Universidade Estadual de Londrina

WALISON AUGUSTO DA SILVA BRITO

AVALIAÇÃO DAS RESPOSTAS BIOLÓGICAS FRENTE À EXPOSIÇÃO AOS NANO- E MICRO-PLÁSTICOS

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Orientadora:

Profa. Dra. Alessandra Lourenço Cecchini Armani

Coorientador:

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“A menos que modifiquemos a nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo”.

(Einstein, Albert).

BRITO, Walison Augusto da Silva. **Avaliação das respostas biológicas frente à exposição aos nano- e micro-plásticos.** 2023. 99 páginas. Tese de Doutorado (Programa de Pós-Graduação em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2023.

RESUMO

A presença de partículas de nano e micro-plásticos (NMPs) é uma preocupação ambiental e ecológica cada vez maior devido aos seus possíveis impactos negativos sobre a saúde humana. Os seres humanos são expostos diariamente aos NMPs por ingestão, inalação e contato tópico. No entanto, devido às limitações nas metodologias de amostragem, detecção e quantificação da exposição aos NMPs em matrizes biológicas e complexas, os roedores e os modelos de cultura de células são ferramentas importantes para a avaliação toxicológica. Estudos *in vitro* e *in vivo* descreveram a bioacumulação, o estresse oxidativo, a inflamação e a perturbação metabólica após a exposição aos NMPs como as principais respostas biológicas. Os NMPs ambientais são difíceis de serem amostrados e identificados, portanto, os NMPs disponíveis comercialmente têm sido amplamente utilizados em estudos toxicológicos. No entanto, esses NMPs geralmente apresentam propriedades físico-químicas homogêneas, diferindo das misturas heterogêneas dos NMPs encontrados no meio ambiente, que apresentam diversos tipos de polímeros, tamanhos e formas. Portanto, é necessário desenvolver novos métodos para produzir NMPs ambientalmente relevantes para servir como materiais de referência para pesquisas toxicológicas e investigar seus possíveis efeitos na saúde humana. Considerando isso, produzimos NMPs de polietileno tereftalato (PET) usando um tratamento de ultrassom em condições alcalinas, adotando algumas modificações ao protocolo convencional. Além disso, nosso estudo teve como objetivo investigar os possíveis efeitos biológicos da exposição aguda de PET NMPs em duas linhas de células humanas, A549 e HaCaT, abordando a inalação e as rotas de exposição humana tópica *in vitro*. O tratamento por ultrassom em condições alcalinas induziu a degradação do polímero em PET NMPs menores, formando fragmentos heterogêneos em forma e, em geral, semelhanças nas superfícies químicas entre o PET antes e depois da sonicação. Em um sistema aquoso, os PET NMPs apresentaram um tamanho médio de 781 nm, uma suspensão polidispersa e uma carga de superfície negativa. Suspenção monodispersa de poliestireno amino-modificado (PS-NH_2) de 55 nm foi utilizado como controle positivo para as análise *in vitro*. Após a exposição aguda de PET NMPs, o metabolismo das células A549 foi ligeiramente aumentado, enquanto o HaCaT não sofreu alteração. Ambas as linhas celulares interagiram e absorveram PET NMPs, o que foi observado pelo aumento da granularidade celular. Além disso, a viabilidade celular foi reduzida e o aumento da necrose foi observado apenas nas células A549. O aumento da formação de micronúcleos em alta concentração foi observado nas células HaCaT. A exposição aos PET NMPs não foi capaz de alterar os níveis de ERO intracelulares, em contraste com os PS-NH_2 NMPs. No entanto, a redução dos níveis de tiois foram observadas somente nas células HaCaT após exposição. Além disso, em alta concentração, os PET NMPs induziram a ativação do Nrf2 em ambas as linhagens celulares. Os PET NMPs induziram diferentes perfis de secreção de citocinas e os principais efeitos observados ocorreram em alta concentração e em citocinas pró-inflamatórias. Assim, ambas as linhagens celulares apresentaram alterações significativas com relação à ativação do NF-κB. Os PET NMPs induziram alterações na morfologia celular apenas nas células HaCaT. Ainda,

os marcadores de autofagia (mTOR, ULK1, ATG5 e LC3B) foram afetados de formas diferentes em ambas as linhagens celulares, sendo que marcadores iniciais (mTOR e ULK1) estavam significativamente reduzidos. Curiosamente, as análise de componentes principais revelaram que as alterações relacionadas à autofagia ocorreram independentemente da concentração. Em conclusão, os PET NMPs ambientalmente relevantes produzidos por sonicação foram internalizados pelas linhagens celulares humanas e afetaram de forma diferente as respostas biológicas em relação à linhagem celular.

Palavras-chave: Avaliação da toxicidade; absorção de partículas; estresse oxidativo, resposta relacionada à inflamação; autofagia.

BRITO, Walison Augusto da Silva. **Evaluation of biological responses through nano- and micro-plastics exposure.** 2023. 99 pages. Doctoral thesis (Postgraduate Program in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2023.

ABSTRACT

The presence of nano- and micro-plastics particles (NMPs) is a growing environmental and ecological concern due to their potential negative impacts on human health. Humans are exposed daily to NMPs by ingestion, inhalation and topic contact. However, due to limitations in methodologies for sampling, detection and quantification of NMPs exposure in biological and complexes matrices, rodents and cell culture models are important tools for toxicological assessment. *In vitro* and *in vivo* studies have described bioaccumulation, oxidative stress, inflammation, and metabolic disruption upon NMPs exposure as main biological responses. Environmental NMPs are difficult to sample and identify, thus, commercially available NMP have been used extensively in toxicological studies. However, those NMP generally present homogenous physico-chemical properties, differing from heterogenous NMPs mixtures found in the environment, presenting diverse polymer types, sizes and shapes. Therefore, it is necessary to develop new methods for producing environmentally relevant NMPs to serve as reference materials for toxicological research and investigate their potential effects on human health. Considering that, we produced polyethylene terephthalate (PET) NMPs using an ultrasound treatment in alkaline conditions, adopting a few modifications. Further, our study aimed to investigate the possible biological effects upon acute PET NMPs exposure in two human cell lines, A549 and HaCaT, addressing inhalation and topic human exposure routes *in vitro*. Ultrasound treatment in alkaline conditions induced polymer degradation into smaller PET NMPs, forming heterogeneous fragments in shape and in general, surface chemical similarities between PET before and after sonication. In aqueous system, PET NMPs showed an average size of 781 nm, a polydisperse suspension and a negative surface charge. Monodisperse suspension of 55 nm polystyrene amino-modified (PS-NH_2) was used as positive control for *in vitro* analysis. After PET NMPs acute exposure, A549 cells metabolism was slightly increased while HaCaT did not alter. Both cell lines interacted and took PET NMPs up, observed by increased cellular granularity. In addition, cell viability was reduced and increased necrosis was observed only in A549 cells. Increased micronuclei formation at high concentration was observed in HaCaT cells. PET NMPs exposure was unable to change intracellular ROS levels contrasting to PS-NH_2 NMPs. However, reduced thiol content was observed only in HaCaT cells after exposure. Moreover, at high concentration, PET NMPs induced activation of Nrf2 in both cell lines. PET NMPs induced different cytokines' secretion profiles and mainly observed effects occurred at high concentration and on proinflammatory cytokines. Thus, both cell lines showed significant alterations regarding activation of NF-κB. PET NMPs induced cell morphology changes only on HaCaT cells. In addition, autophagy markers (mTOR, ULK1, ATG5 and LC3B) were affected in different ways in both cell lines, with initial markers (mTOR and ULK1) being significantly reduced. Interestingly, principal component analysis revealed that the autophagy-related alterations occurred regardless of concentration. In conclusion, environmentally relevant PET NMPs produced by sonication was internalized by human cell lines and differently affected biological responses in regard to cell line.

Keywords: Toxicity assessment; particle uptake; oxidative stress, inflammation-related response; autophagy.

LISTA DE ABREVIATURAS E SIGLAS

µm	Micrômetros
ATG5	Proteína relacionada à autofagia 5 (*Autophagy related 5*)
BPA	Bisfenol-A
ERO	Espécies reativas de oxigênio
IL-	Interleucina-
LC3B	Proteína associadas a microtúbulos cadeia leve B (*microtubule-associated light chain 3 B*)
MP	Microplásticos
mTOR	Alvo mamífero da rapamicina (*mammalyan target of rapamycin*)
NF-κB	Fator Nuclear kappa B (*Nuclear factor kappa B*)
nm	Nanômetros
NMPs	Nano- e micro-plásticos
NP	Nanoplásticos
Nrf2	Fator nuclear eritroide 2 (*Nuclear factor erythroid 2-related factor 2*)
PE	Polietileno
PET	Polietileno tereftalato
PP	Polipropileno
PS	Poliestireno
PS-NH_2	Poliestireno amino-modificado
PUR	Poliuretano
PVC	Policloreto de vinila
ULK1	Kinase ativadora de autofagia 1 do tipo Unc-51 (*UNC-51-Like Kinase 1*)

SUMÁRIO

1 INTRODUÇÃO

1.1 NANO- E MICRO-PLÁSTICOS (NMPS)

Desde o início da produção em larga escala nos anos 1950, os mais diversos polímeros plásticos são sintetizados, acumulados, contaminando o meio ambiente (WARING; HARRIS; MITCHELL, 2018). Com o passar dos anos, bilhões de toneladas de plásticos já foram produzidas, e em 2021 a produção global atingiu quase 391 milhões de toneladas após um período de estagnação decorrente da pandemia do COVID-19 (PLASTICSEUROPE, 2022). Atualmente, a China detém 32% da produção global, sendo a maior produtora de plásticos, enquanto a América Latina detém apenas 4% da produção global (PLASTICSEUROPE, 2022). No entanto, o Brasil é o terceiro maior produtor de lixo no mundo, recicláveis ou não, assim como um dos mercados emergentes mais importantes, participando diretamente da persistência dos plásticos contaminando o meio ambiente (CITI, 2018).

O manejo do lixo plástico pode ser executado através da reutilização, reciclagem, incineração e aterramento (CHEN; AWASTHI; WEI; TAN *et al*., 2021). Mesmo com todas as iniciativas para reciclagem e legislação banindo os plásticos de uso único, diversos polímeros são encontrados em diversos lugares no planeta (CHEN; AWASTHI; WEI; TAN *et al.*, 2021). Partículas plásticas já foram encontradas em oceanos, rios e sistemas agrícolas, meio urbano, na atmosfera e até mesmo em áreas remotas como o Monte Everest (NAPPER; DAVIES; CLIFFORD; ELVIN *et al.*, 2020; ROCHMAN, 2018; WRIGHT; ULKE; FONT; CHAN *et al.*, 2020). O lixo plástico pode causar grande impacto ambiental como inundações, interferência no ecossistema, danos e morte de animais (CHEN; AWASTHI; WEI; TAN *et al*., 2021). Além disso, o potencial impacto ambiental também está relacionado com as pequenas partículas plásticas, definidas como microplásticos (MP; diâmetro menor que 5 mm) e nanoplásticos (NP; diâmetro menor que 100 nm) (EFSA, 2016; THOMPSON; OLSEN; MITCHELL; DAVIS *et al.*, 2004).

Os NMPs podem variar em concentração e serem classificadas baseadas no tamanho, forma, tipo de polímero e origem (AUTA; EMENIKE; FAUZIAH, 2017; CHEN; AWASTHI; WEI; TAN *et al.*, 2021; WARING; HARRIS; MITCHELL, 2018; WRIGHT; KELLY, 2017). Considerando a origem dos NMPs, podem ser tanto intensionalmente manufaturadas (NMPs primárias) ou derivados a partir de plásticos grandes no meio ambiente pela ação de radiação ultravioleta, abrasão mecânica ou

degradação por microrganismos (NMPs secundárias) (AUTA; EMENIKE; FAUZIAH, 2017; CHEN; AWASTHI; WEI; TAN *et al.*, 2021).

Os nano- e micro-plásticos (NMPs) são um problema ambiental multifacetado, não apenas se restringindo aos ambientes aquáticos (AUTA; EMENIKE; FAUZIAH, 2017; ROCHMAN, 2018; THOMPSON; OLSEN; MITCHELL; DAVIS *et al.*, 2004) ou presença em alimentos (VAN RAAMSDONK; VAN DER ZANDE; KOELMANS; HOOGENBOOM *et al.*, 2020; WARING; HARRIS; MITCHELL, 2018) como fontes primárias de exposição, mas também pelas suas consequências epidemiológicas (RIST; CARNEY ALMROTH; HARTMANN; KARLSSON, 2018; RODRIGUES; ABRANTES; GONCALVES; NOGUEIRA *et al.*, 2019; RUBIO; MARCOS; HERNANDEZ, 2020) como poluição ambiental, impacto em ecossistemas terrestres e aquáticos, desafios para a gestão de resíduos. Além disso, tais partículas geram grande preocupação por serem persistentes na natureza, de forma a contaminá-la, mas também podem ser biomagnificados na cadeia alimentar (RAI; LEE; BROWN; KIM, 2021; RIST; CARNEY ALMROTH; HARTMANN; KARLSSON, 2018; WRIGHT; KELLY, 2017). Portanto, por serem destinados ao consumo humano, têm o potencial de causar efeitos tóxicos, servir como veículos para patógenos e outras substâncias poluentes, além de liberarem compostos nocivos utilizados em sua fabricação (CHEN; AWASTHI; WEI; TAN *et al.*, 2021; RAI; LEE; BROWN; KIM, 2021; RIST; CARNEY ALMROTH; HARTMANN; KARLSSON, 2018).

Além de ser considerado um dos mais importantes poluentes ambientais, os polímeros plásticos podem conter aditivos, que por sua vez, geralmente são substâncias tóxicas (GRUBER; STADLBAUER; PICHLER; RESCH-FAUSTER *et al.*, 2022). Dentre os principais aditivos utilizados se destacam os plastificantes (bisfenol-A; BPA e ftalatos), estabilizadores, retardadores de chama, corantes, antioxidantes e outros compostos orgânicos ou inorgânicos (CHEN; WU; SHERRELL; CHEN *et al.*, 2022; GRUBER; STADLBAUER; PICHLER; RESCH-FAUSTER *et al.*, 2022). O problema mais grave associado com a utilização dos aditivos na polimerização dos plásticos é a liberação de tais compostos potencialmente tóxicos durante a fragmentação dos plásticos no ambiente (WANG; BAI; NING; FAN *et al.*, 2020). O BPA e ftalatos por exemplo, podem causar grande impacto, principalmente disruptura do sistema endócrino dos animais (DENG; ZHANG; QIAO; BONILLA *et al.*, 2018; GRUBER; STADLBAUER; PICHLER; RESCH-

FAUSTER *et al.*, 2022), além de serem considerados substâncias carcinogênicas (GRUBER; STADLBAUER; PICHLER; RESCH-FAUSTER *et al.*, 2022; WANG; BAI; NING; FAN *et al.*, 2020).

1.2 Características Físico-Químicas

Os plásticos são polímeros sintéticos produzidos a partir dos combustíveis fósseis ou biomassa (PLASTICSEUROPE, 2020). Os principais tipos de polímeros são: polietileno tereftalato (PET), polietileno (PE), polipropileno (PP), policloreto de vinila (PVC), poliestireno (PS), poliuretano (PUR) e resinas fenólicas (CHEN; AWASTHI; WEI; TAN *et al.*, 2021; PHUONG; ZALOUK-VERGNOUX; POIRIER; KAMARI *et al.*, 2016; PLASTICSEUROPE, 2022). Os polímeros mais utilizados diariamente são PP e PE, sendo especialmente utilizados para empacotamento e garrafas de água descartáveis (CHEN; AWASTHI; WEI; TAN *et al.*, 2021). Além disso, os mais diversos polímeros plásticos também podem ser aplicados na contrução civil, agropecuária e medicina, devido a sua alta versatilidade, durabilidade e resistência (GRUBER; STADLBAUER; PICHLER; RESCH-FAUSTER *et al.*, 2022).

Diante da alta versatilidade e diferentes composições químicas, os NMPs podem exibir diversas formas, o que também pode contribuir para seus potenciais efeitos tóxicos (QIAO; DENG; ZHANG; WOLOSKER *et al.*, 2019). Os NMPs podem se apresentar como esferas, fragmentos, fibras e filamentos (QIAO; DENG; ZHANG; WOLOSKER *et al.*, 2019). Tal diversidade de formas podem influenciar principalmente na interação dos NMPs e potencial internalização pelas células (MA; MA; LI; WANG *et al.*, 2013). Tendo isso em vista, a manipulação dessas formas contribuem para uma aplicação muito interessante na medicina e farmacologia, que é no desenvolvimento de NMPs carregadores de drogas para o tratamento de doenças (GENG; DALHAIMER; CAI; TSAI *et al.*, 2007). No entanto, as formas diversas podem exibir efeitos tóxicos, por possuírem diferentes tempos de retenção, acúmulo nos tecidos, além de causar danos físicos (QIAO; DENG; ZHANG; WOLOSKER *et al.*, 2019).

1.3 Exposição dos Seres Humanos

Considerando a grande quantidade de características fisico-químicas dos NMPs, e sua presença em todos os ambientes, a preocupação com os potenciais efeitos em seres humanos vem crescendo (DA SILVA BRITO; MUTTER; WENDE;

CECCHINI *et al.*, 2022; DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023; SCHMIDT; MUHL; BRITO; SINGER *et al.*, 2023). Os seres humanos são diariamente expostos aos NMPs, seja diretamente ou indiretamente (AUTA; EMENIKE; FAUZIAH, 2017; DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022; VAN RAAMSDONK; VAN DER ZANDE; KOELMANS; HOOGENBOOM *et al.*, 2020). Os seres humanos podem ser expostos aos NMPs pela ingestão, inalação e contato tópico (PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020).

Até o momento, a ingestão é a principal via de exposição dos NMPs tanto em seres humanos quanto em outros animais (PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020; RODRIGUES; ABRANTES; GONCALVES; NOGUEIRA *et al.*, 2019). Boa parte dessa afirmação é devido a presença dos NMPs em todos os ambientes, tanto aquáticos quanto terrestres, e principalmente a contaminação dos alimentos destinados ao consumo de seres humanos (PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020; VAN RAAMSDONK; VAN DER ZANDE; KOELMANS; HOOGENBOOM *et al.*, 2020). Já foi reportado a contaminação por NMPs de alimentos como sal de cozinha, frutos do mar (como ostras, moluscos, camarão), peixes, e até mesmo na água mineral engarrafada e outras bebidas, corroborando para sua possível ingestão (AUTA; EMENIKE; FAUZIAH, 2017; EFSA, 2016; PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020; RODRIGUES; ABRANTES; GONCALVES; NOGUEIRA *et al.*, 2019). Sendo assim, os NMPs podem ser ingeridos na dieta e consumo de água contaminados ou pelo movimento de limpeza mucociliar do trato respiratório após inalação (PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020).

Toneladas de NMPs são liberadas na atmosfera do mundo todo, seja pela evaporação de água contendo tais partículas ou a partir de danos ou atrito de objetos plásticos (PRATA, 2018; SCHMIDT; MUHL; BRITO; SINGER *et al.*, 2023). Animais e seres humanos podem inalar o ar circulante contendo NMPs provenientes principalmente da abrasão da borracha de pneus de automóveis ou produtos têxteis (GASPERI; WRIGHT; DRIS; COLLARD *et al.*, 2018; PRATA, 2018). Os NMPs também estão em contato com a pele, seja através do ar, produtos têxteis ou até mesmo formulações farmacêuticas e cosméticas (PRATA; DA COSTA; LOPES; DUARTE *et al.*, 2020; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023). A pele é o maior orgão do corpo humano e tem como uma de suas funções, a barreira

primária contra possíveis patógenos e substâncias tóxicas (SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023).

1.4 Respostas Biológicas Frente à Exposição de NMPs

Interação dos NMPs com consequente internalização celular são os primeiros eventos biológicos que podem ocorrer após a exposição aos NMPs (FORTE; IACHETTA; TUSSELLINO; CAROTENUTO *et al.*, 2016; LIU; XU; ZHANG; YE *et al.*, 2021). A internalização dos NMPs pelas células involve diversos mecanismos e fatores que influenciam as respostas biológicas (DONAHUE; ACAR; WILHELM, 2019; KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014; MA; MA; LI; WANG *et al.*, 2013). As membranas celulares funcionam como uma barreira seletiva, e diversos mecanismos tem sido descritos para os NMPs atravessarem elas, como a fagocitose e pinocitose (DONAHUE; ACAR; WILHELM, 2019; FIRDESSA; OELSCHLAEGER; MOLL, 2014). Fagocitose é um mecanismo de internalização celular induzidos por ligantes restritos aos fagócitos profissionais (células imunológicas), como macrófagos, células dendríticas e polimorfonucleares, embora tais células também realizam pinocitose (DONAHUE; ACAR; WILHELM, 2019; KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014). É assumido que a fagocitose ocorre em partículas sólidas entre 0.5 μm e 10 μm, enquanto as pequenas partículas são internalizadas por pinocitose (KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014; MA; MA; LI; WANG *et al.*, 2013).

Tanto o tamanho das vesículas quanto as proteínas involvidas na sua formação são importantes fatores para a pinocitose (KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014). Estes fatores dividem a pinocitose em: 1) macropinocitose, via não-específica e dependente de actina que forma vesículas com tamanho de até 5 μm de diâmetro (MA; MA; LI; WANG *et al.*, 2013); 2) endocitose mediada por clatrina, via dependente de receptores que resulta em vesículas cobertas com clatrina com tamanho entre 100 e 500 nm (DONAHUE; ACAR; WILHELM, 2019); 3) endocitose mediada por caveolina, via dependente de receptores que resulta em vesículas cobertas com caveolina com tamanho entre 50 e 100 nm (DONAHUE; ACAR; WILHELM, 2019); 4) endocitose independente de clatrina e caveolina, via que pode envolver balsas lipídicas e outras vias, formando vesículas de 50 nm ou menores (DONAHUE; ACAR; WILHELM, 2019; KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014).

