPK signaling pathways, especially the ERK pathway, in A549 lung epithelial cell line (55).

On the other hand, elevated expression of proinflammatory cytokines can stimulate mucin production, but abnormal glycosylation of these proteins may lead to defective mucus layer formation (28). The immune cell networks play crucial roles in the immune system's function. While direct studies on the immunotoxicity of plastics on the intestinal immune system are limited, in vivo evidence regarding the immunotoxicity of MPNPs suggests that immune cells could indeed be susceptible to damage induced by plastics. This susceptibility arises due to their compromise upon exposure to MPNPs (40). Following the ingestion of MPs, immune cells experience swift and substantial alterations in gene expression levels, influencing enzyme activities and the release of cytokines. The principal toxicological pathway after MP exposure involves the generation of ROS, which can in turn stimulate the synthesis of danger-associated molecular patterns (DAMPs). Conversely, DAMPs have the ability to activate toll-like receptors (TLRs), which play a key role in mediating innate immunity. This activation triggers a cascade of inflammatory responses in immune cells, including the production of cytokines (56). (Figure 8).

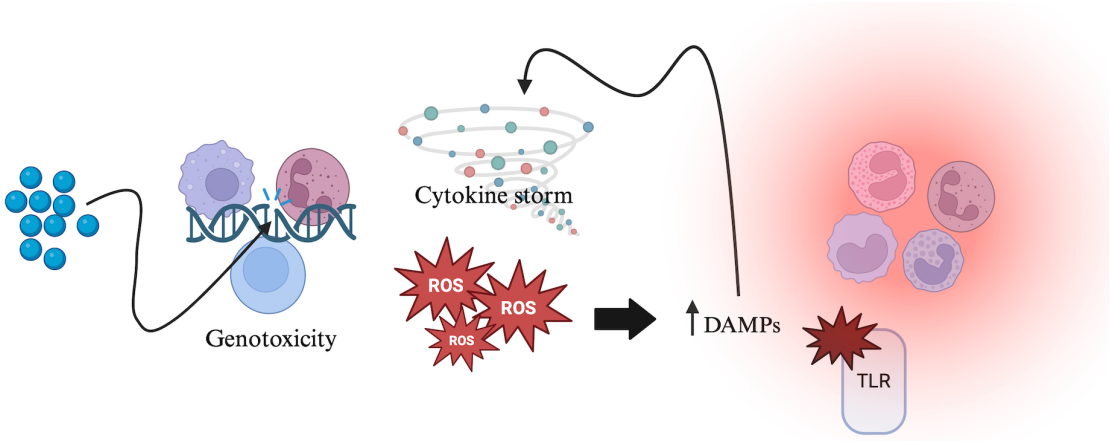

Figure 8. In vivo evidence of immunotoxicity induced by MPNPs on immune cells. This figure provides an overview of significant findings concerning the potential risks linked to exposure to MPNPs. It illustrates how MPNPs can exert genotoxic effects on immune cells, initiating a cascade of inflammatory and oxidative reactions. Created with BioRender.com.

Apart from its oxidative and proinflammatory activity, MPs pose a risk because they can physically block tissues, while NPs can interact with a wide range of substances in the gastrointestinal tract, including proteins, lipids, and nucleic acids. Recent studies suggest that the main source of toxicity for larger particles like MPs is their physical presence, which restricts their interaction with the surrounding medium. Conversely, smaller particles like NPs primarily cause harm through chemical means, such as by generating ROS (39).

All in all, human food and water sources can be contaminated by MPs, posing risks of inhalation or ingestion by humans. Consequently, MPs can harm organisms through their physical presence, causing abrasive effects that lead to inflammation, oxidative stress, and cytotoxicity. Additionally, their chemical burden and interactions with microbiota communities can further contribute to adverse effects on organisms (57).

2. HYPOTHESIS AND OBJECTIVES

Based on the preclinical studies previously stated suggesting the role of MPNPs in CRC development, our hypothesis is that MPNPs contribute to CRC progression through their impact on cell viability, as well as their capacity to activate proinflammatory and pro-survival intracellular signaling pathways. To demonstrate these hypotheses, the following objectives were established:

1. To study the effect of MPNPs exposure on the viability of the CRC cell line HT-29, using PE microspheres of 6 and 54 µm and PS nanospheres of 50 and 100 nm.