Os mecanismos pelos quais os NMPs são internalizados pelas células podem variar dependendo do tipo celular e principalmente das propriedades fisico-químicas, como tamanho, carga de superfície, forma, e hidrofilia (HWANG; CHOI; HAN; JUNG *et al.*, 2020; KETTLER; VELTMAN; VAN DE MEENT; VAN WEZEL *et al.*, 2014; LIU; XU; ZHANG; YE *et al.*, 2021). Alguns estudos tem investigado os efeitos biológicos da exposição aos NMPs em uma vasta faixa de tamanhos (de 20 nm até 200 µm) (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; STOCK; BOHMERT; LISICKI; BLOCK *et al.*, 2019; STOCK; LAURISCH; FRANKE; DONMEZ *et al.*, 2021; ZAUNER; FARROW; HAINES, 2001). Acredita-se que células não-fagocíticas dificilmente internalizariam NMPs maiores que 1 µm por endocitose, enquanto muitos tipos celulares mieloides o fazem por meio da fagocitose (LIU; XU; ZHANG; YE *et al.*, 2021).

Dentre as propriedades dos NMPs, a carga de superfície pode influenciar na internalização celular, onde NMPs positivamente carregados interagem facilmente com os lipídeos para penetrar as membranas celulares (MA; MA; LI; WANG *et al.*, 2013). Isto ocorre principalmente pela carga da superfície da membrana plasmática externa ser negativamente carregada e sua afinidade subsequente aos NMPs positivamente carregados, enquanto NMPs neutro ou negativamente carregados possuirem menor afinidade (DONAHUE; ACAR; WILHELM, 2019; MA; MA; LI; WANG *et al.*, 2013). No entanto, NMPs neutros ou negativamente podem reverter tais cargas e serem internalizados pelas células (DONAHUE; ACAR; WILHELM, 2019). Além disso, modificações nas superfícies dos NMPs podem influenciar na formação da coroa proteica e sua composição, assim, também afetando as respostas biológicas (ANGUISSOLA; GARRY; SALVATI; O'BRIEN *et al.*, 2014; DONAHUE; ACAR; WILHELM, 2019).

Considerando os processos de internalização celular, com a formação de vesículas, consequentemente podem levar a possível tentativa de degradação por meio dos lisossomos (LI; JU, 2018). Ainda, os processos de internalização celular e tentativa de degradação dos NMPs mal sucedida, podem levar a bioacumulação de NMPs em tecidos e órgãos após exposição (LIANG; ZHONG; HUANG; LIN *et al.*, 2021). Além disso, a tentativa de degradação dos NMPs pelos lisossomos pode estar correlacionada com outros efeitos biológicos subsequentes, como alterações do metabolismo celular, morte e processos adaptativos, ou até mesmo não causar danos

e as células continuarem a se dividir (ANGUISSOLA; GARRY; SALVATI; O'BRIEN *et al.*, 2014; LI; JU, 2018).

Tanto as possíveis alterações no metabolismo celular quanto na viabilidade e mecanismos de morte celular são de extrema importância para os estudos toxicológicos da exposição por NMPs. Alterações no metabolismo de diferentes linhagens celulares já foram reportadas após a exposição aos NMPs, em diversas condições e características fisico-químicas (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; SHARMA; GOREY; CASEY, 2019). Ainda, alguns NMPs, como o PS-NH_2 (PS amino-modificado) por exemplo, são capazes de induzir apoptose *in vitro* após exposição, ou seja, apresentam citotoxicidade (ANGUISSOLA; GARRY; SALVATI; O'BRIEN *et al.*, 2014; DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; SHARMA; GOREY; CASEY, 2019).

Um potencial mecanismo para a exposição de NMPs provocar morte celular é pela geração de espécies reativas de oxigênio (ERO) e induzindo o estresse oxidativo (SHARMA; GOREY; CASEY, 2019). Muitos estudos demonstraram que NMPs induziram a geração de ERO como uma das principais respostas biológicas após exposição *in vitro* e *in vivo* (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; DENG; YAN; SHEN; HUANG *et al.*, 2021; LI; SHI; WANG; XIAO *et al.*, 2021; VISALLI; FACCIOLA; PRUITI CIARELLO; DE MARCO *et al.*, 2021; WU; WU; LIU; WANG *et al.*, 2019; WU; WU; TIAN; QIU *et al.*, 2020). O estresse oxidativo causado pela exposição de NMPs pode afetar o ciclo celular, o metabolismo e até induzir as células à apoptose (SHARMA; GOREY; CASEY, 2019). Em resposta as ERO elevadas, as barreiras antioxidantes podem ser ativadas afim de neutralizá-las (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023). Interessantemente, após exposição de células aos NMPs, foram observadas alterações na via de sinalização responsiva ao estresse oxidativo, a via do fator nuclear eritroide 2 (Nrf2) e seus produtos posteriores (LI; SHI; WANG; XIAO *et al.*, 2021; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023; SCHMIDT; MUHL; BRITO; SINGER *et al.*, 2023). A indução de apoptose mediada por estresse oxidativo, geralmente está relacionada aos danos oxidativos nas membranas celulares das organelas, em especial, da mitocôndria (LIU; HOU; WANG; YANG, 2022; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023). Assim, com a membrana mitocondrial lesada, moléculas da família das Bcl-2 que regulam a ativação intríseca da apoptose podem ser alteradas

(SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023). A exposição de células aos NMPs tanto podem induzir estresse oxidativo, danos às membranas e desregular a apoptose (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; LIU; HOU; WANG; YANG, 2022; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023).

A resposta inflamatória também é outro possível efeito biológico seguido da exposição por NMPs (CHOI; BANG; KIM; OH *et al.*, 2020; CHOI; HWANG; BANG; HAN *et al.*, 2021). Estudos que expuseram cultura de diversas linhagens celulares vem demonstrando aumento da produção de citocinas próinflamatórias (CHOI; BANG; KIM; OH *et al.*, 2020; CHOI; HWANG; BANG; HAN *et al.*, 2021; DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; SCHMIDT; DA SILVA BRITO; SINGER; MUHL *et al.*, 2023; SCHMIDT; MUHL; BRITO; SINGER *et al.*, 2023). Ainda, vias de ativação do inflamassoma NLRP3 com consequente aumento de citocinas proinflamatórias em suas formas ativas como IL-1β e IL-18 após exposição pelos NMPs (HOU; LEI; CUI; HOU *et al.*, 2021). A resposta inflamatória também está relacionada com outras respostas biológicas como a proliferação, sobrevivência e morte celular (CHRISTIAN; SMITH; CARMODY, 2016; MITCHELL; VARGAS; HOFFMANN, 2016). Na exposição por NMPs, a ativação da resposta imunológica muita das vezes está relacionada com a ativação das células do sistema imunológico para a possível eliminação de tais partículas estranhas (WOLFF; SINGER; SCHMIDT; BEKESCHUS, 2023). No entanto, células mononucleares de sangue periférico humano expostas aos NMPs apresentaram tanto respostas proinflamatórias quanto antiinflamatórias, complicando a categorização dos efeitos biológicos, principalmente pela extensa variedade de propriedades dos NMPs (WOLFF; SINGER; SCHMIDT; BEKESCHUS, 2023). *In vivo*, a inflamação local também pode ocorrer em decorrência do dano mecânico nos tecidos causados pelos NMPs de tamanho grandes e com formas pontiagudas e/ou fragmentadas (DENG; ZHANG; LEMOS; REN, 2017; DENG; ZHANG; QIAO; BONILLA *et al.*, 2018).

Uma vez que geralmente a internalização dos NMPs estão relacionados com mecanismos que induzem seu acúmulo nos lisossomos, sem a geração de efeitos tóxicos, podemos inferir que processos adaptativos podem estar ocorrendo (LI; JU, 2018). A autofagia é um processo altamente conservado que regula a degradação de organelas e moléculas fisiologicamente ou que pode ser induzida por falta de nutrientes, estresse oxidativo, compostos tóxicos e doenças (YE; ZHOU;

ZHANG, 2018). Autofagia é um processo muito importante para o crescimento e proliferação celular, sobrevivência, adaptação e morte celular (LI; JU, 2018). Contudo, a autofagia pode exercer importante função na exposição aos NMPs, por meio da adaptação e capacidade protetiva em condições de estresse, ou até mesmo promover a morte (LI; JU, 2018; XU; FENG; HAN; YAO *et al.*, 2023). Alguns estudos demonstraram a participação da autofagia como um efeito biológico decorrente da exposição por NMPs (LEE; AMARAKOON; WEI; CHOI *et al.*, 2021; LIU; HOU; WANG; YANG, 2022; XU; FENG; HAN; YAO *et al.*, 2023). A ativação de vias autofágicas na toxicidade dos NMPs pode estar relacionada principalmente com a disfunção ou alterações em organelas como a mitocôndria, lisossomos e retículo endoplasmático (LI; JU, 2018).

1.5 Modelos Experimentais Relevantes aos Humanos

A avaliação toxicológica da exposição de seres humanos aos NMPs são limitadas devido a falta de métodos de detecção sensíveis e não destrutivos que evidencie a presença de NMPs em tecidos e matrizes mais complexas (VAN RAAMSDONK; VAN DER ZANDE; KOELMANS; HOOGENBOOM *et al.*, 2020). Contudo, modelos experientais com desenhos de estudo relevantes à exposição dos seres humanos são amplamente utilizados, como a exposição de roedores e de culturas de linhagens celulares humanas (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; RUBIO; MARCOS; HERNANDEZ, 2020).

A utilização de modelos animais com roedores oferece uma valiosa ferramenta de estudo para avaliar os potenciais efeitos da exposição de NMPs e extrapolar para os humanos (DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022). Os roedores mais utilizados são camundongos e ratos, pela fácil manipulação, principalmente na avaliação da via de exposição oral dos NMPs, geralmente realizada via gavagem ou suspensão de NMPs na água para beber (DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022). A ingestão e a absorção via trato gastrointestinal tem sido o foco da maioria dos estudos *in vivo* da exposição aos NMPs, enquanto poucos modelos focaram nas outras duas vias de exposição pertinentes aos humanos, inalatória e tópica (DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022; ENYOH; SHAFEA; VERLA; VERLA *et al.*, 2020; LIM; JEONG; SONG; SUNG *et al.*, 2021; STOCK; BOHMERT; LISICKI; BLOCK *et al.*, 2019).

O principal efeito biológico investigado é a bioacumulação nos tecidos após exposição por NMPs e se os NMPs são capazes de serem absorvidos e transportados a outros tecidos distantes daquele que teve contato direto (LIANG; ZHONG; HUANG; LIN *et al.*, 2021). O bioacúmulo de NMPs foi demonstrado em órgaos como fígado, rins, cérebro, baço e órgaos reprodutivos (WALCZAK; HENDRIKSEN; WOUTERSEN; VAN DER ZANDE *et al.*, 2015). Para a avaliação da bioacumulação nos tecidos, a utilização de NMPs marcados com fluoróforos vem sendo uma estratégia extensamente empregada, uma vez que os NMPs são de difícil detecção em matrizes biológicas (SCHUR; RIST; BAUN; MAYER *et al.*, 2019; XU; CHEONG; LIU; HERNANDEZ *et al.*, 2020).

Outros efeitos biológicos tem sido descritos na literatura, como inflamação local consistente com a via de exposição aplicada, como nos tratos gastrointestinais (LI; DING; CHENG; SHENG *et al.*, 2020) e respiratórios (LIM; JEONG; SONG; SUNG *et al.*, 2021). Além disso, quando acumulados em tecidos distantes, os NMPs também são capazes de induzir resposta inflamatória, como foi evidenciado no fígado (LI; SHI; WANG; XIAO *et al.*, 2021), nos testículos (XIE; DENG; DUAN; XIE *et al.*, 2020) e ovários (HOU; LEI; CUI; HOU *et al.*, 2021). Ainda, o estresse oxidativo também pode ser induzido após exposição por NMPs, e a participação deste efeito biológico tem sido considerado como um importante mecanismo para a toxicidade dos NMPs e relacionado com outros potenciais efeitos (LI; SHI; WANG; XIAO *et al.*, 2021). Dentre outros potencias efeitos da exposição por NMPs se destacam a alteração da microbiota (FACKELMANN; SOMMER, 2019), alterações metabólicas (LU; WAN; LUO; FU *et al.*, 2018), hepatotoxicidade (LI; SHI; WANG; XIAO *et al.*, 2021), disfunções reprodutivas (AMEREH; BABAEI; ESLAMI; FAZELIPOUR *et al.*, 2020; LEI; WU; LU; LIU *et al.*, 2018), neurotoxicidade (LEI; LIU; SONG; LU *et al.*, 2018) e alterações endócrinas (AMEREH; BABAEI; ESLAMI; FAZELIPOUR *et al.*, 2020).

A avaliação da toxicidade dos NMPs e seus possíveis mecanimos são principalmente realizados em modelos *in vitro*, utilizando as mais diversas metodologias para cultura de linhagens celulares humanas (DONG; CHEN; CHEN; CHEN *et al.*, 2020; HE; LI; CHEN; MIAO *et al.*, 2020; STOCK; LAURISCH; FRANKE; DONMEZ *et al.*, 2021). Assim como em estudos *in vivo*, a exposição de NMPs no trato gastrointestinal é a mais investigada, usando células em cultura de monocamada, co-

cultura e até mesmo cultura invertida para NMPs que flutuam no meio de cultura (STOCK; BOHMERT; LISICKI; BLOCK *et al.*, 2019; STOCK; LAURISCH; FRANKE; DONMEZ *et al.*, 2021). Além disso, a investigação *in vitro* permite que os mais diversos tipos celulares sejam utilizados como modelos, assim como as células do sistema imunológico (JEON; LEE; JEONG; YANG *et al.*, 2021; WOLFF; SINGER; SCHMIDT; BEKESCHUS, 2023), células do trato respiratório (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023) e células epiteliais da pele (SAADATI; DA SILVA BRITO; EMMERT; BEKESCHUS, 2022) e de mucosas (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023), ou outros tecidos (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; HE; LI; CHEN; MIAO *et al.*, 2020; TOLARDO; MAGRI; FUMAGALLI; CASSANO *et al.*, 2022). Dentre os principais efeitos biológicos e seus mecanismos se destacam principalmente os processos de internalização celular (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; GOPINATH; TWAYANA; RAVANAN; JOHN *et al.*, 2021), indução de estresse oxidativo (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; GAUTAM; JO; ACHARYA; MAHARJAN *et al.*, 2022; GOPINATH; TWAYANA; RAVANAN; JOHN *et al.*, 2021), resposta inflamatória (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; GAUTAM; JO; ACHARYA; MAHARJAN *et al.*, 2022; WOLFF; SINGER; SCHMIDT; BEKESCHUS, 2023), indução de morte celular (DA SILVA BRITO; SINGER; MIEBACH; SAADATI *et al.*, 2023; GAUTAM; JO; ACHARYA; MAHARJAN *et al.*, 2022) e processos adaptativos (GOPINATH; TWAYANA; RAVANAN; JOHN *et al.*, 2021), além de genotoxidade (ROURSGAARD; HEZAREH ROTHMANN; SCHULTE; KARADIMOU *et al.*, 2022; SAADATI; DA SILVA BRITO; EMMERT; BEKESCHUS, 2022).

No entanto, a maioria dos efeitos biológicos observados tanto nos estudos *in vivo* quanto *in vitro* foram em decorrência das altas doses aplicadas, seguindo o princípio da precaução para facilitar a observação de qualquer efeito da exposição de NMPs (LESLIE; DEPLEDGE, 2020). Isto ocorre principalmente pela falta de estimativas da real concentração de NMPs presentes na natureza, principalmente pela dificultade nas metodologias para amostragem e detecção dos NMPs, principalmente aqueles na escala nanométrica (COX; COVERNTON; DAVIES; DOWER *et al.*, 2019; DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022). Outra questão muito debatida é sobre a cronicidade com o qual os modelos

experimentais devem ser aplicados e em desenhos de estudo padronizados que levem em consideração os potenciais efeitos ao longo do tempo (DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022).

Geralmente os estudos toxicológicos utilizam NMPs produzidos e disponibilizados comercialmente, porém, tais NMPs se apresentam na maioria das vezes em forma esférica, monodispersos, de tamanhos específicos e de um único tipo de polímero (GIGAULT; PEDRONO; MAXIT; TER HALLE, 2016; KEFER; FRIEDENAUER; LANGOWSKI, 2022; RUBIO; MARCOS; HERNANDEZ, 2020). No entanto, NMPs comerciais são muito diferentes dos NMPs encontrados na natureza (GIGAULT; PEDRONO; MAXIT; TER HALLE, 2016; KEFER; FRIEDENAUER; LANGOWSKI, 2022; VAUTHIER; BOUCHEMAL, 2009). Misturas heterogêneas de NMPs são encontradas na natureza, ou seja, NMPs que possuem as mais diversas propriedades fisico-químicas (BLAIR; WALDRON; PHOENIX; GAUCHOTTE-LINDSAY, 2017; RUBIO; MARCOS; HERNANDEZ, 2020; WAGNER; REEMTSMA, 2019). A utilização de NMPs provenientes da natureza em estudos toxicológicos se torna muito difícil devido as limitações de metodologias padronizadas de amostragem, detecção e quantificação dos NMPs (DA SILVA BRITO; MUTTER; WENDE; CECCHINI *et al.*, 2022; KEFER; FRIEDENAUER; LANGOWSKI, 2022; MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018).

O método mais utilizado para produzir NMPs é o *bottom-up*, no qual NMPs são produzidas em solução a partir da polimerização dos monômeros e polímeros (KEFER; FRIEDENAUER; LANGOWSKI, 2022). No entanto, esse método muita das vezes pode estar relacionado com a presença de solventes e compostos químicos conhecidos por serem tóxicos, assim, podendo influenciar nas respostas biológicas observadas (MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018; RUBIO; MARCOS; HERNANDEZ, 2020). Ainda, o polímero mais utilizado em estudos toxicológicos, o PS NMPs são produzidos por este método, devido principalmente da não disponibilidade comercial de outros polímeros (BALAKRISHNAN; DENIEL; NICOLAI; CHASSENIEUX *et al.*, 2019). Contudo, novas metodologias para a produção de NMPs com características mais relevantes e que possam ser utilizados nos estudos toxicológicos vem sendo encorajados (KEFER; FRIEDENAUER; LANGOWSKI, 2022; MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018). Um exemplo disso foi a dissolução de PE em solventes orgânicos com a adição de

surfactantes para aumentar a emulsificação em solução aquosa e sua estabilidade (BALAKRISHNAN; DENIEL; NICOLAI; CHASSENIEUX *et al.*, 2019). Considerando os potenciais efeitos de “cavalo-de-Troia” dos NMPs por se associarem e absorverem substâncias tóxicas, outro método foi proposto, consistindo na polimerização em emulsão livre de surfactantes e metais para produzir PS NMPs em solução aquosa (PESSONI; VECLIN; EL HADRI; CUGNET *et al.*, 2019).

As metodologias *top-down* são baseadas no princípio de produção dos NMPs secundários, que são formadas a partir da degradação física e/ou mecânica dos materiais existentes (KEFER; FRIEDENAUER; LANGOWSKI, 2022). A moagem criogênica é um método bastante utilizado para produzir NMPs com uma larga faixa de tamanhos, aproximadamente de 1 µm até 200 µm (EITZEN; PAUL; BRAUN; ALTMANN *et al.*, 2019). Devido aos pontos de temperatura de transição vítrea de alguns polímeros, a aplicabilidade deste método se torna difícil e não pode ser utilizada (EITZEN; PAUL; BRAUN; ALTMANN *et al.*, 2019). Outra limitação deste método é que os NMPs produzidos não são suspensos facilmente em soluções aquosas, sendo necessários elevados períodos de tempo e etapas como filtração, fracionação e uso de estabilizadores como surfactantes (EITZEN; PAUL; BRAUN; ALTMANN *et al.*, 2019; HUFFER; PRAETORIUS; WAGNER; VON DER KAMMER *et al.*, 2017).

Um outro método, a abrasão à laser, baseia-se na degradação foto-química e foto-oxidação de polímeros em solução aquosa, produzindo quase que exclusivamente partículas em nanoescala (KEFER; FRIEDENAUER; LANGOWSKI, 2022; MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018). Este método é capaz de produzir NMPs que mimetizam algumas propriedades fisico-químicas similares aos NMPs secundários produzidos a partir da foto-oxidação induzidas por radiação ultravioleta, como por exemplo formas irregulares e polidispersos em solução (MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018). A principal vantagem deste método seria a produção de NMPs com características relevantes aos encontrados na natureza sem a necessidade de utilização de compostos químicos e aditivos considerados tóxicos (MAGRI; SANCHEZ-MORENO; CAPUTO; GATTO *et al.*, 2018). Outro método que também leva em consideração a não necessidade de utilização de aditivos, seria a combinação do ultrassom em solução altamente alcalina (VON DER ESCH; LANZINGER; KOHLES; SCHWAFERTS *et al.*, 2020). Este método

baseia-se na combinação da fragmentação física e degradação dos radicais poliméricos com forte hidrólise e oxidação, produzindo NMPs com caracteristicas envelhecidas (KEFER; FRIEDENAUER; LANGOWSKI, 2022; VON DER ESCH; LANZINGER; KOHLES; SCHWAFERTS *et al.*, 2020).

A utilização de plásticos tende a aumentar cada vez mais, e mesmo com todas as iniciativas para controlar este crescimento e todos as preocupações como o principal contaminante do meio ambiente, sua ampla aplicabilidade é vantajosa e traz benefícios para a humanidade. No entanto, pouco se sabe sobre os potenciais efeitos biológicos da exposição aos NMPs, principalmente decorrente de limitações metodológicas na amostragem, detecção e quantificação dos NMPs presentes na natureza e em matrizes biológicas e complexas. Além disso, a investigação da toxicidade dos NMPs com propriedades mais relevantes daqueles encontrados na natureza também se torna escassa. Para superar estas limitações, o desenvolvimento de novas metodogias para a produção de NMPs relevantes ambientalmente são necessárias para a investigação toxicológica e potenciais efeitos biológicos.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos tóxicos resultantes da exposição aos NMPs através de uma revisão bibliográfica em modelos *in vivo*, além de produzir e caracterizar NMPs em laboratório para compreender melhor suas respostas biológicas na experimentação *in vitro*.

2.2 Objetivos Específicos

- Realizar uma revisão na literatura dos principais achados referentes à exposição *in vivo* de roedores aos NMPs, explorando diferentes desenhos de estudo, vias de administração e os principais efeitos biológicos resultantes dessa exposição;
- Produzir em laboratório e caracterizar NMPs que mimetizem as características fisico-químicas observadas no meio ambiente e que possa ser utilizados como material de referência para estudos toxicológicos;
- Avaliar os efeitos biológicos da exposição aguda dos NMPs *in vitro* por meio de cultura de linhagens celulares humanas.

3 RESULTADOS

O projeto de pesquisa foi realizado no Leibniz Institute for Plasma Science and Technology (INP; Greifwald, Alemanha), no grupo de pesquisa ZIK *plasmatis* 'Plasma-Redox-Effects', em parceria com o Laboratório de Patologia Molecular da Universidade Estadual de Londrina. O projeto foi fomentado pelo Ministério Federal de Educação e Pesquisa da Alemanha (BMBF nº 03Z22DN11, 03Z22DN12, 03Z22Di1, e 03Z22D511) e fez parte do consórcio 'MetaZIK PlasMark' em parceria com outras instituições de pesquisa alemãs.

Os resultados do presente trabalho foram apresentados em dois artigos científicos.

1) "Consequences of nano and microplastic exposure in rodent models: the known and unknow". Artigo de revisão de escopo publicado na revista Particle and Fibre Toxicology em 2022, fator de impacto 10.0.
2) "Application of sonicated polyethylene terephthalate for nano- and micro-plastic particle toxicity and genotoxicity research". Artigo experimental submetido à revista ACS Applied Materials & Interfaces em 2023, fator de impacto 9.5.

ARTIGO 1

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Particle and Fibre Toxicology

REVIEW Open Access

Consequences of nano and microplastic exposure in rodent models: the known and unknown

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Abstract

The ubiquitous nature of micro- (MP) and nanoplastics (NP) is a growing environmental concern. However, their potential impact on human health remains unknown. Research increasingly focused on using rodent models to understand the effects of exposure to individual plastic polymers. In vivo data showed critical exposure effects depending on particle size, polymer, shape, charge, concentration, and exposure routes. Those effects included local inflammation, oxidative stress, and metabolic disruption, leading to gastrointestinal toxicity, hepatotoxicity, reproduction disorders, and neurotoxic effects. This review distillates the current knowledge regarding rodent models exposed to MP and NP with different experimental designs assessing biodistribution, bioaccumulation, and biological responses. Rodents exposed to MP and NP showed particle accumulation in several tissues. Critical responses included local inflammation and oxidative stress, leading to microbiota dysbiosis, metabolic, hepatic, and reproductive disorders, and diseases exacerbation. Most studies used MP and NP commercially provided and doses higher than found in environmental exposure. Hence, standardized sampling techniques and improved characterization of environmental MP and NP are needed and may help in toxicity assessments of relevant particle mixtures, filling knowledge gaps in the literature.

Keywords: Animal, Environment, Mice, Particles, Polymers, Rats, Toxicity

Introduction

Plastic debris is a growing environmental concern. In 2019, 368 million tons of plastic were produced globally [1]. Furthermore, pandemic-related single-use plastics (i.e., surgical masks) have worsened the scenario [2]. Despite recycling initiatives and legislation to ban single-use plastics, different plastic particles have been found in oceans, fresh water and agricultural systems, urban environments, the atmosphere, and remote areas such as the Mount Everest [3–5]. Small plastic particles are defined as microplastics (MP) (less than 5 mm diameter) and nanoplastics (NP) (less than 100 nm) [6, 7] and can vary in size, shape, type of polymer, and concentration [1, 3, 8, 9]. Regarding the sources, these are either deliberately manufactured (primary MP/NP) or derived from larger plastics during environmental exposure such as UV irradiation, mechanical abrasion, or microbial degradation (secondary MP/NP) [8].

Plastic particles are far-reaching and a multifaceted problem. The focus is not only on food [10, 11] or aquatic systems [4, 7, 8] as primary sources of plastic exposure but also on its epidemiological consequences [9, 12–14]. Small volume but large surface area facilitates chemical reactions with body fluids and tissues in direct contact with particle surfaces. These particles are of particular

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concern due to their persistence, bioaccumulation in the food chain and in wildlife destined for human consumption, potential toxicity, and ability to act as vectors for pathogens and co-pollutants [9, 12]. Marine organisms have also presented toxic effects of MP and NP exposure, depending on the type of organism, ultimately affecting bioaccumulation, metabolic changes, inflammation, reproduction effects, behavior, and ecosystem interactions [8, 15]. In addition, fish exposed to NP by environmentally relevant exposure route (contaminated prey ingestion) showed NP accumulated in different fish tissues and affected innate immune gene signatures. This exposure may compromise their ability to survive in nature [16].

Humans are exposed either directly to MP and NP in drinking water, sea salt, and the atmosphere or indirectly through the food chain [8–11]. Debris from plastic prosthetic implants is also a source of exposure to MP and NP in humans [9]. Moreover, the accumulation of particles in all trophic levels may expose humans to more particles in food sources [10, 13]. In a recent systematic review about MP content in American food sources, a caloric intake-based calculation was used to estimate human ingestion of a large number of particles (>50,000) per year, significantly rising if drinking bottled water was included [17]. Such studies are necessary to raise public awareness about the constant uptake of plastic into the human body. It remains a matter of debate, however, which types of particles or their size or cargo as well as location may be critical in driving specific health-related conditions and diseases.

Continuous sources of less-concentrated MP (food containers and drinking water) are also a concern. Regulators (EFSA/WHO) state that MP and NP exposure in humans present few adverse effects, although this statement may be due to little evidence rather than a lack of effects. Preliminary signs of harm are still arising. The precautionary principle recommends and supports initiatives to develop better analytical methods before concluding that MP and NP exposure is entirely safe after all [18].

Current estimations of plastic particle exposure in humans are limited due to the lack of an established method to provide non-destructive evidence of MP and NP presence in tissue [10]. Ultra-thin sections of tissue, often used in medical research, cannot clarify the possible involvement of plastic in disease processes, as plastic is technically challenging to identify due to its small size and chemical inertness. Assessment of MP and NP exposure in rodent models offers a valuable tool to assess health risk of plastic exposure to animals and parallel it to humans. In addition, many established rodent models of human diseases offer the possibility to assess the sensitivity of specific pathologies to MP and NP exposure. We review recent findings from MP exposure within in vivo rodents model systems intending to give an outlook on them beyond the highlighted gastrointestinal and respiratory tract possible effects and fill knowledge gaps within other systems as well.

Searching methods

In this scoping review, we used different combinations of keywords in the Google Scholar database between 2001 and 2021: "microplastics"; "nanoplastics"; "exposure"; "oral administration"; "inhalation"; "rodents"; "mice"; "rats"; "accumulation"; "toxicity" and "toxic effects". Inclusion criteria were original studies published in peer-reviewed journals and performed by exposing rodents (mice and rats) to MP and NP, assessing the accumulation of particles in tissues and/or toxic effects. With that, 31 original studies were included and described in Table 1. The remaining manuscripts were included as complementary information.

Discussion

Plastics utilized in rodent models

Plastics are synthetic polymers derived from fossil fuels or biomass. The most common polymers produced globally include polyethylene terephthalate (PET), polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS), and polyurethane (PUR) [19]. Heterogeneous plastic mixtures contaminate environmental sources such as water [20, 21], in which environmental fragmentation and degradation may hinder their classification, generating products with different shapes, sizes, chemical compositions, and densities [14]. However, most rodent studies used one plastic entity (Table 1) and not with heterogeneous mixtures as found in the environment.

Commercially available particles are uniform spheres with pristine or functionalized surfaces. Despite the characterization of exposure effects of a particular polymer, commercial specifications do not reflect environmental exposure accurately [14]. To this end, Estrela and colleagues assessed acute exposure to the combination of zinc oxide nanoparticles and PS NP in mice [22]. Although pathophysiological changes were observed from exposure to PS NP (Table 1), no additive or synergistic effects were observed when administered in combination. Moreover, Liang and colleagues found that MP and NP mixtures with different sizes facilitate biodistribution in mice's tissues [23].

Secondary MP and NP exhibit diverse shapes and surfaces from environmental weathering that may influence biodistribution. For example, an assessment of tritiated polyethylene glycol (PEG)ylated PS in a

Table 1 Assessment of plastic particles in rodent models

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Oral administration								
Liang et al. [23]	C57BL/6 mice	PS (pristine or fluorescent)	50, 500, and 5000 nm, alone or in mixture	Oral gavage	Double distilled water	2.5, 25, 50, 125, 250, 500 mg/kg bw	Single dose, 24 h. Daily, 28 days	Single-dose: bioaccumulation over time in intestines (IVIS). Bioavailability in other organs was size-dependent, with small particles in the gut, liver, spleen, kidneys, heart, lungs, testis, epididymis, brain, blood, ovaries, and uterus. Larger particles were found in the blood and gut but not in other organs. They have altered mucus production in the gut. Co-exposure with different sizes increased biodistribution in organs and increased ROS generation, epithelium apoptosis, and permeability in the intestines. Antioxidant pretreatment was able to reverse the effects. The 28-day repeated exposure model showed the same effects

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Amereh et al. [30]	Wistar rats	PS	25 and 50 nm as mixture	Oral gavage	Distilled water	1, 3, 6, 10 mg/kg bw-day	Daily, 35 days	Decreased serum testosterone, luteinizing hormone, follicle-stimulating hormone, altered sperm concentration, motility, morphology, DNA damage. Histopathological signs of testes atrophy and degeneration and particles accumulation
Deng et al. [35]	CD-1 mice	PE (coated with phthalate esters)	0.4 to 3.2 µm	Oral gavage	Pure water, phthalate esters, virgin MP	100 mg/kg bw	Daily, 30 days	Phthalate ester accumulation in gut > liver > testes. Testes: reduced sperm count and viability, increased oxidative stress (SOD, MDA), increased spermatogenesis disorder markers LDH and ACP by MP contaminated with phthalate ester
Stock et al. [38]	C57BL/6 HOTT reporter mice	PS (fluorescent carboxyl or sulphate coated)	1, 4, and 10 µm in mixture	Oral gavage	0.5% (m/v) carboxymethylcellulose	1.25 – 34 mg/kg bw adjusted for surface area	Three times a week, 28 days	No effects observed: no Hmox1 reporter response or changes in body or organ weights and low intestinal particle retention. No pathological changes were measured by histology, and very low concentrations of particles in the intestines

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Deng et al. [41]	ICR mice	PS (pristine or fluorescent)	5 and 20 µm	Oral gavage	Water	0.01–0.5 mg/day	Up to 28 days	Accumulation in gut, liver, and kidney. Liver inflammation, hepatic lipid accumulation, oxidative stress, decreased AChE activity, altered lipid profile, and impairment of energy metabolism (reduction in ATP levels)
Li et al. [48]	C57BL/6 mice	PE	10–150 µm	Diet	Basal feed with no special preparation	6, 60, and 600 µg/day, adjusted for 3 g consumption/animal	35 days	Increased IL-1α in serum and different serum cytokine profiles depending on concentration. Small intestinal inflammation: increased TLR4, AP-1, and IRF5 protein (IF) and increased microbial diversity and abundance from fecal samples at the highest concentration of MP
Ding et al. [46]	SPF grade C57BL/6 mice	PS (fluorescent)	60 nm	Oral gavage	Double distilled water	50 µg/mL (500 µL)	Single-dose, 3 days	Particle accumulation in the stomach, intestines, and liver. No accumulation was observed in the heart, spleen, and lungs

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Jin et al. [49]	ICR mice	PS (pristine or fluorescent)	5 µm	Drinking water	Reverse osmosis purewater	100 and 1000 µg/L	42 days	Accumulation in the gut was followed by gut microbiota dysbiosis and decreased mucous secretion. Intestinal barrier dysfunction. Significant increase in hepatic total bile acid (ns increases in serum) and altered bile acid metabolites. Altered amino acid metabolism: increased serum arginine, tyrosine, and succinylacetone
Luo et al. [50]	ICR mice	PS	5 µm	Drinking water	Water	100 and 1000 µg/L	During pregnancy and lactation (about 6 weeks)	Altered serum and hepatic metabolic markers; different levels of genes related to glycolipids and energy metabolism in dams, F1 and F2 offspring. No influences on F1 and F2 growth rate. Dams: hepatic ballooning degeneration. Altered gut microbiota and decreased mucus secretion. F1: altered serum metabolites. Adult female F1: lipid accumulation in the liver

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Luo et al. [51]	ICR mice	PS	0.5 and 5 µm	Drinking water	Water	100 and 1000 µg/L	During pregnancy (about 6 weeks)	Increased risk of fatty acid metabolism disruption in offspring: In both sexes, 5 µm particle exposure reduced β-oxidation and fatty acid synthesis. Amino acid metabolism is reduced in females
Walczak et al. [52]	Fischer 344 rats	PS (fluorescent non-coated, aminated and carboxyl-modified)	50 nm	Oral gavage	Deionized water	125 mg/kg bw	Single dose, 6 h	All particles were observed in the lung, heart, kidneys, brain, stomach, and intestines. Negative NP was also in the liver. Estimated bioavailability: from 0.2 to 1.7%. No histopathological changes
Li et al. [69]	C57BL/6 mice	PS (fluorescent)	5 µm	Drinking water	Reverse osmosis water	20 mg/kg/day bw	30 days	Particle accumulation in the liver. Vacuolar degeneration, chronic inflammatory infiltration, and hepatocellular edema (histologically). Increased IL-1β and TNF-α mRNA (hepatic). Signs of apoptosis (TEM). Increased Nrf2 and Keap1 hepatic protein. Liver oxidative stress: decreased SOD and GSH, increased MDA

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Deng et al. [70]	CD-1 mice	Suspended PE and PS in organic flame retardants (OFR)	0.5 to 1.0 µm	Drinking water	Water	2 mg/L (PE or PS) in 10 or 100 µg/L (OFR in aqueous solution)	90 days	Accumulation in liver and gut, with local inflammation and lipid droplets (H&E). Hepatic oxidative stress and LDH increased in MP + OFR, decreased AChE in the brain, and altered metabolomics in serum and liver
Jin et al. [71]	BALB/C mice	PS (fluorescent)	0.5, 4, and 10 µm	Oral gavage	Double distilled water	10 mg/mL	Daily, 28 days	Testicular accumulation followed by local inflammation. Reduced sperm quality and testosterone levels. Disruption of blood-testis barrier and disordered arrangement of spermatogenic cells with the presence of multinucleated gonocytes (H&E)
Lu et al. [79]	ICR mice	PS	0.5 and 50 µm	Drinking water	Reverse osmosis water	100 and 1000 µg/L	35 days	Decreased body, liver, and epididymis fat weights. Decreased mucus secretion in the gut. Altered biochemical serum markers. Changes in microbiota, hepatic lipid profile, and expression of some genes related to lipid metabolism decreased triglyceride synthesis markers mRNA in fat tissue

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Silva et al. [84]	Swiss mice	PUR	250 nm	Oral gavage and IP	0.9% NaCl	2, 5, and 10 mg/ kg bw	10 days	Oral gavage: increased visceral fat accumulation, glomerular atrophy, and increased serum TNF-α and ALP. IP: glomerular necrosis and inflammatory infiltrate in adipose tissue on the high dose. Both administration routes: lung inflammation, liver vascular congestion, and hepatocytes vacuolization. Increased ALT levels and serum IL-6
Zheng et al. [85]	C57BL/6 mice	PS	5 µm	Drinking water	Distilled water	500 µg/L	28 days	Exacerbated acute colitis model: increased intestinal permeability, lipid and liver metabolites disruption, triglyceride accumulation, and lipid peroxidation in the liver. Increased serum IL-1β, TNF-α, and INF-γ in mice exposed only to MP. In addition, MP exacerbated serum cytokines in the colitis model

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Xie et al. [87]	BALB/c	PS	5.0–5.9 μm	Oral gavage	0.9% NaCl	0.01, 0.1 and 1 mg/d or 1 mg/d + NAC or p38 MAPK inhibitor	Daily, 42 days	Decreased sperm number, motility, metabolism markers LDH and SDH, serum testosterone, and GSH. Increased sperm deformity rate, ROS, MDA, apoptosis, and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). Rescued by N-acetylcysteine and SB203580
Hou et al. [88]	ICR mice	PS	5 μm	Drinking water	Water	100, 1000 and 10,000 μg/L	35 days	Sperm count decreased, and deformities increased. Disordered arrangement of spermatic cells. Increased NF-κB, IL-1β, IL-6, and testicular apoptosis. Decreased HO-1 protein and Nrf2 protein and mRNA
Hou et al. [90]	Wistar rats	PS	0.5 μm	Drinking water	Deionized water	0.015, 0.15 and 1.5 mg/kg/d	90 days	Increased thickness of granulosa layer with some thinner secondary follicles (H&E) and decreased number of growing follicles. Decreased antioxidant defenses (GPx, SOD, and CAT). Increased MDA in ovaries, and NLRP3 and caspase-1 in ovarian granulosa cells (high dose). IL-1β and IL-18 increased, and anti-Müllerian hormone decreased

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
An et al. [91]	Wistar rats	PS	0.5 μm	Drinking water	Water	0.015, 0.15 and 1.5 mg/d	90 days	Decreased number and volume of growing follicles and ovary fibrosis in high concentration. Decreased anti-Müllerian hormone and decreased ovarian reserve capacity. Increased MDA and decreased antioxidant enzymes (SOD, CAT, GPx). Increased apoptosis, Wnt, and TGF-β in ovaries
Park et al. [93]	ICR mice	PE (containing surface modification with acid and hydroxyl groups)	40 to 48 μm	Oral gavage	Water	3.75, 15 and 60 mg/kg body weight	Daily, 90 days, some females exposed more than 21 days (lactation period)	90 days exposed males: decreased body weight gain, changes in hematological parameters. 90 days exposed females: altered hematological parameters and spleen immune response parameters, and increased serum IgA. 90 days exposed mice: hypertrophy/hyperplasia of stomachs mucosa. No adverse symptoms were observed in dams during gestation or lactation. Pups: altered sex ratio and growth rate, altered spleen immune response parameters

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Rafiee et al. [98]	Wistar rats	PS	25 and 50 nm	Drinking water	Distilled water, dispersing reagent (surrounding medium)	1, 3, 6, 10 mg/kg body weight	Daily, 5 weeks	No effects observed: no differences in body weight. Neurobehavioral assessment alone. No cognitive changes
Li et al. [100]	Wistar rats	PS	0.5 µm	Drinking water	Deionized water	0.5, 5 and 50 mg/L	Daily, 90 days	Myocardium vascular congestion and accumulation of MP. Thinner and ruptured tissue in high dose followed by increased serum cardiac damage markers CK-MB and Troponin I. Increased MDA and reduced antioxidant enzymes in the heart. Increased myocardium apoptosis and fibrosis mediated by Wnt/β-catenin pathway activation
Amereh et al. [101]	Wistar rats	PS	25 and 50 nm mixture	Oral gavage	Distilled water	1, 3, 6 and 10 mg/kg body weight/day	Daily, 5 weeks	There were no effects on T3 and T4 hormones in serum; however, circulating active forms of thyroid hormones (FT3 and FT4) were decreased in rats. Increased TSH levels in high-dose. Changes in cholesterol serum markers and increased levels of liver damage markers (ALT and AST)

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Inhalation/airways								
Eyles et al. [27]	BALB/c mice	Scandium-46 labelled styrene-divinyl benzene	7 µm	Intranasal instil-lation	Absent control group	0.250 mg (47.5 kBq) in 50 or 10 µl PBS	24 days	50 µL dose: sub-stantial bronchopul-monary deposition, accumulation on liver and spleen. 10 µL dose: accumulation in nasopharyngeal regions only
Lim et al. [58]	SD rats	PS	0.1 µm	Inhalation	Fresh air control	0.68×10^5, 1.38×10^5 and 2.82×10^5 particles/cm^3	6 h each day, 5 days/week for 14 days (Modified OECD TG 412)	Serum AST and lung inspiratory time decreased in males. Respiratory frequency increased and inspira-tory/expiratory time decreased in females. In females, reduced leukocytes count. Inflammatory mark-ers: TGF-β and TNF-α increased in lung dose-dependently in both sexes. No changes in body weight or food con-sumption. No con-centration–response was observed
Fournier et al. [59]	SD rats	PS (fluorescent)	20 nm	Intratra-cheal instilla-tion	0.9% NaCl	2.64×10^{14} particles	24 h	Accumulation in maternal lungs, heart, and spleen. Fetal liver, lungs, heat kidney, and brain. Signifi-cantly lower fetal and placental weights when adjusted for litter size variation. No differences in mater-nal weight or number of fetuses per litter

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/ effects
Other routes								
Estrela et al. [22]	Swiss mice	PS (fluorescent) and/or ZnO	PS NP: 23 nm ZnO: 69 nm	IP	Water	14.6 ng/kg	3 days	In separate, both particles induced cognitive impairment, redox imbalance (increased nitric oxide levels and thiobarbituric acid reactive species), and suppressed acetylcholinesterase activity. Systemic DNA damage was observed in separate and combined injections of particles
Kaga et al. [24]	Athymic nude mice	Radiolabelled PEGylated PS	Spherical: 21 and 33 nm, rod-like: 37 nm diamet., 350–500 nm length, worm-like: 45 nm diamet., 1–2 µm length	IV	Absent control group	0.1 mg in 50 µL PBS (2 mg/mL)	48 h	All particles accumulated in the liver, spleen, kidneys, heart, lungs, pancreas, thigh muscle, and tumor with different biodistribution
Hu et al. [94]	C57BL/6-mated BALB/c mice	PS	10 µm	IP	0.9% NaCl	250 µg in 200 µL saline	Pregnant mice on embryonic days 5.5 and 7.5	Increased embryo resorption rate and decreased number and diameter of uterine arterioles in the placenta of MP. Decreased leukocytes in blood, spleen, and placenta of dams. Decreased NK cells and macrophages in the placenta. Changes in macrophages polarization favoring M2-subtype, increased T CD4 + cells in the placenta, and changed cytokines secretion

Table 1 (continued)

Ref	Model	Polymer	Size	Route	Controls	Dose	Duration	Accumulation/effects
Nie et al. [95]	ICR mice	PS	60 and 900 nm	IV	0.9% NaCl	300 µg	Pregnant mice on embryonic days 8, 9,10 and 15	No effects on number of embryos. Decreased body weight of embryos. 60 nm NP: decreased placental diameter, extravasation in fetus and placenta

PS, polystyrene; IVIS, in vivo image system; bw, body weight; MP, microplastics; ACP, acid phosphatase; HOTT, Heme-oxygenase triple transgenic; Hmox1, heme oxygenase-1; PE, polyethylene; IF, immunofluorescence; NP, nanoplastics; TEM, transmission electron microscopy; OFR, organic flames retardants; PUR, polyurethane; SDH, succinate dehydrogenase; SB203580, p38 MAPK inhibitor; CAT, catalase; ZnO, zinc oxide nanoparticles; IP, intraperitoneal; PEG, polyethylene glycol; IV, intravenous

tumor model nude mouse highlighted the accumulation of rod/worm-like particles in the liver and spleen compared with retention of small spherical particles in tumor masses [24]. However, further work is needed to determine the effects of polydisperse environmental secondary particles. In addition, the development of improved sampling methods to accurately characterize 'natural' particles is necessary [20, 21].