2. To investigate the activation of the MAPK/ERK signaling pathway following treatment with PE microspheres of 6 and 54 µm.

3. MATERIAL

3.1. HUMAN COLORECTAL ADENOCARCINOMA CELL LINE HT-29

HT-29 human colorectal adenocarcinoma cell line was used in this study. HT-29 cells derived from a human colon tumor (grade II adenocarcinoma). HT-29 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, high glucose, pyruvate) (Gibco®, Thermo Fisher Scientific), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Reactiva®) treated at 56ºC for 45 minutes and 1% penicillin-streptomycin (Thermo Fisher Scientific). These cells have a doubling time of 24 hours under these conditions. They exhibit negative RER phenotype and *TP53* protein mutation involving an amino acid substitution from Arg to His at codon 273. Moreover, the p21 protein is non-functional in this cell line. Additionally, there is a mutation (V600E) in the *BRAF* oncogene, although not in the KRAS protein.

3.2. MICROPLASTICS AND NANOPLASTICS

For the experiments, MP particles consisting of PE microspheres (Polysciences, Inc) with sizes of 6 and 54 μm were utilized. Additionally, NP particles composed of PS nanospheres (Polysciences, Inc) with sizes of 50 and 100 nm were employed. These particles were chosen to represent a range of sizes and plastic types commonly found in environmental samples and to assess the potential effects of both MPNPs on cellular response. PE microspheres and PS nanospheres serve as model particles to simulate environmental exposure scenarios and investigate their impact on cellular cytotoxicity and inflammatory pathway activation.

4. METHODOLOGY

4.1. MTT ASSAY

To determine the cytotoxicity induced by MPNPs, the MTT cell viability detection method was employed. This assay relies on the ability of viable, metabolically active cells to metabolize the yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-]-2,5-diphenyltetrazolium bromide (MTT)) into a purple formazan salt. This salt is solubilized with a solution containing 0.1% sodium dodecyl sulfate (SDS) in 0.01M hydrogen chloride (HCl), resulting in a quantifiable purple solution using a conventional automated ELISA reader. The peak absorbance of the formazan salt corresponds to a wavelength between 500 and 600 nm, thus readings were taken at 590 nm. The optical density (OD) obtained is proportional to the number of viable cells.

Initially, tumor cells were seeded in 96-well plates (Nunc, Labclinics) at an optimal density to avoid saturation of OD in control wells ($1.5 \leq OD \leq 1.8$). Considering the doubling time of the HT29 cell line and the duration of the experiments, cells were seeded

at a density of 2,000 cells/well in a final volume of 100 µL per well. After 24 hours (time required for cell adherence to the bottom of the wells), the culture medium was removed, and cells were treated with different concentrations of the MPNPs under study.

Treatment with MPNPs was conducted for 72 hours. Then, the culture medium was removed and the MTT solution was added following the manufacturer's instructions (MTT 10% (Roche) and 90% DMEM w/o red phenol (Gibco®, Thermo Fisher Scientific) not supplemented with FBS, in a final volume of 100 µL for each well. The MTT solution was incubated for 2 hours at 37°C and 5% CO_2, and subsequently the formazan salts formed at the bottom of the wells were resuspended with 100 µL/well of solubilizing solution (0.1% SDS + 0.01M HCl). Once solubilized, plates were incubated overnight at 37°C and 5% CO_2 in a humid atmosphere until they were read in a microplate reader at 590 nm the next day.

In each MTT experiment, the survival fraction (f) was calculated as the ratio of the OD of cells treated with different drug doses to the OD of untreated control cells. The data obtained was analyzed using the median effect lines method. This method involves graphically representing log10((1/fn)-1) (where fn represents the different viability fractions obtained) against the log10 (drug dose). From this point, the regression line was calculated using the least squares method; lines with a regression coefficient r2 greater than 0.95 were accepted. Through these lines, referred to as median effect lines, the slope (m) and IC50 (half-maximal inhibitory concentration) dose could be calculated. With these parameters, the drug concentrations necessary to inhibit a specific cell fraction, between 10 and 90%, were evaluated according to the equation:

$$\text{Drug dose} = \text{IC50 dose } (1/f\text{-}1)1/m$$

4.2. WESTERN BLOT

To detect changes in the levels of specific proteins present in the samples, the Western Blot technique was used. This technique involves the separation of proteins present in a sample by electrophoresis under denaturing conditions according to their molecular weight, followed by the transfer of these proteins to a polyvinylidene difluoride (PVDF) membrane (blot), and finally the detection of the protein(s) of interest using specific antibodies (Figure 9).