According to our literature review, label-free determination of plastic in cells and human-relevant systems has not yet been successful, although innovative microscopic or spectroscopic methods (e.g., UV light spectrum, infrared light spectrum, and Raman spectrum) are still emerging [25]. Radio-labeled plastic particles are used to include quantitative whole-body radiography in marine organisms and determine the mass balance in mice [24, 26, 27]. Fluorescently-labeled MP and NP facilitate direct quantification of bioaccumulation in tissues. Also, many commercial particles are produced with internalized fluorescence, avoiding dye-specific interactions on the particle surface. Nonetheless, possible effects of label leaching over time must be considered [28, 29].

Quantifying particle deposition within tissues helps determine whether responses are due to direct interactions with particles or indirect secondary effects [28, 29]. Monitoring labeled polymers non-invasively offer the potential for real-time measurements. For instance, Amereh and colleagues observed the accumulation of a mixture between 25 and 50 nm polystyrene particles in testes of Wistar rats using in vivo imaging system (IVIS) [30]. Another study using IVIS showed accumulation over time in the intestines of mice exposed to MP and NP [23]. However, longitudinal monitoring of fluorescent probes is hampered in deep tissues by signal penetration and tissue autofluorescence. Also, due to the low resolution, positive fluorescent signals are likely to be aggregates rather than being dispersed particles. Those difficulties may justify the observation of particle fluorescence only in peripheral tissues.

Plastic contaminants should not be viewed as isolated particles as several organic and non-organic molecules can adhere to them. Proteins can, for example, form a protein corona around particles [31, 32]. However, it is unclear whether these are human-relevant proteins and their effect. Other toxic molecules can also bind to plastic (some of them already during the manufacture of plastic products) and are slowly released later into the environment or the body [33]. Moreover, plastic binds to lipids or changes their composition in cell membranes, which may occur in freshwater algae [34]. However, we did not find any information on such phenomena in rodents or human-relevant systems.

Due to synthetic production and environmental degradation, plastics are in close contact with several types of additives and pollutants, such as phthalates, bisphenol analogs, surfactants, and pigments, all associated with potential toxic effects [14]. For example, Deng and colleagues demonstrated phthalate ester accumulation in the gut, liver, and testes following exposure to PE MP by oral gavage [35]. Moreover, several chemicals can act as endocrine disruptors, i.e., affecting hormones pathways or acting as pseudo-hormones themselves [36, 37].

In summary, improved sampling methods to determine the most common environmental particle properties will help to streamline the systematic characterization of the effects of individual polymers of different shapes, sizes, and associated coronas. In addition, the experimental utilization of heterogeneous mixtures of particle combinations and environmental plastic samples may contribute to a better understanding of the potential additive effects and effects of chemicals that come as cargo with MP and NP exposure.

Dosage

The environmental relevant dose of MP and NP exposure is heavily debated. Many studies use MP and NP concentrations far greater than current human exposure estimates (Table 1). Estimations are that human consumption of up to 0.06 mg/kg/day of particles occurs via drinking water [30]. Administration of a high single dose of particles followed by substantial recovery or constant exposure of concentrated particles is unlikely to reflect real-world scenarios. To this end, Stock and colleagues used a dosing regimen of PS MP at less than 34 mg/kg body weight thrice weekly for four weeks [38]. They found minimal particle uptake into intestinal tissue and no toxic effects.

Conversely, high concentrations reflect the combination of multiple exposure routes in nature [39] and emulate increases in microplastic pollution in the future. Current limitations in methods to detect MP and NP accurately hinder estimations of environmental concentrations unreliable [40]. Therefore, determining the threshold at which MP and NP exposure is associated with adverse events remains critical.

Polymer exposure routes

Oral ingestion of plastic and absorption via the gastrointestinal tract has so far been the focus of MP/NP research [38]. However, reports in which plastic particles sized up to 20 µm are ingested [41] do not seem comprehensible according to the assessment of the German Federal Institute for Risk Assessment (BfR) [42]. Although microparticles up to 150 µm can translocate across mammals' intestinal barriers [43], the

absorption rate is below 0.3%. From the rate, mostly particles sized up to 10 µm should be able to penetrate all organs, including the brain, with unexplored consequences [44].

Low absorption of MP and NP through intestinal epithelium could be related to particles properties and efficiency of the mucus barrier to interact and maintain MP and NP in the intestinal lumen. By being maintained, MP and NP can be excreted in the feces or deposited, which may cause local irritation or release of toxic additives [44]. Also, MP and NP can be internalized by intestinal epithelium and be re-released into the intestinal lumen due to a turnover of approximately 3 days, thus not reaching the bloodstream [45]. Currently, some studies assume that toxic effects are expected in the digestive tract and liver due to continuous plastic accumulation (Table 1) [46, 47]. A murine model fed with PE particles showed increased inflammation in small intestines followed by changes in microbiota and increased systemic pro-inflammatory markers [48].

Another route for human exposure to MP and NP is drinking water, as plastic particles were detected in tap and bottled water [17]. Some studies used this administration route to expose rodents models to MP and NP (Table 1). However, water consumption was not assessed for particle intake calculations [49–51]. Additionally, this route is not appropriate for assessing buoyant polymers such as PP and PE and may be inefficient considering particle sedimentation over time for MP and NP suspensions. Another limitation of the oral uptake route (drinking water, diet, and oral gavage) might be bioavailability, which was estimated to range from 0.2 to 1.7% with different types of NP in vivo [52].

Plastic is not only absorbed by food through the digestive tract [53]. It can also be inhaled through fine air dust (e.g., abrasion from car tires or clothing [54, 55] and release chemical additives [56] once within the body [57]). Occupational diseases associated with textiles have been extensively reviewed [54]. Fragments and fibers are the most common forms of atmospheric MP and NP. However, estimations of human exposure levels are limited by the lack of sensitivity of current methods to detect small particles [5, 58].

Clearance of inhaled particles can be through mucociliary transport resulting in negligible deposition in airways or phagocytosis by alveolar macrophages or lymphatic transport [54]. MP and NP may avoid these mechanisms, accumulating in the lungs and entering systemic circulation [27, 58, 59]. Inhaled nanoparticles can also reach the central nervous system (CNS) through the olfactory bulb [60]. A recent 14-day repeat inhalation study in rats highlighted lung inflammation and decreased inspiratory rate following exposure to 100 nm PS particles [58]. Also, a single intratracheal dose during gestation resulted in maternal-to-fetal translocation of PS NP [59].

Topical exposure to MP and NP from microbeads in personal hygiene products and contaminated water may directly affect the skin. Epidermal cells exposed to MP and NP in vitro exhibited oxidative stress [61]. However, uptake across the outermost skin layer, the *stratum corneum*, is considered restricted to nanoparticles smaller than 100 nm [43]. Minimal uptake was observed following ex vivo administration of 20–200 µm fluorescent particles to pig ears both with and without compromised barrier function [62]. Particle weathering and aging may enhance topical uptake, as observed in mice with quantum dot nanoparticles [63]. To our knowledge, topical plastic exposure has not been extensively characterized in rodent models.

Various exposure routes have been utilized in animal models. Oral and inhalation routes are considered the main exposure routes in humans. The influence of a particular administration route on particle characteristics (e.g., accompanying corona or ability to release toxic chemicals) is not well understood.

In vivo effects of polymer exposure

Despite being considered chemically inert compared to plastic monomers, toxicity following MP and NP exposure was described (Fig. 1, Table 1). MP and NP toxicity may result from their persistent physical presence in tissues. Size-dependent effects have been demonstrated in vitro with PS spheres [61, 64]. Small and positively charged particles may have greater bioavailability in mammals [65]. Particle accumulation has been demonstrated in organs such as the liver, kidneys, brain, spleen, and reproductive organs (Fig. 1, Table 1), although it was independent of the functionalized surface coating in high concentration [52].

Disruption, penetration, absorption, and endocytosis mechanisms, which may be toxic [66], are currently being discussed as possible ways plastic particles can enter and interact with cells and tissues [67, 68]. Possible toxic consequences may not only be due to MP and NP exposure, as most commercially available particles used in studies in vivo are provided in aqueous suspensions with dispersant and conservant solvents. Walczak and collaborators centrifuged the particles for conservant and surfactant removal before usage, controlling possible effects found after exposure [52]. Thus, evaluating additional compounds as control groups and not only test vehicle solutions is essential.

Direct effects and underlying mechanisms

In mice exposed to fluorescently labeled particles, localized inflammation at the site of particle accumulation

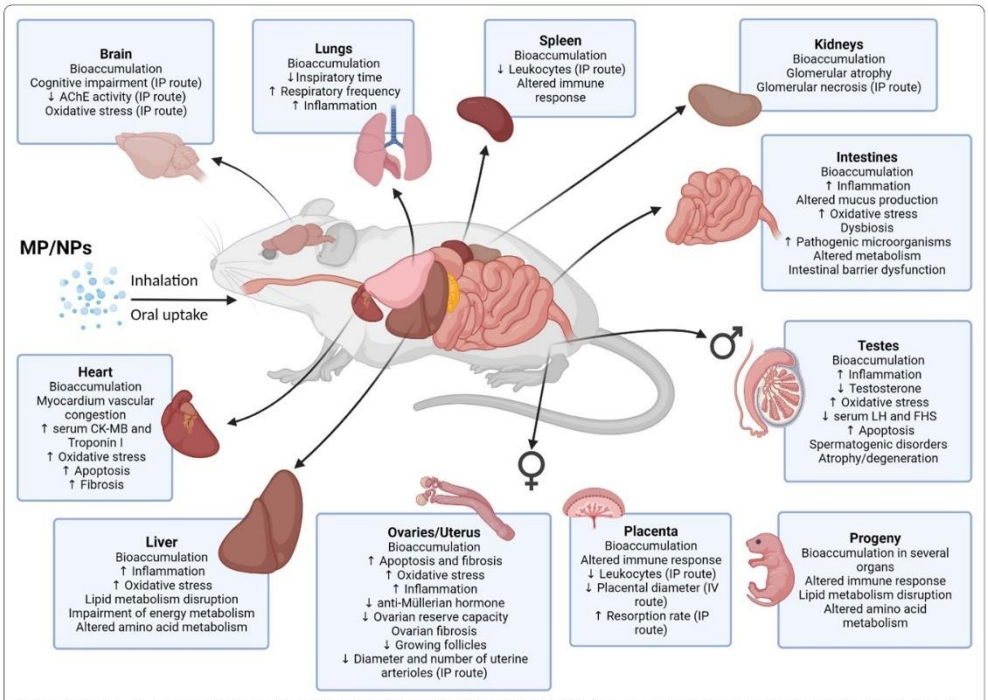

Fig. 1 Biological effects observed in rodents exposed to MP and NP. *Abbreviations* MP/NPs, micro and nanoplastics; AChE, acetylcholinesterase; IP, intraperitoneal; LH, luteinizing hormone; FSH, follicle-stimulating hormone; IV, intravenous. Created with BioRender.com

has been confirmed in the liver [41, 69, 70] and testes [71]. However, fluorescent dye leaching from MP and NP could also contribute to the exposure effects observed. Interestingly, few studies evaluated the fluorescent dye leaching of particles under conditions such as simulated gastric and intestinal fluids, and fluorescence leaching was negligible [23, 52]. Moreover, fluorescent MP and NP are mainly used only for bioaccumulation and biodistribution assessments into tissues, and non-fluorescent for toxicity evaluations [23, 41]. Exposure to non-fluorescent particles resulted in increased inflammation in primary absorption sites consistent with the exposure route, such as the gut [48] and lungs [58].

One proposed central mechanism for MP and NP toxicity is the induction of oxidative stress, which has been extensively observed in vitro [72]. However, another study found the opposite effect, a reduction of plastic-induced oxidative stress in cells in vitro [73]. In addition, some cell types can actively excrete plastic particles [64], possibly influencing the response to oxidative stress [74]. Mice exposed to drinking water with high concentrations of MP showed impaired antioxidant defenses, such as decreased superoxide dismutase (SOD) and glutathione (GSH) expression and increased malondialdehyde (MDA) formation (a product from lipid peroxidation). In addition, increased activity of the Nrf2/Keap1 pathway was observed, suggesting plastic-induced oxidative stress and its relation with inflammation in the tissue microenvironment [69].

Regarding additives and pollutants leached from plastic particles, mice exposed to MP and NP (PS and PE) by drinking water with organic flame retardants presented more pronounced oxidative stress in the liver [70]. Testes of mice exposed to oral gavage with PE coated with phthalate esters also showed oxidative stress responses [35]. However, these effects may be due to additives released in the solution and not to MP and NP exposure, as no information was provided regarding solutions stability over time or whether they were used as fresh preparations [35, 70]. Mice exposed to a single dose of MP and NP mixtures with different sizes by oral gavage showed increased ROS generation, intestinal epithelium

apoptosis, and intestinal permeability, and pre-treatment with antioxidants reversed the effects [23].

Current studies do not indicate genotoxicity or mutagenicity of everyday plastics, as shown for PS [75]. In contrast, an in vitro study in human fibroblasts [76] and an investigation into the damage to cell-free DNA [77] indicated corresponding genotoxicity. However, other types of plastic and rodents models have hardly been investigated to confirm effects on a broader species scale.

Gastrointestinal toxicity

Plastic exposure in the intestines of mice induces local inflammation [48], alters microbiomes [78] especially favoring facultative pathogenic *S. aureus* strains [48], provokes metabolic dysfunction [49], influences liver lipid metabolism [79, 80], and modifies host–pathogen interactions [81]. Although these results seem relevant for humans [82], most effects occurred with high MP and NP doses in unspecific endpoints not simulating environmental conditions.

Changes to the intestinal microbiota contribute to metabolic disorders, including obesity and diseases such as colorectal carcinoma [82, 83]. Li and colleagues observed increased microbial load and diversity in fecal samples of mice fed with PE particles (600 µg/day for 35 days) [48]. Gut dysbiosis coincided with increased hepatic bile acid levels and altered serum bile- and amino acid-related metabolites in mice exposed to high concentrations of 5 µm PS MP (100 and 1000 µg/L) in drinking water for six weeks [49].

Hepatotoxicity

In response to oral exposure to MP and NP, multiple groups showed altered gut microbiome and disruption of serum and hepatic markers of amino acid synthesis and metabolism, energy, and lipid metabolism [49–51, 79], followed by liver inflammation [41, 69]. Hepatocellular edema and inflammatory cell infiltration were observed with increased hepatic IL-1β and TNF-α mRNA following exposure to 5 µm PS particles (20 mg/kg/day body weight) in drinking water for 30 days [69]. The extent of hepatotoxic insult was not sufficient to alter serum markers of liver function (alanine transaminase [ALT] and aspartate aminotransferase [AST]) after the exposure period. However, mice exposed to 250 nm PUR particles by oral gavage for 10 days showed increased serum ALT, alkaline phosphatase (ALP), IL-6, and TNF-α levels, followed by liver vascular congestion and hepatocytes vacuolization [84]. Accumulation quantification of fluorescent particles was hindered by extensive tissue autofluorescence, hampering to conclude whether the effects were associated with the presence of hepatic particles.

Stock and colleagues treated heme oxygenase-1 (HO-1) triple transgenic (HOTT) reporter mice with a mixture of 1, 4, and 10 µm PS particles by oral gavage [38]. These animals expressed a LacZ reporter sensitive to oxidative stress and inflammation. However, the study found no positive responses or pathological changes to the liver or other organs, possibly due to the low concentrations of particles (1.25–34.0 mg/kg body weight for particles mixture every 3 days for 28 days).

The liver is the primary site for lipid metabolism and is sensitive to pathologies such as nonalcoholic fatty liver disease (NAFLD) that manifest as an accumulation of fatty vesicles combined with elevated circulatory cholesterol and triglycerides [83]. Lipid disruption in response to MP/NP exposure in rodents has been observed by multiple groups [50, 51, 82, 85]. Luo and colleagues observed hepatic ballooning (characteristic of apoptosis), increased hepatic triglycerides, total cholesterol, and decreased PPARα and PPARγ mRNA in maternal mice after exposure to 5 µm PS MP (100 and 1000 µg/L) by drinking water during gestation and lactation [50]. Disrupted PPAR signaling and decreased hepatic triglycerides and total cholesterol were also observed in F1 offspring. The lipid-sensitive nuclear receptor PPARα regulates fatty acid catabolism and clearance and is thought to have anti-inflammatory effects (NF-κB suppression) [86]. Therefore, the extent of hepatic PPARα downregulation is predictive of NAFLD severity.

PPARγ is also downregulated during hepatic stellate cell activation, resulting in fibrosis [86]. At lower concentrations of 5 µm PS (500 µg/L), hepatic fatty vacuoles were observed in male C57BL/6 wild-type mice exposed to MP by drinking water for 28 days, without changes to hepatic triglyceride or PPARγ at the protein level [85]. This result indicates potential strain and/or gender differences, although a lack of water intake assessment may have resulted in different particle exposure between individuals. However, Lu and colleagues observed decreased liver weights and hepatic and circulatory levels of total cholesterol and triglycerides with downregulation of hepatic triglyceride synthesis in male mice exposed to 0.5 and 50 µm PS MP (100 and 1000 µg/L) by drinking water for 35 days. At the mRNA level, increased PPARα and decreased PPARγ expression were identified [79].

Changes in lipid metabolism are thought to be dependent on particle size. F1 offspring from dams exposed to 0.5 and 5 µm PS particles (100 and 1000 µg/L) in drinking water during gestation exhibited decreased hepatic total cholesterol and triglycerides in a particle dose- and size-dependent manner [51]. In addition, decreased PPARα hepatic mRNA expression was observed in groups exposed to 5 µm MP alone. Whether these effects are due to altered maternal metabolism, making offspring more

susceptible to disease, or particles transferred to the fetus directly affecting the next generation remains unclear.

Reproductive dysfunction

MP and NP have been shown to accumulate in reproductive tissues [23, 30] and cross the placental barrier [59]. Accumulation of MP and NP in testes of rodents corresponded with histological changes followed by local inflammation and DNA damage in germ cells [30, 71, 87]. Also, rodents exposed to MP and NP by oral gavage showed decreased serum testosterone levels, a hormone essential for spermatogenic cells development [30, 71, 87]. These observed effects were alleviated in male mice treated with ROS scavenging compounds because oxidative stress was induced through p38 MAPK signaling pathway activation after MP exposure [87]. This pathway is also involved in inflammation, which could explain increased levels of pro-inflammatory cytokines in testes of mice exposed to MP and NP [71, 87, 88]. Additionally, mice exposed to MP by drinking water demonstrated increased NF-κB followed by decreased Nrf2 and HO-1 in testes, suggesting this increased pro-inflammatory profile may be due to reduced Nrf2/HO-1-mediated NF-κB inhibition pathways [88].

Plastic exposure of mice dams caused far-reaching effects on milk ingress [50] and generally metabolic syndromes [51] in first and second-generation offspring of the first and second generation, regardless of sex [89]. In ovaries, exposure to MP by drinking water for 90 days reduced the number and volume of growing follicles and anti-Müllerian hormone levels and induced oxidative stress in rats [90, 91]. In addition, oxidative stress triggered cell death mechanisms, inflammation [90], and fibrosis through Wnt/β-catenin pathway activation in ovaries [91]. Changes in the uterus due to plastic exposure were also observed [92], with altered number and gender ratio of offspring of parents exposed to PE MP by oral gavage during pregnancy. However, tendencies were not dose-dependent [93].

Exposure to PE MP in dams by oral gavage during pregnancy and lactation altered the development and number of T cells in spleens in offspring of both sexes. Also, the maturation of dendritic cells was inhibited in males and enhanced in female pups [93]. Furthermore, in an allogeneic mating murine model, pregnant mice exposed to PS MP by IP administration showed increased resorption rates of embryos, decreased number and diameter of uterine arterioles, and disturbances of maternal–fetal immune microenvironment, which compromises embryos development [94].