Cells were homogenized in RIPA plus buffer [PBS; NP-40 1%; Sodium deoxycholate 0.5%; SDS 0.1%; Ethylenediaminetetraacetic acid (EDTA) 1 mM; Sodium fluoride (NaF) 50 mM; Sodium orthovanadate (NaVO3) 5 mM] supplemented with a cocktail of EDTA-free protease inhibitors (Roche). The protein concentration was determined using the Bradford method with the DC™ Protein Assay kit (Bio-Rad), employing bovine serum albumin as a standard. Protein homogenates were thawed on ice, and an appropriate volume, depending on the protein concentration, was used to load 50 µg of protein. Subsequently, homogenates were mixed with NuPAGE lithium dodecyl sulfate (LDS)

sample buffer (Thermo Fisher Scientific), supplemented with a reducing agent (Thermo Fisher Scientific), and incubated at 70°C for 10 minutes in a thermoblock to denature the proteins. The protein samples were then loaded onto 10% SDS-PAGE gels (NUPAGE Novex Bis-Tris Gel; Thermo Fisher Scientific) and transferred (wet transfer) onto 0.45 µm pore PVDF membranes (Immobilon-FL, Millipore) previously activated with methanol. Electrophoresis was conducted using 1X 3-(N-morpholino) propanesulfonic acid (MOPS)/SDS electrophoresis buffer [50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH = 7.7], and protein transfer was carried out using 1X transfer buffer [25 mM Tris, 192 mM Glycine, and 20% methanol]. Transfer was performed for 1 hour at 100 V and 4°C.

Following a 1-hour blocking step with Tris-buffered saline (TBS) Odyssey Blocking buffer (LI-COR Biosciences), membranes were incubated overnight at 4°C with specific primary antibodies against phosphorylated ERK1/2 (Thr202/Tyr204) (Cell Signaling, 1:1000) and ERK (Cell Signaling, 1:1000) diluted in TBS Odyssey Blocking buffer. Mouse monoclonal anti-α-tubulin antibody (Sigma Aldrich, 1:20000) served as the internal control. After overnight incubation, membranes underwent three 5-minute washes with washing buffer (TBS + 0.1% Tween-20), followed by a 50-minute incubation with IRDye rabbit and mouse secondary antibodies conjugated with fluorophores (1:10000) (LI-COR Biosciences), protected from light. Finally, membranes were washed three times for 5 minutes each with a washing buffer and once for 10 minutes with PBS or TBS. Membranes were scanned using an Odyssey Imaging System based on near-infrared fluorescence detection and analyzed with Odyssey v2.0 software (LI-COR Biosciences). Densitometric values of bands corresponding to the proteins of interest were normalized with corresponding alpha-tubulin/actin bands (internal control).

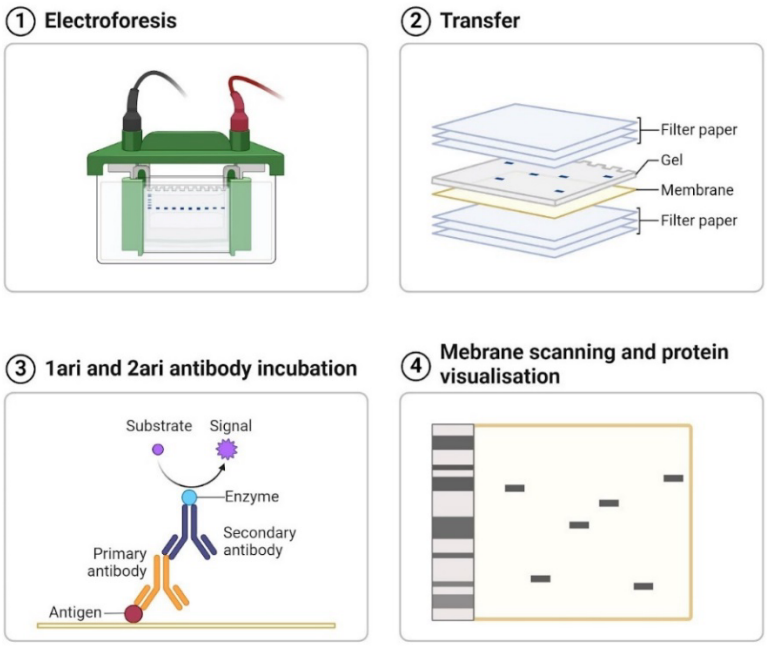

Figure 9. Schematic representation of the Western Blot process. The procedure involves several steps including protein extraction, electrophoresis separation, protein transfer to a membrane, blocking, antibody incubation, washing, and detection. Created with Biorender.com.

4.3. STATISTICAL ANALYSIS

For viability assays, data are presented as mean ± SEM of at least 3 independent biological replicates with six internal technical replicates and the statistical analysis was performed with Graphpad Prism V.4 software. Statistical differences in cell viability were determined by graphic representation and p-values were calculated using a two-tailed Student's t-test. p-values ≤ 0.05 were considered significant.

5. RESULTS

5.1. EFFECT OF POLYETHYLENE MICROSPHERES EXPOSURE ON HT-29 CELL VIABILITY

We first investigated the effect of PE exposure on HT-29 cell viability. HT-29 cells were treated with increasing doses (0 to 1000 µg/mL) of PE microspheres (6 and 54µm) for 72 hours. As shown in Figure 10, exposure to 6 µm PE microspheres resulted in a dose-dependent decrease in cell viability, with statistically significant reductions observed at doses of 100 µg/mL ($p = 0.039$) and higher. The most pronounced effect was observed at 1000 µg/mL dose ($p = 0.003$), resulting in a 60% decrease on cell viability (Figure 10A). In contrast, exposure to 54 µm PE microspheres at 1000 µg/mL caused only a slight decrease in cell viability ($p = 0.009$) (Figure 10B).

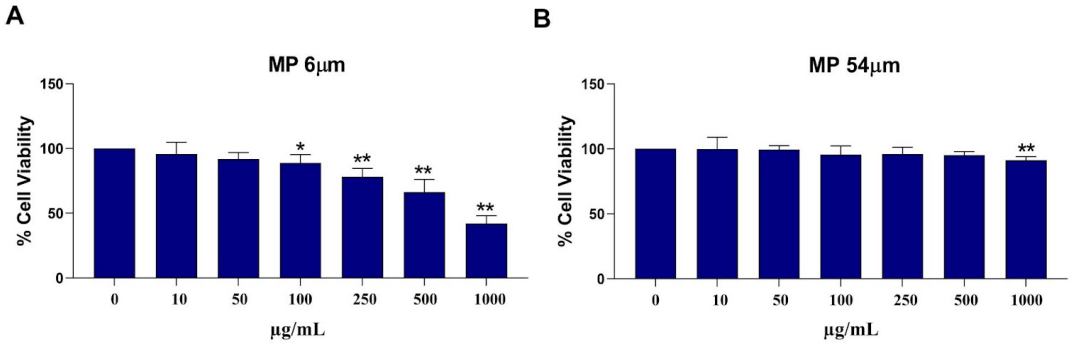

Figure 10. Effect of PE microspheres exposure on HT-29 cell viability. (A) Bar graphs representing mean ± SEM percentage of cell viability after 72-hours exposure with 6 µm PE microspheres (MP) at the indicated doses in HT-29 cells. **(B)** Bar graphs representing mean ± SEM percentage of cell viability after 72-hours exposure with 54 µm PE microspheres at the indicated doses in HT-29 cells. Results shown were obtained from at least three independent biological replicates. p-values were calculated using a two-tailed Student's t-test. *$p \leq 0.05$; **$p \leq 0.01$, relative to viability of non-treated cells.

5.2. EFFECT OF POLYSTYRENE NANOSPHERES EXPOSURE ON HT-29 CELL VIABILITY

Subsequently, we assessed the impact of PS nanospheres exposure on the viability of the HT-29 cells. Cells were treated with increasing doses (0 to 1000 µg/mL) of PS nanospheres (50 and 100 nm) for 72 h. As depicted in Figures 11A and 11B, exposure of

HT-29 cells to 50 and 100 nm PS nanospheres did not result in significant changes in cellular viability compared to the control conditions. However, there was a noticeable trend towards increased cellular viability following exposure to PS nanospheres.