Metabolic disorders were also observed in offspring of dams exposed to PS MP by drinking water during pregnancy [50, 51] and lactation [50]. To evaluate the long-term effects of MP and NP exposure, Luo and colleagues analyzed physiological, pathological, and metabolism indicators of adult F1 offspring (40-weeks old) of dams exposed to PS MP during pregnancy and lactation. Adult female F1 offspring showed increased lipid accumulation in the liver [50]. Furthermore, pregnant mice exposed to MP and NP by IV administration showed decreased embryo body weight, although not affecting the number of embryos [95]. In addition, mice dams exposed to 60 nm NP showed decreased placental diameter and extravasation in fetuses and placenta [95].

Neurotoxicity

Nanoplastics can cross the blood–brain barrier in a size-dependent manner [96]. Bioaccumulation, altered lipid peroxidation, and disrupted activity of neurotransmitters have been reported in the brains of marine organisms and fish [96, 97]. However, plastic-mediated neurotoxicity in rodents has been poorly investigated so far [97]. While no significant differences in cognitive function were observed in rats exposed to PS NP for five weeks by drinking water, the authors noted that the small sample size ($n=6$) and limited testing unlikely reflected subtle, transient effects [98].

Estrela and colleagues observed impaired object recognition in response to PS NP exposure, coinciding with redox changes, reduced acetylcholinesterase (AChE) activity, and accumulation of particles in the brain [22]. Nonetheless, administration of particles systemically (IP) does not reflect the first-pass effect and is not considered a relevant exposure route for environmental MP and NP. Furthermore, altered neurotransmitter activity following MP and NP accumulation was observed in organs besides the brain, such as the liver [41], highlighting the potential for particles to damage CNS function in multiple tissues. In addition, indirect effects of particle exposure, such as pro-inflammatory mediators from other accumulation sites, may also result in neurotoxicity [99].

Other effects

The potential effects of MP and NP exposure in other tissues are still poorly investigated in rodent models. For example, rats exposed daily to MP for 90 days by drinking water showed myocardium alterations, such as vascular congestion, areas with thinner muscle fibers and ruptures, and increased serum heart damage markers (CK-MB and Troponin I) [100]. Also, increased apoptosis and oxidative stress in the heart were observed, which triggered activation of the Wnt/β-catenin signaling pathway related to myocardium fibrosis [100].

Another concern is the potential toxicity in endocrine tissues caused by plastics. For example, rats exposed daily to PS NP for five weeks by oral gavage showed decreased

active forms of thyroid hormones (FT3 and FT4) in circulation and increased levels of TSH with high doses of NP, followed by changes in cholesterol serum markers and more liver damage. Hence, PS NP administration could interfere with lipid metabolism by disrupting the thyroid endocrine system [101].

The pathophysiology of chronic inflammatory diseases and co-morbidities of metabolic syndrome may be exacerbated in individuals exposed to excessive MP and NP levels. Administration of 5 µm PS particles by drinking water in a murine acute colitis model enhanced hepatic lipid disruption and intestinal barrier dysfunction [85]. Serum inflammatory markers were higher in mice with colitis than in control animals exposed to MP, indicating the potential for sensitization of individuals with substantial plastic loads to chronic diseases.

Future perspectives

New studies are continuously published regarding possible harmful effects in terrestrial mammalian organisms caused by plastic particles. However, most studies have a set of inherent challenges that need to be overcome. Considering plastic particles are found everywhere, the first challenge is the presence of contaminants during analysis. Contaminants were described in detecting plastic particles in controls, possibly from contact with air and plastic released from clothing and laboratory materials. In addition, the high diversity of plastic properties, such as insolubility to non-harmful solvents and buoyancy, can compromise the main experimental models to assess toxicity.

Another challenge is the availability of environmental plastics, like heterogeneous mixtures compared to commercially available plastics used in studies, which cannot be extrapolated to reality. This lack of studies on environmental plastics is mainly related to poor improvement in sampling, processing, and detection of plastics loads, which also compromises estimations of MP and NP doses found in the environment. This issue converges with another challenge: doses applied in many in vivo studies do not correspond to plastics concentrations found in the environment, and studies using environmentally relevant doses showed no effects, diverging from high doses experiments.

Many variables and conditions are applied in different studies designs; thus, considering multiple testing problems that could be related to data and performing proper adjustments for each case is needed for satisfactory conclusions and suggestions. Studies may use the precautionary principle as an argument for evaluating exposure to high doses of MP and NP before assessing the environmental dose. However, literature bias may occur for publications demonstrating effects, conflicting with the studies using low doses, as they might show different results or absence of effects. Furthermore, low incentives for studies with no effects may further compromise a critical debate regarding exposure to MP and NP.

Although plastics are compounds that can be in nature for a long time, longitudinal monitoring for plastic toxicology remains poorly explored. Experimental chronic models assessing only one terminal endpoint may not show effects, hence questioning the exposure period required to observe effects. Additionally, improvement in experimental designs for long-term and chronic studies may help comprehend immunogenic responses to prolonged plastic exposure.

Several knowledge gaps were addressed in this review: synergistic or antagonistic effects of particle mixtures on uptake, biodistribution, bioaccumulation, clearance, and biological responses; standardized method(s) of assessment of particle combinations or environmental plastics is vital for appropriate risk assessment of reliable exposure concentrations and time; lack of non-invasive or non-destructive estimation of particle load and biodistribution at an adequate resolution. These knowledge gaps may be filled by improving sampling, processing, and detection in optimal resolution, leading to better estimations and the development of experimental designs closer to the environment.

Conclusion

Understanding cytotoxic effects of plastic exposure requires more progress in several fields. First, standardized sampling techniques and improved characterization of environmental MP and NP are needed. Second, will there is a good body of evidence on acute plastic exposure, chronic exposure over longer time frames in higher organisms is understudied. Third, consensus on the effects and methodological tools on the presence of plastic in vertebrates in different types of organs are lacking to better understand potential relationships to chronic inflammation and disease. More research is needed to shed light on those aspects to better understand the consequences of plastic exposure in human health and environmental risks.

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Publisher's Note

ARTIGO 2

Application of sonicated polyethylene terephthalate in nano- and micro-plastic particle toxicity and genotoxicity research

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Running head: Sonicated PET as microplastic research tool

ABSTRACT

The environmental presence of nano- and micro-plastic particles (NMPs) is suspected to have a negative impact on human health. Environmental NMPs are difficult to sample and use in life science research, while commercially available plastic particles are too morphologically uniform. Therefore, developing new methods for producing heterogenous NMPs as observed in the environment is important as reference materials for research. To this end, we produced polyethylene terephthalate (PET) NMPs using an ultrasound protocol and characterized the particles by dynamic light scattering and scanning electron microscopy. Ultrasound treatment induced polymer degradation into smaller and heterogeneous PET NMPs shape fragments with similar surface chemistry before and after treatment. A polydisperse suspension of PET NMPs with 781 nm in average size and negative surface charge was generated. Then, the PET NMPs were cultured with two human cell lines, A549 (lung) and HaCaT (skin), addressing inhalation and topical exposure routes. Both cell lines interacted with and have taken up PET NMPs as quantified via cellular granularity assay. A549 but not HaCaT cell metabolism, viability, and cell death were affected by PET NMPs. In HaCaT keratinocytes, large PET NMPs provoked genotoxic effects. In both cell lines, PET NMPs exposure affected oxidative stress, cytokine release, and cell morphology, independently of concentration, which we could relate mechanistically to Nrf2 and autophagy activation. Collectively, we present a new PET NMP generation model suitable for studying the environmental and biological consequences of exposure to this polymer.

INTRODUCTION

The presence of nano- and micro-plastics particles (NMPs) in the environment is of great concern because of their potential negative impacts on human health [1]. Humans are exposed to NMPs by ingestion, inhalation, and topical contact [2-4]. Many NMP toxicity studies focused on ingestion-related models, while less is known about inhalation or topical exposure routes [1,5-8]. NMPs are constantly released into the atmosphere from different human-made processes, making respiratory uptake an important underexplored route [9]. Additionally, NMPs are in contact with skin through air, clothes and pharmaceutical/cosmetic formulations with potential consequences [4]. Yet, environmental NMPs are difficult to sample, separate, concentrate, and identify [9,10]. Hence, artificial model systems have been used to study their interactions with biological systems and other compounds [11,12]. Most reports have used monodisperse polystyrene (PS) NMPs synthesized by bottom-up colloid chemistry, producing homogenous physico-chemical compositions and morphologies that differ from environmental NMPs and could skew results [12-14]. In addition, the NMPs synthesized using bottom-up methodology could contain hazardous solvent residues used during synthesis, which may influence their interactions, affecting the biological responses [11]. Since only a few polymer types of NMPs are commercially available [15] and environmental samples are difficult to obtain [11], there is a need to develop new NMPs production methods to serve as reference materials for research on the biological effects of NMPs. A few methodologies have been used to produce NMPs with greater environmental relevance, mimicking some physico-chemical characteristics [15,16]. Surfactants addition [15], soap-free emulsion polymerization [16], laser ablation [11], and cryo-milling [17] are some of the examples. However, in some methodologies, the NMPs produced are not easily suspendable or require extensive time and preparation, such as filtration or fractionation [15,18].

Ultrasonic treatments were reported to produce NMPs in a wide size range and using lengthy (five-day) sonication fragmentation protocols [19]. It was suggested that combining the dissolution of polyethylene in toluene via sonication with subsequent emulsification in water could also be applied to other polymers if using volatile solvents [15]. Recently, it was proposed to use ultrasound under alkaline conditions for the facile and controlled production of chemically aged NMPs from disposable plastic items [20]. Here, we report a modified ultrasonic protocol to generate heterogeneous polyethylene terephthalate (PET) NMPs suspensions and studied particle characteristics and

biological effects in two human cell lines, addressing inhalation and topic exposure routes *in vitro*.

MATERIALS AND METHODS

Plastic particle preparation and characterization

To generate PET NMPs, free-additives PET film (Goodfellow Cambridge, USA) randomly cut in pieces (ca. 10 mg) was sonicated in hydrolytic conditions (10 mL, 0.1 M KOH, pH 13.0) for 15 h at 35 kHz at room temperature by an ultrasonic machine (Ultrasonic cleaner USC 500TH; VWR, Germany) (**Fig. 1a**). The resulted particles were recovered from alkaline suspension by centrifugation (3,000 rpm, 20 °C, 30 min), removal of the supernatant, and resuspension in MilliQ water. Subsequent washes were performed until pH 7.0 was reached. The sonicated PET NMPs were centrifuged, the supernatant removed, and the pellet weighed and resuspended in MilliQ water (*q.s.*) to prepare a 2 mg/mL stock suspension. For *in vitro* toxicity assessment, PET NMPs were filtered with a 0.8 µm pore size gold-coated polycarbonate filter prior to centrifugation and stock suspension preparations. PET particle surface morphology was examined before and after sonication treatment by high-resolution scanning electron microscopy (SEM; JSM-7500F; JEOL, Japan). In addition, PET NMPs' surface chemistry was investigated using X-ray photoelectron spectrometry (XPS; AXIS Supra spectrometer; Kratos Analytical, UK). Both PET film and sonicated PET NMPs were fixed on gold and steel substrates for the experiments. Amine-modified PS ($PS\text{-}NH_2$) NMPs with 50 nm diameter were purchased (Polysciences Europe, Germany) and used as positive control for *in vitro* toxicity assessment, as previously reported [21,22]. Hydrodynamic sizes, polydispersity indices, and zeta-potential of both sonicated PET and purchased $PS\text{-}NH_2$ NMPs suspensions were determined by Dynamic Light Scattering (DLS) using Zetasizer Ultra (Malvern Panalytical, Germany).

Cell culture and particle exposure design

Epithelial cell lines from human lung carcinoma A549 (ATCC: CRM-CCL-185) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Pan-Biotech, Germany) with high glucose (4.5 g/L). Immortalized human keratinocytes HaCaT (Cell Line Service, CLS300493CP) were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640; Pan-Biotech, Germany). Both cell culture media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin and streptomycin (Sigma-Aldrich, Germany). The cells were grown in cell culture flasks (Sarstedt, Germany) in a humidified incubator at 37 °C with 5% CO_2 [23]. For experiments, A549 and HaCaT cells (1×10^4 cells per well) were seeded in 96-well flat bottom plates (Nunc Edge;

NUNC, Denmark) and incubated at 37 °C for 24 h for adherence. The plates were equipped with a rim filled with deionized water to provide edge effect protection. In the following, 10× concentrated NMPs suspensions were added into wells once, reaching 1× final concentration (1, 10, and 100 µg/mL), and cells were exposed to the particles at 37 °C for 24 h, as indicated (**Fig. 1b**).

Metabolic activity

Cellular metabolism after NMPs exposure was determined using resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one), which is converted by metabolically active cells to resorufin (7-hydroxyphenoxazin-3-one) exhibiting high fluorescence [24]. After 22 h of NMPs exposure, resazurin (100 µM final concentration; Alfa Aesar, Germany) was added into wells and incubated at 37 °C for 2 h for metabolization. After an NMPs exposure time of 24 h, fluorescence intensity was measured using a multimode plate reader (F200; Tecan, Switzerland) at λ_{ex} 535 nm and λ_{em} 590 nm. After background subtraction, data normalization was performed against vehicle groups and presented as percentage (%) of metabolic activity.

Flow Cytometry

Flow cytometry experiments were performed to determine the cells' viability, intracellular ROS, and thiol content NMP exposure. Cell viability was determined 24 h after NMPs exposure using AlexaFluor (AF) 647-conjugated Annexin V and DAPI (4',6-diamidino-2-phenylindole dihydrochloride) staining (both BioLegend, The Netherlands). Intracellular ROS levels were assessed by H_2-DCF-DA staining (final concentration: 2 µM). Single-cell acquisition was performed using multicolor flow cytometry (CytoFLEX S; Beckman-Coulter, Germany). Thiol content was determined 24 h after MP exposure using a glutathione detection reagent (ThiolTracker Violet; Thermo Fisher, Germany) and Zombie NIR (BioLegend, The Netherlands) staining, and cell acquisition was performed using flow cytometry (CytoFLEX LX; Beckman-Coulter, Germany).

OECD-based cytokinesis-block micronucleus genotoxicity assay

Genotoxicity was determined by *in vitro* micronucleus assay performed according to OECD guidelines to visualize and quantify DNA damage in individual cells . Briefly, 1×10^4 HaCaT cells were seeded in 96-well flat bottom plates and allowed to attach for 18 hours. PET NMPs were freshly prepared in culture medium (10× concentrated,

10 and 1000 µg/mL) and applied into wells reaching 1× final concentration (1 and 100 µg/mL). The cells were exposed for 6 h at 37 °C in a humidified incubator (**Fig. 4a**). After treatment with PET NMPs, cells were exposed to cytochalasin B for 48 h to block cytokinesis for identifying micronuclei in cells that had passed nuclei division. Then, accutase was applied for 5 min to separate cells from each other but not from the plate [25]. The cells were fixed with 4 % paraformaldehyde (PFA; Sigma-Aldrich, Germany) at room temperature for 10 min and washed with phosphate-buffered saline (PBS). The fixed cells were permeabilized with 0.25 % Triton X-100, and the actin cytoskeleton was stained with Flash Phalloidin Red 594 (BioLegend, The Netherlands), besides nuclei staining (DAPI) for 45 min. Images were acquired using a high-content imaging system (Operetta CLS; PerkinElmer, Germany) with 20× air objective (NA 0.4; Zeiss, Germany), and the cells were segmented and quantified using Harmony 4.9 (PerkinElmer, Germany) imaging and analysis software, as previously described [25].

High-content imaging immunofluorescence analysis

Immunofluorescence was performed using a high-content imaging system (Operetta CLS; PerkinElmer, Germany). After NMPs exposure for 24 h, cells were fixed with 4 % PFA for 15 min, washed, and permeabilized with 0.25 % Triton X-100 in PBS for 20 min. Unspecific binding blocking was performed by incubating the cells with 1 % BSA for 30 min. The cells were incubated at 4 °C overnight with the following conjugated and unconjugated primary antibodies: Nrf2-AF488 (Santa Cruz Biotechnology, USA; dilution 1:150), NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) p50-AF647 (Santa Cruz Biotechnology, USA; dilution 1:500), phosphorylated-NF-κB p65-PE (Thermo Fisher, Germany; dilution 1:200), ATG5-AF488 (BioLegend, The Netherlands; dilution 1:1000), LC3B-PE (Novus Biologicals, USA; dilution 1:500), phosphorylated-mTOR (Cell Signaling, Germany; dilution 1:500), and ULK1 (Santa Cruz Biotechnology, USA; dilution 1:500). Secondary, AF647-conjugated antibodies (Thermo Fisher, Germany; dilution 1:1000) were applied accordingly at room temperature for 90 min, followed by DAPI as nuclear counterstain. The images were acquired using a 20× air objective (NA 0.4; Zeiss, Germany) and analyzed by Harmony software. After flatfield correction, cell nuclei were detected by DAPI signals, followed by cytoplasm detection using digital phase contrast images. After fluorescence background signal subtraction, the nuclear fluorescence intensities from Nrf2 (nuclear factor erythroid 2-related factor 2), NF-κB

(nuclear factor-κB) p50, and phosphorylated-NF-κB p65 (phospho-p65) were quantified. The cytoplasmic fluorescence intensities from phosphorylated-mTOR (phospho-mTOR), ULK1, ATG5, and LC3B were also quantified. Standard cell morphology measurements were investigated by calculating area, roundness, width, length, and ratio width/length. For autophagy pathway markers (phospho-mTOR, ULK1, ATG5, and LC3B), advanced morphology properties were calculated in each marker by the texture features produced in eight image filters (bright, dark, ridge, valley, spot, hole, saddle, and edge; **Fig. S1a**). The texture features calculations were also segmented into three regions of cells: inner cytoplasmic membrane, cytoplasm, and outer nuclear membrane (**Fig. S1b**).

Multiplex cytokines analyses

The inflammatory secretion profile was measured in supernatants of two cell lines exposed to NMPs after 24 h following the manufacturer's instructions using multiplex cytokine detection technology (LEGENDplex; BioLegend, The Netherlands) [26]. The bead-based sandwich immunoassay was measured using flow cytometry (CytoFLEX LX; Beckman-Coulter, Germany) targeting nine interleukins (IL1β, IL6, IL8, IL10, IL12p70, IL17A, IL18, IL23, and IL33), tumor necrosis factor-alpha (TNFα), interferon (IFN) α and γ, and monocyte chemotactic protein 1 (MCP1 / CCL2). Data analysis was performed using LEGENDplex software for target quantification.

Statistical analysis

Data were represented as mean ± standard deviation (SD) if not indicated otherwise in the figure legends. Graphing and statistical analysis were performed using *Prism* 9.5.1 (GraphPad Software, USA). Principal Component Analysis (PCA) and Spearman’s correlation analysis were also done in this software. Comparisons between more than two groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons against the untreated or vehicle control. Levels of significance were indicated as follows: $\alpha = 0.05$ (*), $\alpha = 0.01$ (**), $\alpha = 0.001$ (***).

RESULTS

Ultrasonic degradation altered surface morphology but not surface chemistry

Ultrasonic treatment of solid polymers led to a changed morphology on the polymer surface, which was examined by scanning electron microscopy (SEM) for the initial film before (**Fig. 2a I**) and after sonication (**Fig. 2a II-IV**). The images showed that the PET film became cloudy after sonication. Moreover, holes and other structures appeared on the surface. The structuring on the surface of the plastic particles suggesting that the polymer chains were broken, and increasing the sonication time broke larger particles into smaller ones. To address whether the surface chemistry of PET was altered during ultrasonic treatment, XPS was performed on PET film and PET NMPs, which were fixed in gold and steel substrates for the experiments. The PET film contained ~73 atomic % C and ~27 atomic % O, and trace amounts of Si and Ca were also detected. The elemental composition of sonicated PET NMPs samples was similar to that of the control sample (**Fig. 2b**), while trace amounts of N were detectable. In addition, the O/C ratio of the control sample was 37 % and did not change after sonication, whereas the N/C ratio of the PET NMPs increased for both substrates (**Fig. 2c**). The percentages of binding in C 1s (**Fig. 2d**) remained unchanged after sonication (~63% C-C/C harom, ~20-21 % C-O, 16-17 % COO), whereas binding in O 1s (**Fig. 2e**) changed after sonication (~53 % O-C in the control sample decreased to ~47 % in the PET NMPs, and ~46 % O=C in the control sample increased to 53 % in the PET NMPs). The binding results indicated the breaking of PET chains in the polymer and the formation of NMPs. In addition, XPS results showed that the sonication method used to prepare the NMPs did not significantly change the surface chemistry of the polymer films. After ultrasonic treatment of PET NMPs, the particle suspensions used for following *in vitro* experiments were characterized by DLS. Sonicated PET NMPs suspensions showed an average size of 781 nm, polydispersity index of 0.5, and negatively surface charged (**Tab. 1**). PS-NH_2 NMPs suspensions, selected as positive control for cytotoxicity, showed an average size of 54.46 nm, polydispersity index of 0.017, and positively surface charged (**Tab. 1**).

PET NMPs altered cell metabolism and viability

Plain, non-quantitative microscopy did not reveal major cell change upon PET NMPs exposure (**Fig. 3a**). Quantitatively, 24 h after PET NMPs exposure, A549 cells showed increased metabolic activity upon PET NMPs at high concentrations, while HaCaT

cells did not show alterations (**Fig. 3b-c**). The granularity of cells (side-scatter intensities measured by flow cytometry) exposed to NMPs can reflect the uptake of non-labeled plastic particles. Both cell lines showed slightly increased SSC intensities at high PET NMPs concentrations (**Fig. 3d-e**), indicating that both cell lines are taking up sonicated PET NMPs. Then, we investigated the cellular responses on a single-cell level using flow cytometry for viability assessment. PS-NH_2 NMPs reduced the viability in both cell lines (**Fig. 3f-h**). PET NMPs exposure slightly reduced A549 cell viability (**Fig. 3f-g**), while HaCaT cells remained unaltered (**Fig. 3h**).