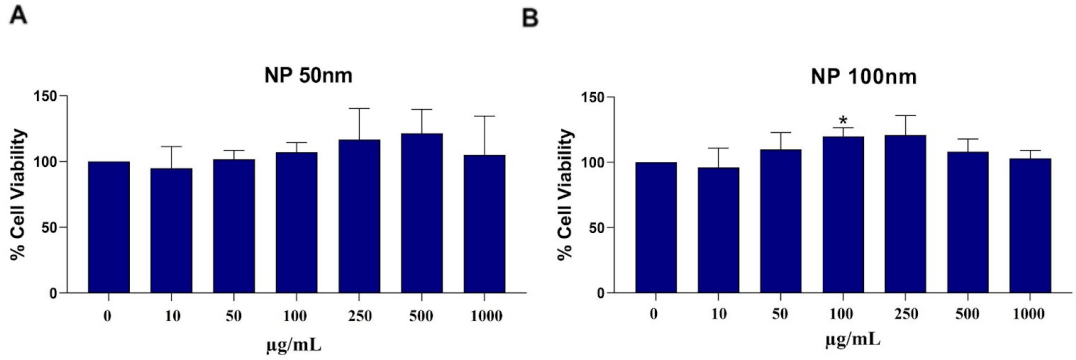

Figure 11. Effect of PS nanospheres exposure on HT-29 cell viability. (A) Bar graphs representing mean ± SEM percentage of cell viability after 72-hours exposure with 50 nm PS nanospheres (NP) at the indicated doses in HT-29 cells. **(B)** Bar graphs representing mean ± SEM percentage of cell viability after 72-hours exposure with 100 nm PS nanospheres at the indicated doses in HT-29 cells. Results shown were obtained from at least three independent biological replicates. p-values were calculated using a two-tailed Student's t-test. *p ≤0.05; **p ≤0.01, relative to viability of non-treated cells.

5.3. EFFECT POLYETHYLENE MICROSPHERES ON THE MAPK/ERK PATHWAY ACTIVATION

In view of our results, we wanted to investigate whether the observed differences in cell viability following exposure to 6 and 54 µm PE microspheres correlated at the molecular level with differential activation of the MAPK/ERK pro-inflammatory and pro-survival signaling pathway. To this end, we assessed the phosphorylation status of ERK1/2 in HT29 cells after 72 hours of exposure to PE microspheres (6 and 54 µm) at doses ranging from 0 to 500 µg/mL. As shown in figure 12, our preliminary results showed a greater increase in ERK1/2 phosphorylation following exposure to 6 µm PE microspheres compared to 54 µme, particularly at the dose of 500 µg/mL.

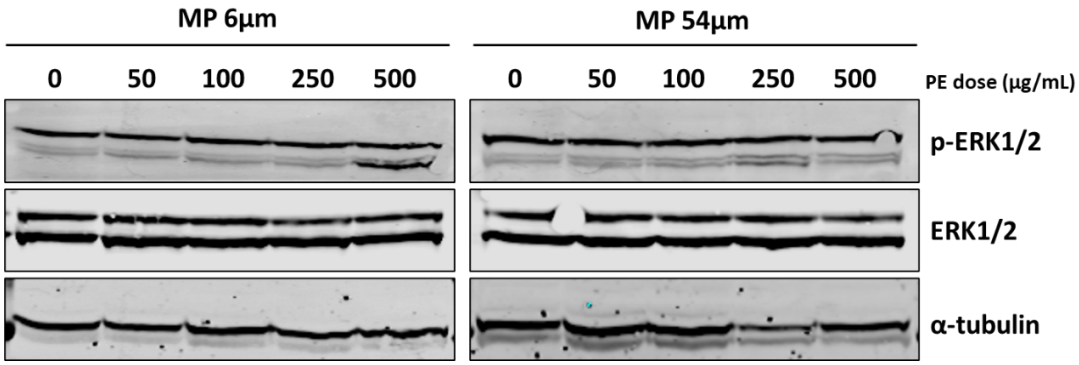

Figure 12. Effect of PE microspheres exposure on MAPK/ERK signaling activation in HT-29 cells. Western blot images (n=1) showing protein expression changes of phosphorylated ERK1/2 (p-ERK1/2 Thr202/Tyr204) in HT-29 cells after treatment with 6 µm or 54 µm PE microspheres (MP) for 72 hours at the indicated doses. Alpha-tubulin was used as endogenous control.

6. DISCUSSION

MPNPs have been monitored in the environment for some years due to their polluting potential, but there is a lack of information about their effects on human health and disease, including CRC. In this study, we conducted a literature review to explore the current understanding of the impact of MPNPs on CRC development. Additionally, we performed a proof-of-concept analysis to study the effect of exposure to PE microspheres and PS nanospheres on cell viability and the activation of the MAPK/ERK pro-inflammatory and pro-survival pathway in the human colorectal adenocarcinoma HT-29 cell line. It is important to highlight that, although HT-29 is a tumor cell line, the inherent difficulties in cultivating normal colon cells in vitro make HT-29 a widely utilized and reliable alternative for toxicological studies. Indeed, HT-29 represents a well-characterized model for studying intestinal epithelial responses to toxicants, as well as to chemical and bacterial infections (58).

Since human exposure to MPNPs is chronic, 48h is the most often used timepoint in toxicity studies with MPs in CRC (41, 45). In this study, however, we used a 72-hour time frame to allow for a longer observation period to assess any potential effects of MPNPs on HT-29 cells. In agreement with other studies (45), our results showed that exposing HT-29 cells to PE microspheres resulted in a MP size dependent-decrease in cell viability. Remarkably, our findings revealed distinct responses to 6 and 54 μm PE microspheres. While exposure to 6 μm PE microspheres resulted in a significant dose-dependent decrease in cell viability, with the most pronounced effect observed at 1000 μg/mL, exposure to 54 μm PE microspheres resulted in only a slight decrease in viability at the same concentration. These findings suggest that the effect of PE microspheres on cell viability may be influenced by particle size, with smaller particles exerting a more significant impact compared to larger ones. In the same line, Saenen et al. demonstrated that the size and shape of MPs has an impact on the cytotoxicity of CRC Caco-2 cells. The uptake clearly depends on the particle size since the PS 200 nm spheres had more profound effects than the 2 μm PM particles (59). Thus, there are various reasons that could explain why exposure to 6 μm MPs might lead to a decrease in cell viability, while exposure to 50 μm MPs does not. On the one hand, smaller MPs can more easily penetrate cell membranes or tissues, which can cause direct cellular damage or activate different signaling pathways (60). On the other hand, smaller molecules have a higher surface-to-volume ratio, allowing for increased interaction among themselves, which can intensify the physical toxic effect on cells by forming denser aggregates. In this context, Summers et al. investigated the agglomeration of MPNP particles in seawater by supplementing seawater with PS spheres of various sizes (50 nm, 1 μm, and 10 μm) up to a concentration of 5 μg/mL. After 24 hours of exposure, they observed that 50 nm PS spheres promoted the formation of larger aggregates (61). Additionally, according to Wang et al., PE infiltrates lipid membranes, leading to significant alterations in dipalmitoyl phosphatidylcholine (DPPC) bilayers, including reduced density, variations in fluidity, and membrane thickening. Their research aimed to assess the impact of PE on DPPC bilayer characteristics using HepG2 cells and found that PE aggregates increased the

organization of phospholipid chains, disrupting the standard structure of the DPPC bilayer by altering its fluidity (62). Based on this evidence, we hypothesize that 6 µm PE microspheres could form larger aggregates compared to 54 µm microspheres and are likely to cause greater disruption to the lipid bilayer of cells, thereby exhibiting higher toxicity and reducing cell viability.