PET NMPs were genotoxic in HaCaT cells

We next applied a recently established method [25] to analyze if sonicated PET NMPs exposure could induce DNA damage by evaluating the micronuclei (MN) formation in HaCaT cells using high-content imaging analysis. Briefly, HaCaT cells were exposed for 6 h to PET NMPs and methyl-methanesulphonate (MMS; as positive control for genotoxicity), followed by cytokinesis inhibition (**Fig. 4a**). The latest step is crucial, as the MN quantification must be performed in bi-nucleated cells (BNCs) only, i.e., single cells that underwent to cell division but not separation, as per OECD guidelines No. 487 [25]. Cytotoxicity was evaluated by quantifying the number of nuclei after image segmentation (**Fig. 4b**). MMS significantly reduced the number of cells, while PET NMPs exposure reduced cell count at low concentrations but modestly reduced the counts at high concentrations (**Fig. 4c**). For MN formation quantification, an algorithm-based method was used consisting of an image-segmentation-initiated detection of BNCs and counting of MN (**Fig. 4d**). PET NMPs at high concentrations reduced BNCs numbers significantly (**Fig. 4e**) and increased the relative number of MN in BNC (**Fig. 4f**), although this was not in the same magnitude as observed with MMS (**Fig. 4e-f**).

PET NMPs altered oxidative stress and inflammatory responses

Oxidative stress has been reported as an effect of NMPs exposure *in vitro* [27]. To this end, we evaluated intracellular ROS production after NMPs exposure in A549 and HaCaT cells. Intracellular ROS levels were significantly elevated with PS-NH_2 but not PET NMPs exposure in both cell lines (**Fig. 5a-b**). We next investigated the cells' antioxidant defense capacity by quantifying thiol content. Thiol levels were significantly elevated after PS-NH_2 but not PET NMPs exposure in both cell lines (**Fig. 5c-d**). By contrast, HaCaT cells exposed to PET NMPs were observed to have reduced thiol

levels (**Fig. 5d**). In response to increased ROS and reduced antioxidant capacity, Nrf2 signaling pathway may be activated. Therefore, we investigated if PET NMPs exposure could affect Nrf2 activation by high-content imaging immunofluorescence analysis (**Fig. 5e**). The nuclear translocation of Nrf2 upon PS-NH_2 NMPs was reduced only in A549 cells but not in HaCaT cells (**Fig. 5f**). By contrast, PET NMPs exposure at high concentration significantly increased Nrf2 nuclear translocation in both cell lines (**Fig. 5f**). Another effect that may occur after PET NMPs exposure is the induction of inflammatory responses by promoting inflammation mediators [27]. To address how those changes affected the inflammatory secretion profiles, we investigated cell supernatants after NMPs exposure. The profiles were significantly altered in both cell lines (**Fig. 6a**). Therefore, we investigated NF-κB activation upon NMPs exposure by high-content imaging immunofluorescence quantification (**Fig. 6b**). After image-segmentation, we quantified the activation of two members of NF-κB family, p50 and phospho-p65, by measuring their nuclear mean fluorescence intensities (**Fig. 6b**). Reduced activation of both p50 and phospho-p65 was observed upon PS-NH_2 NMPs in both cell lines, which was also the case with PET NMPs in A549 cells (**Fig. 6c-d**). By contrast, HaCaT cells showed significantly increased activation of phospho-p65 at high PET NMPs concentration (**Fig. 6d**). Intriguingly, phospho-p65 activation by PET NMPs was dose-dependent (**Fig. 8d**).

PET NMPs affected cell morphology and autophagy pathways

As PET NMPs affected cellular inflammation, we inferred whether this would have an effect on cellular morphology and autophagy, an important pathway for cell survival and adaptation. Regarding cell morphology (**Fig. 7a**), both cell types showed significant alterations upon PS-NH_2 NMPs exposure (**Fig. 7b**). However, with PET NMPs exposure, only HaCaT cell morphology was significantly affected at high concentration (**Fig. 7b**). Cellular metabolism, proliferation, and survival and adaptative responses are closely interconnected. Thus, autophagic events may occur upon NMPs exposure. Therefore, we investigated the autophagy pathway by immunofluorescence quantification using an algorithm-based imaging analysis. To understand the pathway at several points, four targets were investigated: phospho-mTOR, ULK1, ATG5, and LC3B (**Fig. 7c**). NMPs exposure differently affected autophagy in the analyzed cell regions, i.e., cytoplasm (**Fig. 7d**), inner cytoplasmic membrane (**Fig. S1c**), and outer nuclear membrane (**Fig. S1d**). It was also observed that PET NMPs exposure mainly

affected the initiation targets of autophagy, phospho-mTOR and ULK1 (**Fig. 7d; Fig. S1c-d**). Contrasting to PET, PS-NH_2 NMPs exposure mainly affected the final autophagy targets ATG5 and LC3B (**Fig. 7d; Fig. S1c-d**). Principal component analysis (PCA), calculated from autophagy markers in all cell regions for each cell line (**Fig. 8a-b**), revealed that PS-NH_2 and PET NMPs exposure in A549 cells demonstrated some similarities (**Fig. 8a**). By contrast, HaCaT cells' autophagic response was opposite upon PS-NH_2 and PET NMPs (**Fig. 8b**). In addition, no dose-dependence was observed for PET NMPs exposure regarding any autophagy marker (**Fig. 8d**) or cell regions investigated (**Fig**. **8a-b**). Moreover, interesting and different correlations were observed between the biological responses after PET NMPs exposure (**Fig. 8d**).

DISCUSSION

Environmental pollutants are considered rising environmental and human health concerns [2,4,28]. Despite their ubiquitous appearance, environmental sampling for further research in biology has proven difficult [29,30]. To overcome that, new methodologies have been developed to produce more heterogenous NMPs more suitable for risk assessment studies [11,15-17]. Here, we provide a fast generation and comprehensive biological testing protocol for PET NMPs using sonication in an alkaline solution modified from a previous report [20].

Ultrasonic degradation can be an interesting method for NMPs production once ultrasound waves generate cavities in the liquid, leading to collapse, consequently releasing energy and radicals [31,32]. In an aqueous system, oxidizing conditions are promoted by the $OH^{\bullet}$ and $H^{\bullet}$ radicals formed, generating hydrogen peroxide (H_2O_2) [20,32]. Moreover, the rapid movement of solvent molecules and the incapacity of macromolecules in the solvent to follow simultaneously promote friction [20,33]. Therefore, with the induced stress, the bonds within macromolecules eventually break [20,33]. Altogether, those conditions are used to induce polymer cleavages by the combination of physical fracture and radical polymer degradation [33]. Additionally, OH^- ions are accessible in alkaline conditions (KOH 0.1 M, pH = 13), promoting strong hydrolysis/oxidation circumstances for the freshly cleaved polymers and increasing the fragments amount detection [20]. Applying this method with slight modifications, we believe that the PET NMPs produced in our study was similar as the previous report [20]. Moreover, the similarities could include porous PET NMPs and ageing/weathering signatures [10,20]. Therefore, the sonicated PET NMPs, presenting characteristics environmentally relevant, were used for the toxicity and genotoxicity assessment *in vitro*.

Cellular metabolism and viability are important markers for toxicity assessment *in vitro*. We observed PET NMPs increased A549 cell metabolism, while HaCaT showed no significant effect. Zhang and collaborators demonstrated that A549 cells reduced viability after exposure to precipitation-produced PET NMPs with sizes ranging from 120 to 300 nm at high concentrations [34]. However, another study using the same method for PET NMPs production showed increased metabolism of murine macrophages (RAW 264.7 cells) exposed to similar concentrations of PET NMPs as used in our study [35]. NMPs interaction with cellular membranes, followed by uptake, are the first events upon particle exposure [36,37]. To address this, we evaluated the

side scatter intensities by flow cytometry to measure the cell granularity. Nonetheless, flow cytometry is unable to differentiate between the NMPs in contact with the outlayer cell membranes and those uptaken [22,38]. Despite that, side scatter intensities quantification could reflect the particle uptake, and it is suitable for free-labeled and large-sized NMPs as previous reported [22]. The free-labeled sonicated PET NMPs suspensions demonstrated an average hydrodynamic size of 781 nm, which is ideal for side scatter intensity assessment. PET NMPs uptake was observed in both A549 and HaCaT cells, more evidently at high concentrations. This is because uptake mechanisms mostly involve vesicle formation, and larger NMPs are internalized by a single vesicle [39]. Additionally, increased side scatter intensities were observed at high concentrations, inferring the high number of intracellular granules formed after NMPs exposure [22]. It was shown that fluorescent-labeled PET NMPs interacted with cellular membranes [1,40] and were taken up by cells [34,35,40]. Also, it was shown that PET NMPs are colocalized with lysosomes, supporting the role of endocytic mechanisms of PET NMPs uptake with vesicle formation, like other polymers [11].

Following NMPs interaction, cell uptake, and the different metabolic responses, we wanted to investigate if PET NMPs exposure could affect cell viability. We assessed cellular viability by flow cytometry, and only alterations in A549 cell viability were observed. That result is consistent with others using precipitation-produced PET NMPs at high concentrations [34]. In addition, a significant increase in necrosis was observed in A549 cells exposed to sonicated PET NMPs. In contrast to that, apoptotic events were affected only at low concentrations. In another study using different NMPs, increased necrosis events were observed, while apoptosis was rather affected [41]. Different mechanisms, such as oxidative stress and inflammation, can induce cell death. To address whether sonicated PET NMPs could induce oxidative stress, we evaluated intracellular ROS and thiol content after exposure. As expected, increased intracellular ROS and thiol content were observed after PS-NH_2 NMPs exposure in both cell lines, corroborating previous results [22]. Using precipitation-produced PET NMPs, other studies found increased intracellular ROS after exposure [34,35], not observed in our study. Also, reduced thiols were observed in our study, inferring less antioxidant capacity. In response to increased ROS and reduced antioxidant capacity, the Nrf2 signaling pathway may be activated [42]. The Nrf2 activation promotes the transcription of antioxidant-responsive elements to neutralize those species [42]. Here, we observed strong nuclear translocation of Nrf2 in both cell lines following PET NMPs

363 at high concentrations, indicating Nrf2 activation. That result is consistent with other
364 studies using murine primary skin and lung cells following PS NMPs exposure [43,44].
365 It was also shown that PS NMPs exposure altered the downstream targets of the Nrf2
366 signaling pathway, such as heme-oxygenase 1, superoxide dismutase 1, glutathione
367 peroxidase 2 and catalase, suggesting a protective response of cells against ROS
368 [43,44].

369 Cellular morphology is dynamically regulated by changes in cytoskeleton
370 components, which also modulate and participate in several signaling pathways and
371 cellular responses [45,46]. In primary skin and lung cells, it has been shown that
372 cellular architecture and signaling pathways were affected by PS NMPs exposure
373 [43,44]. It was previously reported that cellular morphologic alterations occurred after
374 PS NMPs exposure [37]. Thus, we examined if cell morphology was affected through
375 PET NMPs exposure. Significant cell morphology alterations were observed in both
376 cell lines after PS-NH_2 NMPs exposure, while PET NMPs only altered HaCaT cell
377 morphology at high-concentration conditions. Interestingly, cell morphology
378 parameters showed diverse correlation patterns with cell viability, oxidative stress and
379 inflammation-related parameters, inferring the crosstalk between cellular morphology
380 and biological responses through PET NMPs exposure.

381 Another possible effect of NMPs exposure is inducing inflammatory responses
382 [47,48]. The cytokines secretion in cells' supernatants was significantly altered with
383 different profiles following PET NMPs exposure in our study. Additionally, those
384 alterations were more pronounced in pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6,
385 IL-8, IL-18 and TNF-α), mainly at high-concentration conditions. Those results
386 corroborate other studies where increased pro-inflammatory cytokines were observed
387 after exposure to other NMPs [37,47-50]. Our study also demonstrated that activation
388 of NF-κB is differently affected by PET NMPs exposure. NF-κB is an important
389 transcriptor factor related to inflammation, cell proliferation, survivor and apoptosis
390 [51,52]. NF-κB family consists of protein monomer subunits, such as p50 (also called
391 NF-κB1) and p65 (also called RelA), that form homodimers or heterodimers binding
392 differently to DNA [52]. NF-κB activation is mediated by several different stimuli, such
393 as cytokines, growth factors, oxidative stress and immunoreceptors [51]. The p50:p65
394 heterodimers induce inflammatory-related gene expression, while p50 homodimers
395 may act as transcriptional repressors [53-55]. The p50 and p65 heterodimerization is
396 the main NF-κB activated form by canonical activation pathway [54]. In our study, PET

NMPs at low concentrations induced less nuclear translocation of p50 and phosphorylated-p65 in A549 cells, suggesting a reduced inflammatory response. Decreased IL-6 levels were observed in A549 cells in our study after PET NMPs exposure. That may be due to the regulatory role of NF-κB in IL-6 expression [56]. By contrast, PET NMPs at high concentrations significantly increased the nuclear translocation of phosphorylated-p65 in HaCaT cells. Additionally, the nuclear translocation of phosphorylated-p65 was dose-dependent and strongly positively related to IL-18 levels, suggesting the increased pro-inflammatory response. It has been reported that oxidative stress-induced NF-κB phosphorylation leads to NRLP3 inflammasome activation and pyroptosis [57]. It was reported that PS NMP exposure in rats ovarian granulosa cells induced NRLP3 inflammasome increased caspase-1 activity, consequently, cleavage of pro-IL-1β and pro-IL-18 in IL-1β and IL-18 [57]. Interestingly, in our study, a strong positive correlation between oxidative stress and inflammation was observed by nuclear translocation of Nrf2 and IL-18 levels. Also, a strong negative correlation between thiol content and pro-inflammatory cytokines secretion (IFN-γ, TNF-α and IL-17A). Those results may support the role of oxidative stress-mediated inflammation in PET NMPs exposure.

Genotoxicity assessment has been used as an important biomarker for toxicological effects evaluation through NMPs exposure *in vitro* [58]. Due to many mechanisms, DNA damage may promote mutation, carcinogenesis, cell death and other health impacts [59]. A previous study, using the comet assay for DNA breaks detection, reported that PS NMPs exposure induced DNA damage, mainly associated with oxidative stress, but those effects were cell-line dependent [59]. Another study observed DNA breaks in Caco-2 and HepG2 cell lines after exposure to PET NMPs at high concentration conditions [60]. Differently, precipitation-produced PET NMPs exposure did not induce DNA damage or oxidative stress [40]. Regarding micronucleus formation, another assay that measures the chromosome breaks and/or chromosome loss to detect genotoxic effects, increased MN formation was observed in human cell lines exposed to PS [58] and polyethylene NMPs [61]. Interestingly, our results showed increased MN counts at high concentrations of sonicated PET NMPs, while low concentrations showed no significant alteration. This could be due to prolonged exposure to several NMPs causing changes in the cell membrane, leading to cells' vulnerability to undergo cell division properly, causing cell cycle arrest and possibly cell death [62]. Similarly observed for 1 μm PS NMPs exposed HaCaT cells [25], the BNC

(binucleated cell) count was significantly reduced at high concentrations of PET NMPs (100 µg/mL), suggesting also cell cycle arrest. Additionally, at low concentrations (1 µg/mL), sonicated PET NMPs could not induce any change in BNC count in our study. That result corroborated with a study using grinded-food containers of PET NMPs at a similar concentration range (4-8 µg/mL), where no alterations in cell cycle phases were observed [60].

Autophagy is a highly conservative process of regulated cellular organelles and molecule degradation that occurs physiologically or can be induced by starvation, oxidative stress, toxic compounds, and diseases [63]. Autophagy is critical in cell growth, survival, adaptation, and death [64]. Therefore, the potential importance of autophagy in NMPs toxicity could be the adaptative and protective capacity into stress conditions or cell death promotion [64,65]. Several proteins regulate and participate in autophagy steps, from engulfment of cytoplasmic undesired content forming the autophagosome to lysosome fusion forming the autophagolysosome with consequent degradation [63,64]. Autophagy is mainly regulated by the mammalian target of rapamycin (mTOR), a cellular metabolism sensor consisting of two complexes, mTORC1 and mTORC2, playing different roles in cell signaling [66,67]. The mTORC1 is mainly localized in the cytoplasmic side of lysosomes, controlling their function in recycling unwanted content [66]. In mTORC1 activated form, the phosphorylation of autophagy-related gene (ATG) proteins, ULK1 and ATG13, leads to autophagy inhibition [66]. ULK1 (UNC-51-Like Kinase 1) complex activation is necessary to initiate autophagosome formation [67,68]. ULK1 complex phosphorylates and activates other complexes and proteins involved in signal transduction, autophagosome elongation and maturation [67,68]. For instance, the ATG12-ATG5 and ATG8-LC3-PE (phosphatidylethanolamine) complexes, downstream autophagy factors of ULK1 activation, participate in autophagosome elongation and maturation [63,68,69]. The importance of the ATG12-ATG5 complex is acting as an ubiquitin-like conjugation system E3-like enzyme that promotes the LC3 (microtubule-associated light chain 3) system, and each complex components act differently [63]. Independently of the LC3 system, the ATG12-ATG5 complex can bind directly to the autophagosome membrane and ATG5 alone [63]. Also, the ATG12-ATG5 complex is involved in the autophagosome and lysosome fusion by promoting the binding with TECPR1 located in the lysosome membrane [63]. The ATG8-LC3-PE conjugation system is involved in the autophagosome membrane lipidation of the LC3 family (LC3-A, LC3-B and LC3-

C) to PE decoration and membrane closure [69]. Importantly, the conversion of LC3 (LC3-I) to LC3-PE (LC3-II conjugated form) is a protein marker for autophagy [65,70]. Also, LC3 is an important protein in mature autophagosome intracellular trafficking to lysosomal fusion by interaction with microtubules and recruitment of motor adaptor proteins [69].

Previous studies have reported that PS NMPs exposure in HUVEC cells increased LC3-II, inferring autophagy induction [41,71]. Also, human intestinal epithelial cells (HIEC-6) exposed to PS NMPs showed increased expression of LC3-II and p62/sequestosome 1 (SQSTM1) [65]. The p62 is an adaptor protein important in the selective autophagic protein degradation, although, autophagic degradation inhibition was observed in PS NMPs exposed to HIEC-6 cells by increased p62 expression and aggregation [65,72]. Similarly to PS NMPs, human bronchus epithelial cells (BEAS-2B) exposed to PS-NH_2 NMPs showed increased expression of LC3 and p62 [73]. Our study assessed the autophagy signaling pathway in the regulation, initiation, elongation and maturation steps. Interestingly, PS-NH_2 NMPs exposure induced different alterations in autophagy-related markers (phospho-mTOR, ULK1, ATG5 and LC3-B) related to the cell line. Moreover, the most pronounced alteration was the increased ATG5 and LC3-B in both cell lines, suggesting a role in the final autophagy steps. Sonicated PET NMPs also induced different alterations in autophagy-related markers concerning the type of cell line, although, independently of concentration. In contrast to PS-NH_2 NMPs, PET NMPs induced alterations were more pronounced in decreased phospho-mTOR and ULK1, suggesting a role in the initial autophagy steps.

Curiously, PET NMPs interaction and uptake showed a strong negative correlation with ATG5 and a strong positive correlation with LC3B. That may be due to endocytic mechanisms of PET NMPs internalization and accumulation into lysosomes [11], and both ATG5 and LC3B are involved in the autophagy-lysosome fusion [69]. Also, PET NMPs exposure may cause lysosome and mitochondrial dysfunction, consequently inducing autophagy, cell cycle arrest and cell death [64,73]. Moreover, autophagy-related markers showed different correlations with oxidative stress and inflammation parameters upon PET NMPs exposure. Interestingly, Nrf2 nuclear translocation was negatively correlated with ATG5, which may be due to non-canonical activation-mediated by autophagic degradation of Keap1 [72]. The p62-mediated Keap1-autophagic degradation may result in prolonged Nrf2 activation [64,72], which could be the reason for increased Nrf2 activation in PET NMPs at high concentrations.

Regarding inflammation-related parameters, IL-10 was strongly negatively correlated with phospho-mTOR in our study. Possibly due to IL-10-mediated Akt activation and, consequently, phosphorylation of mTOR, inhibiting autophagy [74]. Additionally, it was observed in our study negative correlation between ULK1 with IL-1β and ATG5 with IL-18 and IL-33, despite a positive correlation between LC3B with IL-18 and IFN-a2. The IL-1 cytokines family, such as IL-1β, IL-18 and IL-33, are produced as precursor proteins, pro-IL-1β, pro-IL-18 and pro-IL-33 [75,76]. The caspase-1 cleavage of pro-IL-1β and pro-IL-18 into active forms is regulated by inflammasome activation [75,76]. IL-33 maturation is independent of caspase-1 cleavage and is mediated by other intracellular proteases [77,78]. Autophagy plays an important role in the regulation of IL-1 family cytokines cleavage by promoting ubiquitinated inflammasome degradation, which is crucial for caspase-1 activity [76]. On the other hand, the signaling cascade of the IL-1 cytokines family activates NF-κB and p38 MAPK (mitogen-associated protein kinase), resulting in inflammatory response and autophagy inhibition [75,79]. In IFN type I, such as IFN-a2, the signaling cascade targets mainly JAK (Janus kinase) and STAT (signal transducers and activators of transcription) proteins involved in cell proliferation and immunomodulation [80]. Besides that, IFN-a2 may activate other kinases like phosphoinositide 3-kinases (PI3K)-Akt and p38 MAPK signaling pathways involved in cell death and survivor responses [80], suggesting a role in autophagy as well by regulating mTOR activity [64,81]. Therefore, the autophagy triggered by NMPs exposure may exert a critical role in cellular adaptation upon stress conditions, but also in an attempt for NMPs degradation, or by regulating key pathways involved in survival or cell death.