A growing body of evidence has demonstrated that activation of the MAPK/ERK signaling pathway is involved in the pathogenesis, progression, and oncogenic behavior of human CRC through the activation of cell proliferation, apoptosis, inflammation, angiogenesis, and metastasis (63). Our results showed that MAPK/ERK pathway activation increased notably following exposure to 6 µm MP compared to 54 µm MP, especially at higher doses. Similarly, Bahadur et al. exposed different cell lines to two different sizes of irregular shape PTFE-MPs with a diameter of 6 or 31.7 µm and confirmed that PTFE-MPs activate the MAPK signaling pathways, especially the MEK-ERK pathway, in A549 and U937 cells, and in the THP-1 dendritic cell line (55). In conclusion, our preliminary findings suggest that exposure to 6 µm MPs leads to a decrease in cell viability while concurrently increasing the activation of survival and tumor progression pathways in the HT29 model. This phenomenon may be attributed to various mechanisms. Firstly, MP exposure may promote the activation of cellular survival signaling pathways, such as MAPK/ERK, as a mechanism to counteract their toxic effects. Additionally, from a Darwinian perspective, MP exposure may select cells that inherently exhibit higher activation of these signaling pathways, thereby enhancing their capacity to survive the toxic insult produced by PE aggregates.

In addition, our study aimed to evaluate the impact of PS nanospheres (50 and 100 nm) exposure on the cellular viability of the HT-29 cell line. Following exposure to 50 and 100 nm PS nanospheres, HT-29 cells did not exhibit significant alterations in cellular viability compared to control conditions, although a noticeable trend towards increased cellular viability was observed. This observation is consistent with the findings of Xu et al., who demonstrated that exposure of A549 cells to PS NPs measuring 25 and 70 µm, at concentrations of 25 µg/mL and 160 µg/mL respectively, led to an upregulation of cyclin D, cyclin E, and Ki67 genes, which are involved in cell proliferation (64).

It is important to note that our study has some limitations that should be taken into account when interpreting the results obtained. One limitation of our study is the relatively short exposure time of MPNPs to cells, which may not fully replicate chronic exposure scenarios commonly encountered in real-world settings. Extending the exposure duration in future studies could yield further insights into the long-term effects of MPNPs on cellular health. Additionally, it is important to note that the MPNPs utilized in this study were free from chemical toxins and pathogens. Consequently, the findings may not fully capture the potential impacts of MPNPs that carry such contaminants, which are frequently encountered in environmental and occupational exposures.

In conclusion, our preliminary results suggest that MPNPs likely contribute to CRC progression through their effects on cell viability and their proinflammatory and pro-

survival properties. Smaller particles, such as 6 μm PE microspheres, have been observed to induce a dose-dependent decrease in cell viability, indicating their potential to induce cellular stress or death. This phenomenon could lead to an elevated rate of cellular turnover and accumulation of mutations, both of which are known factors in cancer progression. Additionally, MPNPs are known to activate proinflammatory pathways, notably the MAPK/ERK pathway, which plays a critical role in cell proliferation, differentiation, and survival. Enhanced activation of this pathway, particularly at higher doses of smaller MPNPs, may result in a chronic inflammatory state within the intestinal microenvironment, a well-established risk factor for CRC. This inflammatory state can promote genetic mutations, alter cellular behavior, and create conditions conducive to tumor growth and survival. Thus, the combined effect of MPNPs in reducing cell viability and promoting inflammation suggests a significant role in CRC progression. By directly damaging cells and fostering an inflammatory environment, MPNPs may accelerate the onset and advancement of CRC. This underscores the need for further research into the mechanisms underlying these effects and emphasizes the importance of considering particle size in the assessment of MPNP toxicity.

7. CONCLUSIONS

In conclusion, our study provides valuable insights into the cellular responses triggered by different sizes and types of MPNPs particles, emphasizing the pivotal role of particle size in determining cytotoxic effects. Concretely, exposure to 6 μm PE microspheres led to a significant dose-dependent decrease in cell viability, whereas 54 μm PE microspheres showed only a slight decrease, underscoring the greater toxicity of smaller particles. Furthermore, cells exposed to 6 μm PE microspheres exhibited a notable increase in MAPK/ERK pathway activation compared to those exposed to 54 μm microspheres, suggesting that smaller MPs may induce stronger cellular stress and pro-survival responses. Conversely, exposure to PS nanospheres (50 nm and 100 nm) did not significantly affect cell viability, indicating potential differences in action mechanisms between MPs and NPs. Further research should focus on elucidating these molecular pathways. Overall, our preliminary findings highlight the role of MPs in CRC development via size-dependent cytotoxic effects and activation of critical signaling pathways, underscoring the need for continued investigation into the long-term implications of chronic MPNPs exposure on CRC progression.

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