Our results suggested that PET NMPs produced by ultrasound treatment is an interesting methodology to generate NMPs relevant for toxicological assessment. Moreover, sonicated PET NMPs demonstrated crutial physico-chemical properties similar to NMPs found in nature, such as diverse shapes and a polydisperse suspension. Thus, using those particles for toxicity assessment bring also an environmental relevance, evenmore, if extrapolated to *in vivo*. In our study, sonicated PET NMPs were uptaken by two human cell lines independently of concentration. In addition, acute PET NMPs exposure affected modestly cell metabolism, viability, oxidative stress, inflammation-related and autophagy-related parameters. Also, the major effects, such as in genotoxicity, were observed under high concentration, which is unlike to be environmentally relevant. Besides the biological responses were

533 independently correlated with PET NMPs concentration, they were dependently
534 associated with cell line. Hence, our study suggests that different biological responses
535 are likely when other cell lines are exposed to sonicated PET NMPs, evenmore, if
536 considered increased exposure times.

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544 **CONFLICT OF INTEREST**

545 The authors have no conflict of interest to declare.

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LEGENDS

Table 1. Characteristics of nano- and micro-plastic particles (NMPs) suspensions. NMPs hydrodynamic size, polydispersity and zeta-potential of sonicated PET and purchased PS-NH_2 particle suspensions are shown. PET: polyethylene terephthalate; PS-NH_2: amine-modified polystyrene

Figure 1. Study setup. (**a**) PET production and characterization design protocol; (**b**) NMPs exposure design protocol.

Figure 2. PET NMPs morphologic and chemical characterization. (**a**) scanning electron microscopy images of PET before (I) and after (II-IV) sonication; (**b-e**) XPS results of PET ctrl and sonicated-NMPs (**b**) elemental fraction, (**c**) elemental ratio, (**d**) binding in C 1s and (**e**) binding in O 1s. Results were presented as mean ± SD. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test (ns: non-significant; *p<0.05, **p<0.01, and *** p<0.001).

Figure 3. Cellular metabolism, granularity, and viability. (**a**) brightfield channel and DAPI (blue) staining representative images of cell lines exposed to NMPs after 24 h (scale bars: 100 μm); (**b**) resazurin-based metabolic activity assay plate representative image of HaCaT cells exposed to NMPs after 24 h; (**c**) metabolic activity of cell lines exposed to NMPs after 24h; (**d**) representative side scatter (SSC) histograms of A549 and HaCaT cells exposed to NMPs; (**e**) NMP interaction (SSC intensity) of cell lines exposed to NMPs; (**f**) representative density-plots of A549 cells exposed to NMPs after 24 h; (**g-h**) viability of A549 (**g**) and HaCaT (**h**) cells after NMPs exposure. Results were normalized to vehicle control and presented as mean ± SD. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test (*p<0.05, **p<0.01, and *** p<0.001).

Figure 4. Toxicity and genotoxicity testing in HaCaT cells after PET NMPs exposure. (**a**) particles exposure scheme; (**b**) representative images of HaCaT cells for genotoxicity assessment; (**c**) number of nuclei after methyl-methanesulphonate (MMS) and PET NMPs exposure; (**d**) representative images of algorithm-based imaging analysis segmentation for genotoxicity assessment; (**e**) number of bi-nucleated cells (BNC) and (**f**) number of micronuclei (MN). Results were normalized to vehicle control and presented as mean ± SD. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test (*p<0.05, **p<0.01, and *** p<0.001). Scale bars: 50 μm.

Figure 5. PET NMPs exposure affected redox balance in human cells. (**a-b**) intracellular ROS histograms (**a**) and heat-map (**b**) of human cell lines exposed to NMPs after 24 h; (**c-d**) thiol content histograms (**c**) and heat-map (**d**) of human cell lines after NMPs exposure; (**e-f**) Nrf2 immunofluorescence representative images (**e**) and nuclear Nrf2 staining algorithm-based imaging quantification (**f**). Results were normalized to vehicle control and presented as mean ± SD or only mean (heat maps). Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test (*p<0.05, **p<0.01, and *** p<0.001). Nrf2 staining (green), cytoplasm (white), nuclei (blue). Scale bars: 100 μm.

Figure 6. Inflammation responses were differently altered upon NMPs exposure. (**a**) volcano plots showing cytokine release profiles 24 h after NMPs exposure normalized to vehicle control; (**b**) NF-κB p50 (red) and phosphorylated-p65 (orange) immunofluorescence representative images; (**c-d**) algorithm-based imaging quantification of nuclear p50 (**c**) and phosphorylated-p65 (**d**). Results were normalized to vehicle control and presented as mean ± SD. Statistical analysis was performed by one-way ANOVA with

Dunnett's multiple comparison test (*p<0.05, **p<0.01, and *** p<0.001). Cytoplasm (white) and nuclei (blue) staining. Scale bars: 100 µm. FDR = false discovery rate; FC = fold-change

Figure 7. Cell morphology and autophagy-related markers were affected by NMPs exposure. (**a**) digital Phase contrast (DPC; grey) channel and DAPI (blue) staining representative images of cell lines exposed to NMPs; (**b**) volcano plots of standard morphology measurements of cell lines after NMPs exposure; (**c**) representative images of autophagy-related markers (phosphorylated-mTOR, ULK1, ATG5, and LC3B) immunofluorescence in A549 cells and nuclei in blue (DAPI staining); (**d**) volcano plots of cytoplasmic SER texture features algorithm-based imaging analysis from autophagy-related markers immunostaining. Results were normalized to vehicle control. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test. FDR = false discovery rate; FC = fold-change

Figure 8. Metadata analysis. (**a-b**) principal component analysis (PCA) calculated from autophagy-related markers and SER texture feature (bright, dark, ridge, valley, spot, hole, saddle, and edge) quantifications and cell regions (inner cytoplasmic membrane, cytoplasm, and outer nuclear membrane) in A549 (**a**) and HaCaT (**b**) cells; (**c**) PCA calculated of all biological results obtained after 24 h of NMPs exposure; (**d**) Spearman's R correlation matrix of PET NMPs data set. Levels of significance were *p<0.05 and **p<0.01.

Supplemental Figure S1. Autophagy-related markers from algorithm-based imaging analysis. (**a**) representative magnified images of SER texture features filters (bright, dark, ridge, valley, spot, hole, saddle, and edge) applied to ATG5 (green) immunostained A549 cells (nuclei: blue; scale bars: 20 µm); (**b**) representative magnified images of HaCaT cell regions (inner cytoplasmic membrane, cytoplasm, and outer nuclear membrane) used for SER texture feature quantification of each autophagy-related marker (scale bars: 50 pixels); (**c-d**) volcano plots of inner cytoplasmic membrane (**c**) and outer nuclear membrane (**d**) SER texture feature from algorithm-based imaging analysis of autophagy-related marker immunostaining. Results were normalized to vehicle control. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test. FDR = false discovery rate; FC = fold-change

854 **TABLES**

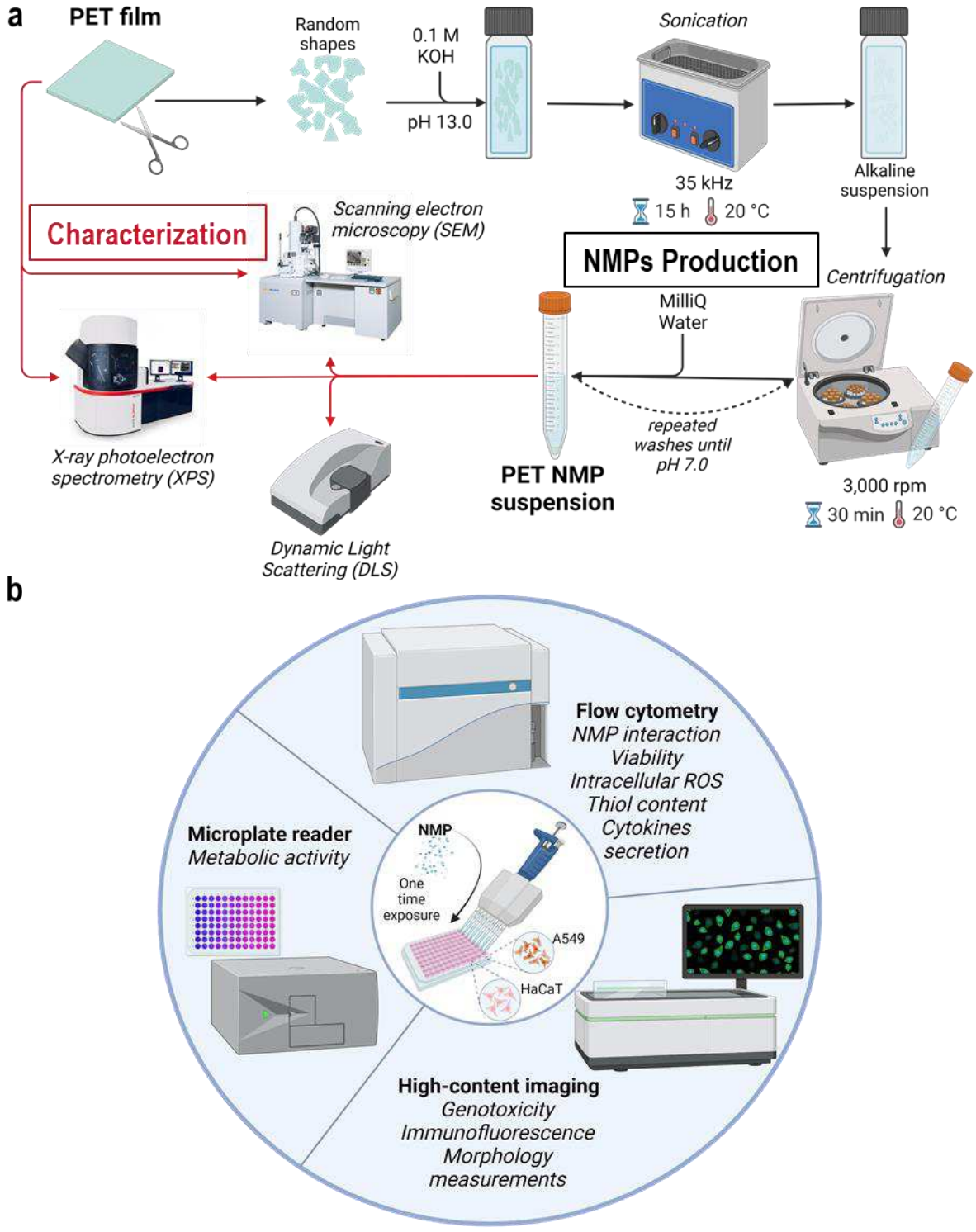
855 *Table 1*

Particle	Z-average diameter size (nm mean ± SD)	Polydispersity Index (mean ± SD)	Zeta-potencial (mV mean ± SD)
PET (sonication)	781.0 ± 153.0	0.500 ± 0.100	-62.00 ± 1.000
PS-NH_2 (purchased)	54.46 ± 0.289	0.017 ± 0.019	+34.55 ± 3.049

856

857 **FIGURES**

858 *Figure 1*



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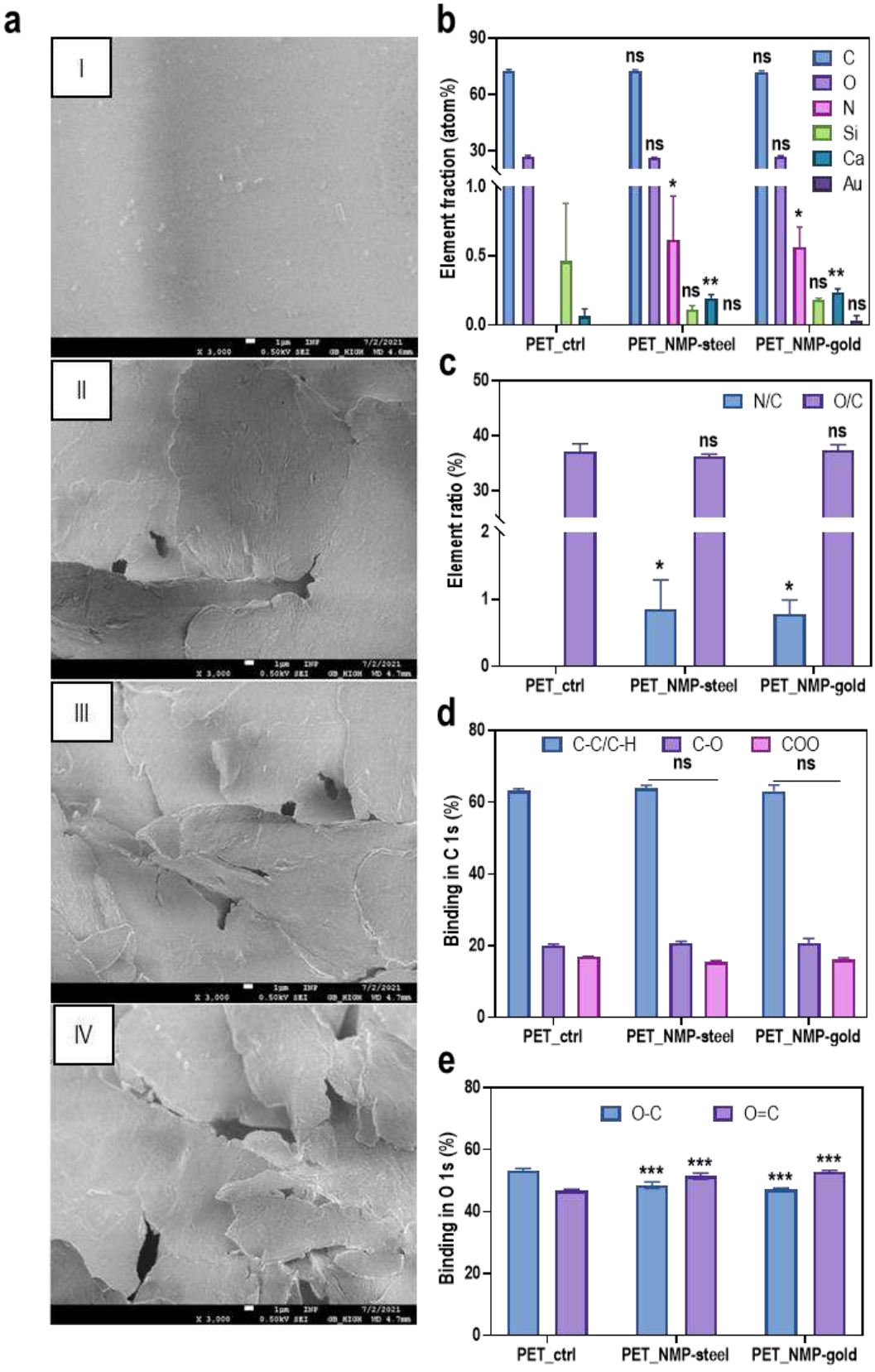

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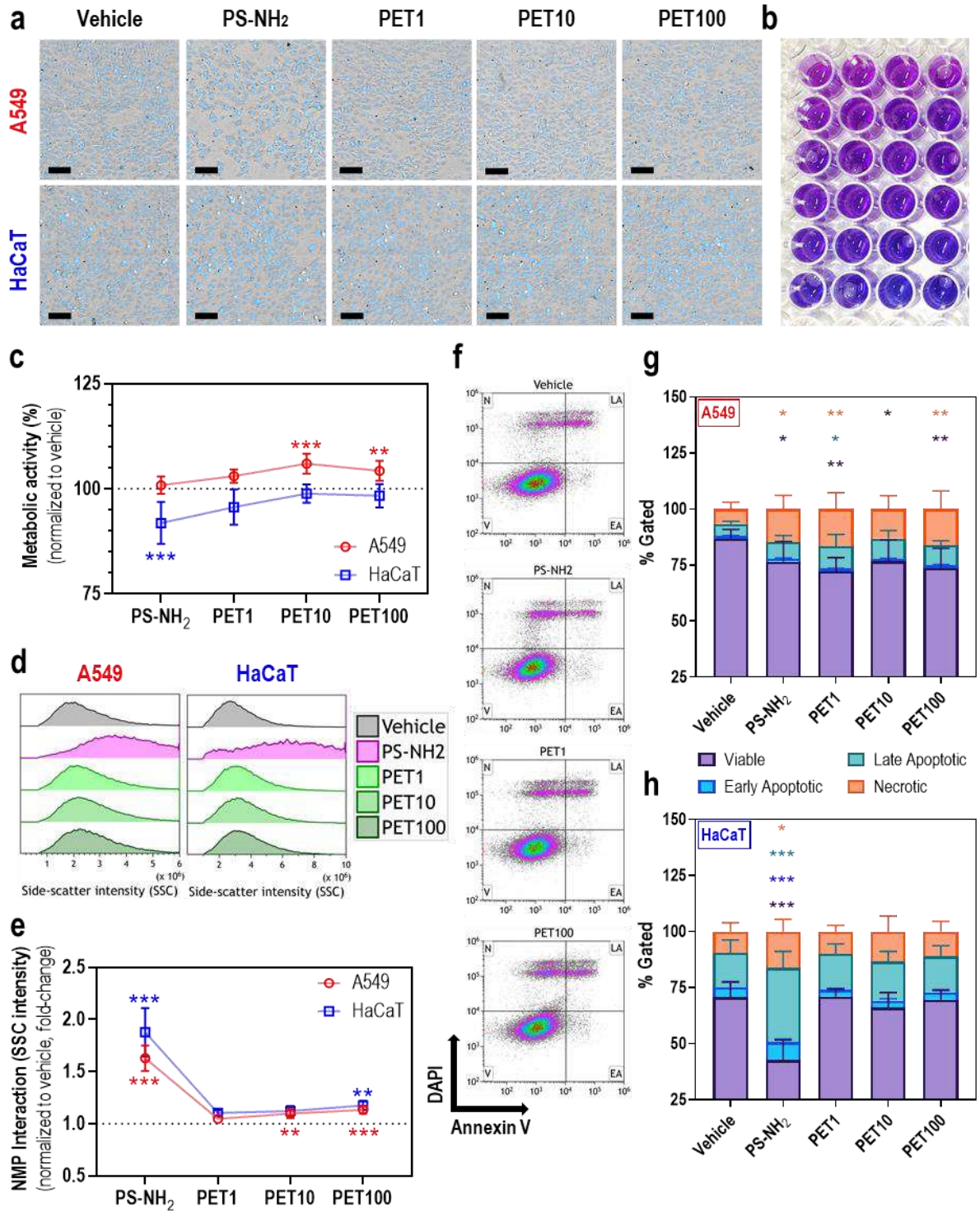

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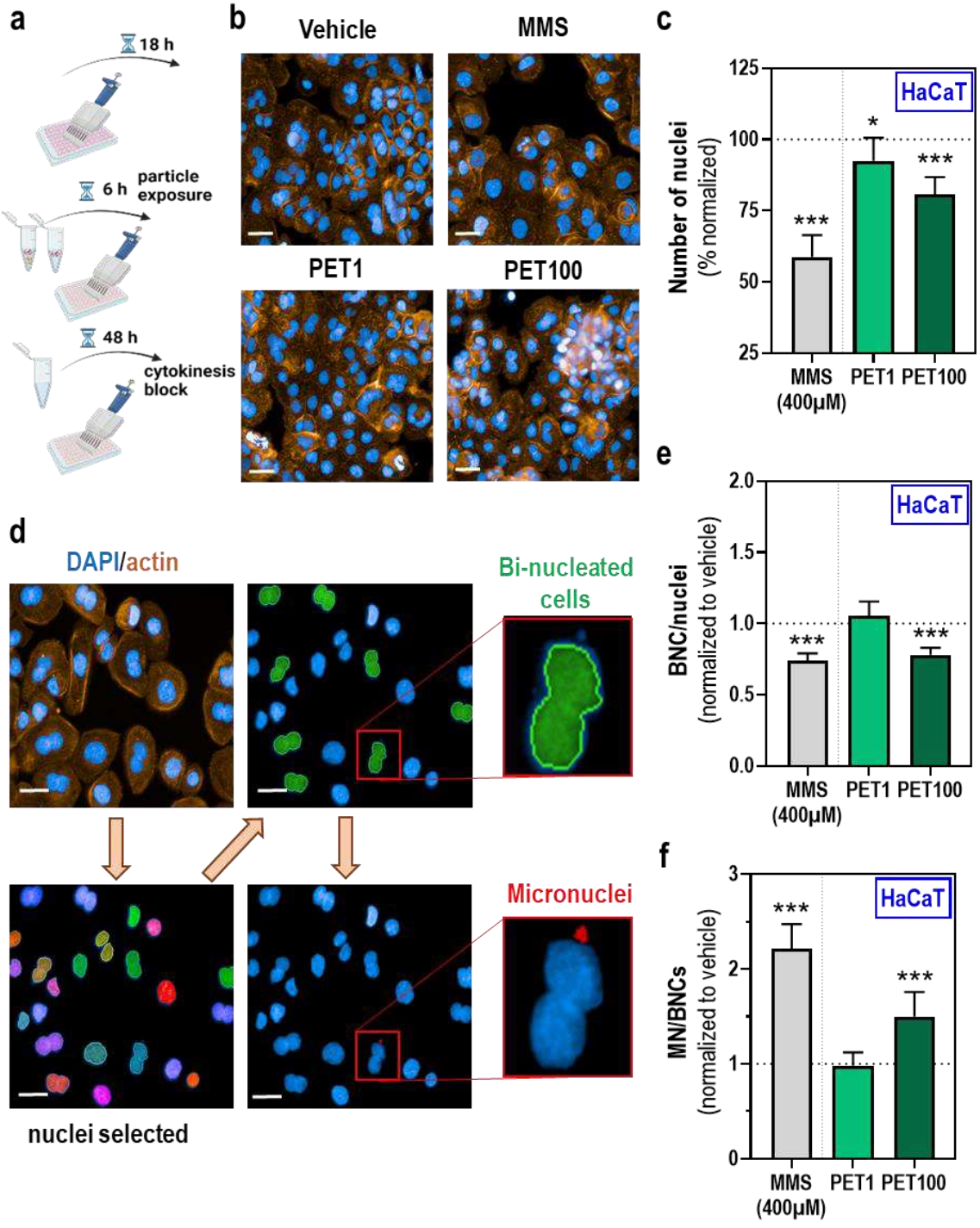
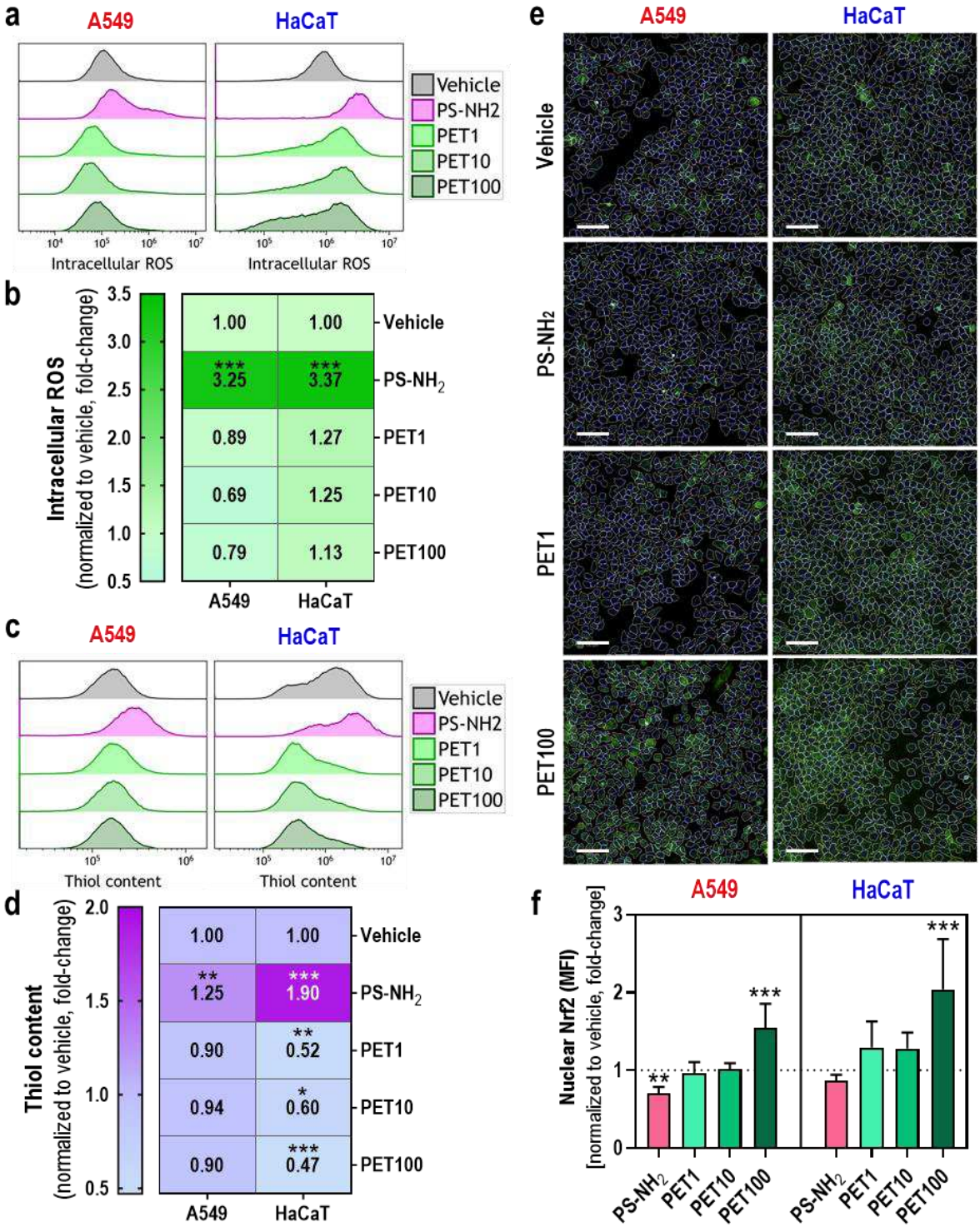
Figure 4

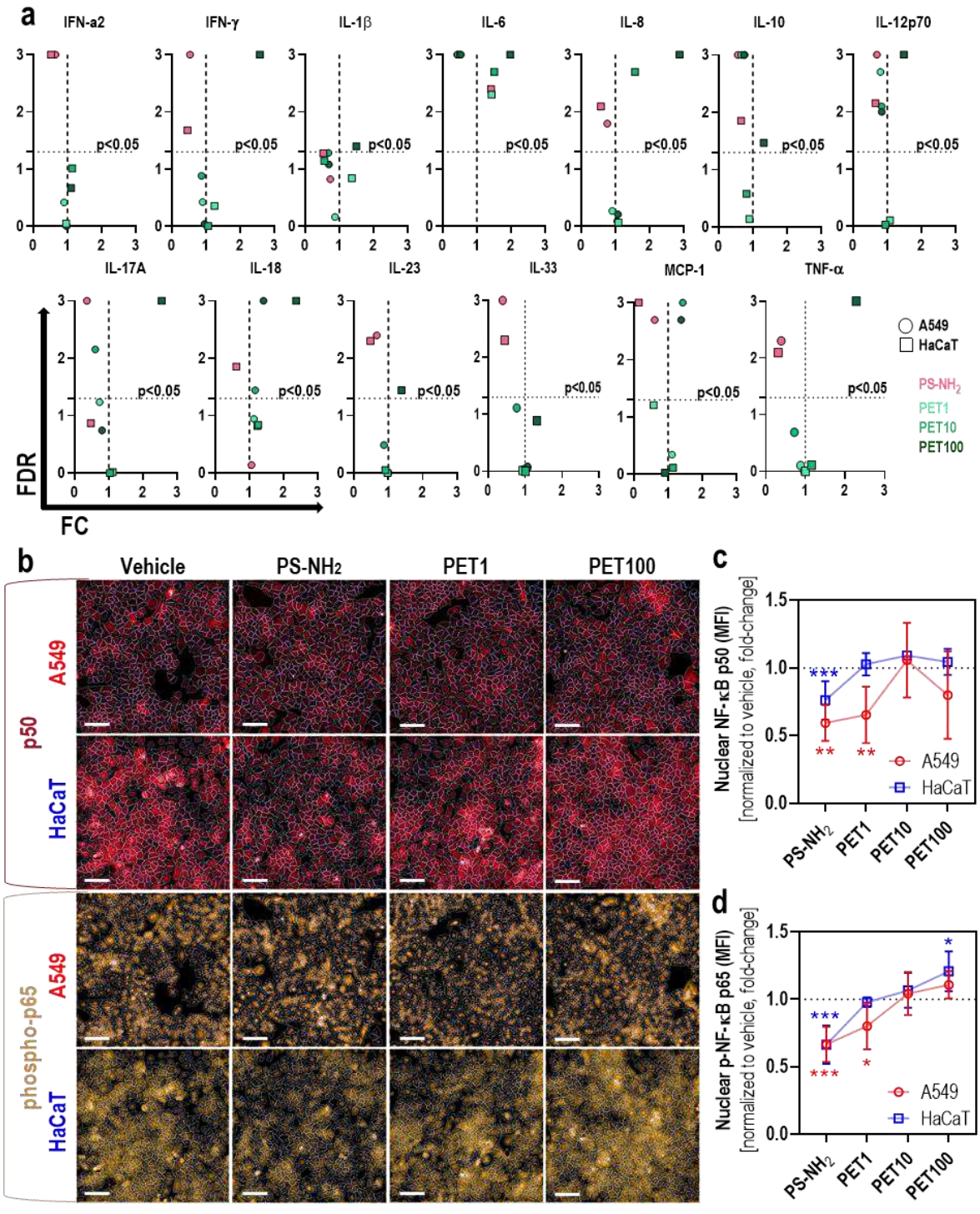

a
18 h
6 h
particle exposure
48 h
cytokinesis block
b
Vehicle
MMS
PET1
PET100
c
HaCaT
Number of nuclei (% normalized)
125
100
75
50
25
*
***
***
MMS (400µM)
PET1
PET100
d
DAPI/actin
Bi-nucleated cells
Micronuclei
nuclei selected
e
HaCaT
BNC/nuclei (normalized to vehicle)
2.0
1.5
1.0
0.5
0.0
***
***
MMS (400µM)
PET1
PET100
f
HaCaT
MN/BNCs (normalized to vehicle)
3
2
1
0
***
***
MMS (400µM)
PET1
PET100

866 *Figure 5*



867

868 *Figure 6*



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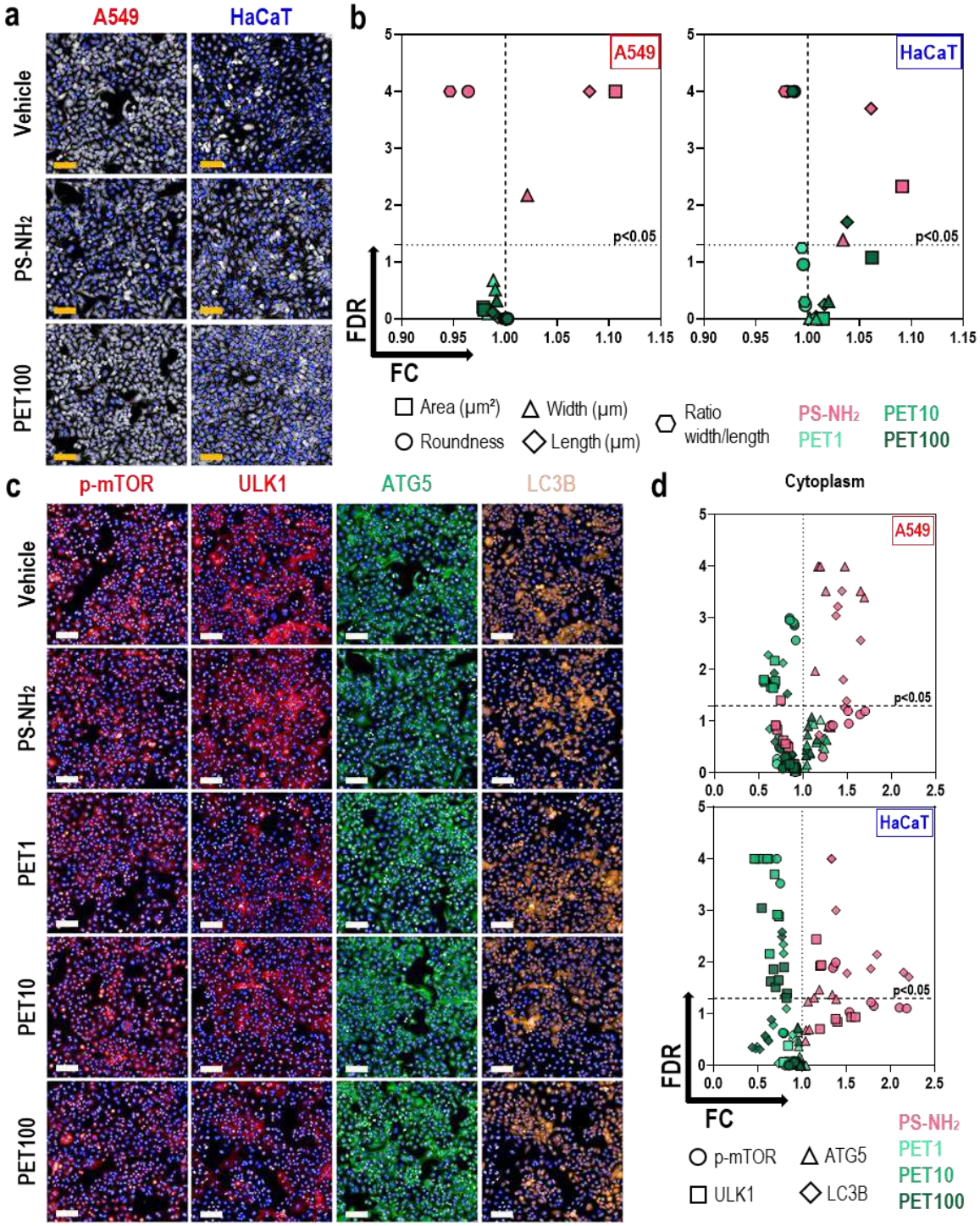

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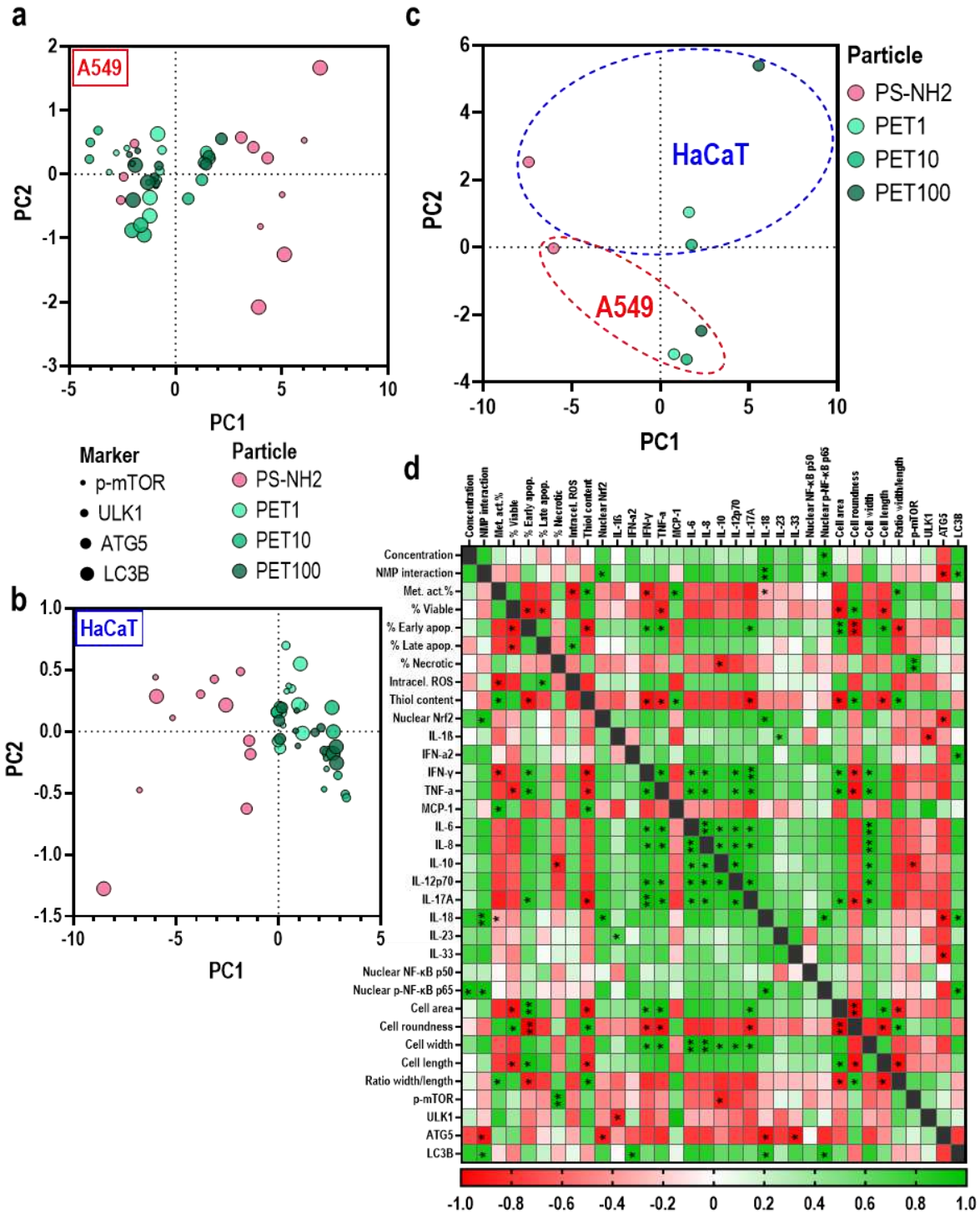
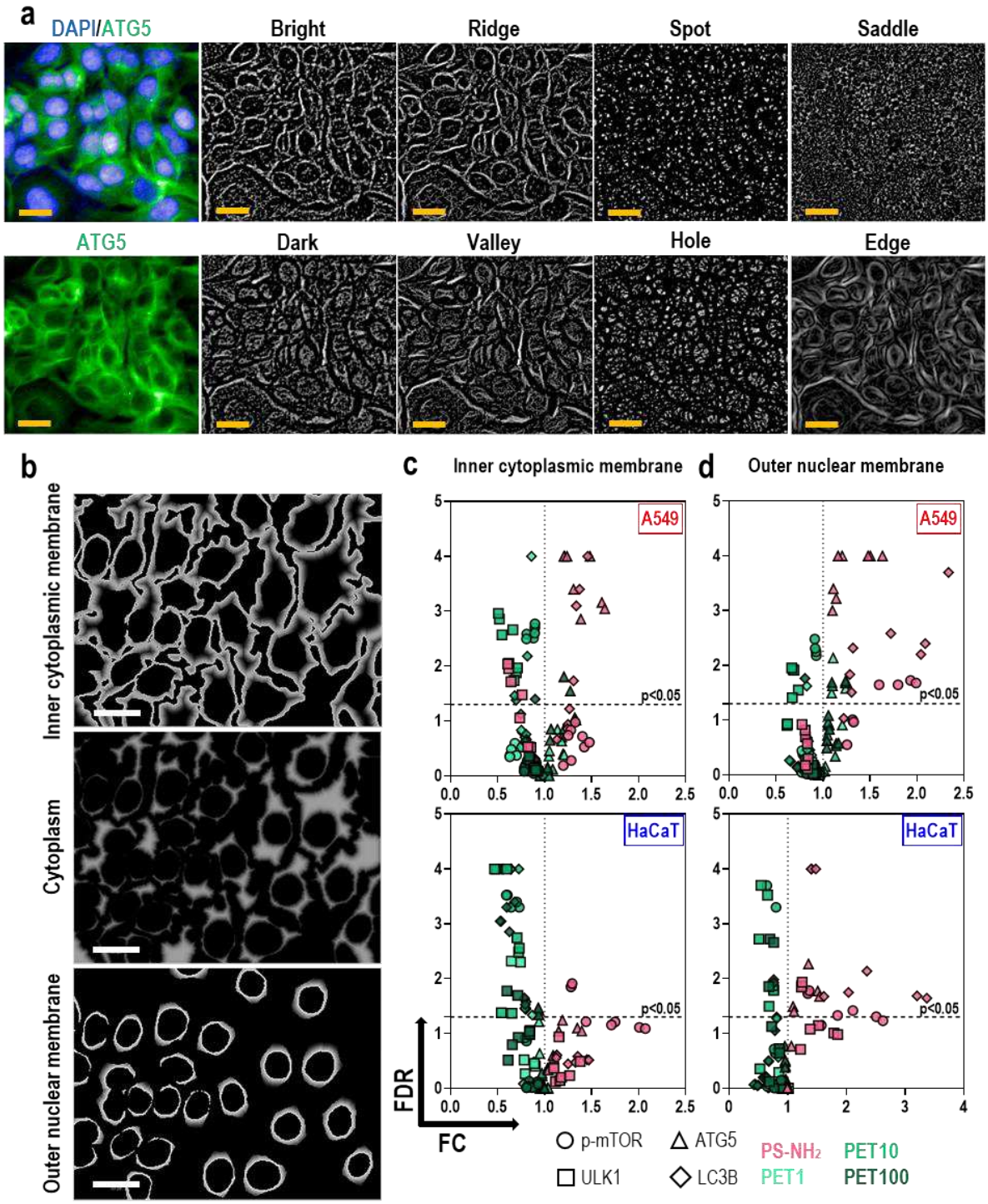
872 *Figure 8*



873

Supplemental Figure S1



4 CONCLUSÃO

- Segundo a literatura, os modelos de roedores mais utilizados são camundongos e ratos, que foram submetidos à exposição de NMPs principalmente pela ingestão oral direta (gavagem) ou NMPs suspensos na água para beber, mas também foram realizados as via inalatória, intraperitoneal e intravenosa, em diferentes desenhos de estudo;
- O polímero mais utilizado foi o PS, pela sua alta disponibilidade comercial, baixo preço de mercado e facilidade em ser suspendido em soluções aquosas, no entanto, outros polímeros como PE e PUR também foram utilizados;
- NMPs de diversos tamanhos foram utilizados nos modelos animais, e os efeitos biológicos mais observados após exposição foram a bioacumulação nos tecidos, inflamação, estresse oxidativo, alterações na microbiota, no metabolismo, disfunção reprodutivas e endócrinas;
- Os NMPs produzidos pelo tratamento por ultrassom em solução alcalina apresentou características fisico-químicas similares as encontradas na natureza e permitiu a investigação os efeitos biológicos *in vitro* da exposição aos PET-NMPs em duas linhagens de células humanas de duas rotas de exposição pouco investigadas, a inalatória (A549) e tópica (HaCaT);
- A exposição aguda de células aos PET NMPs diferentemente afetou as respostas biológicas de cada célula utilizada no estudo, onde ambas células internalizaram PET NMPs, apresentaram alterações no estresse oxidativo, secreção de citocinas e ativação de autofagia, além de diferentes respostas metabólicas e de viabilidade celular, de forma independente da concentração de NMPs.

5 CONSIDERAÇÕES FINAIS

Este trabalho permite a reflexão sobre a utilização de plásticos de maneira descontrolada e sua alta produção a longo prazo podem trazer potenciais danos não apenas para o meio ambiente mas para nós mesmos. O que antes era pensado ser inerte, hoje já se sabe que a exposição aos NMPs pode gerar respostas biológicas mesmo que brandas dependendo da concentração aplicada. No entanto, em um pior cenário, os efeitos são muito evidentes, ainda mais se pensarmos no acúmulo de NMPs ao longo das gerações e pouco se sabe sobre os efeitos a longo prazo. Se torna ainda mais desafiador os estudos toxicológicos, uma vez que é necessário um aprimoramento analítico, de metodologias e de novas tecnologias para que a detecção dos NMPs de sistemas mais complexos como os tecido animais. O desenvolvimento de metodologias para a produção de NMPs com características relevantes ambientalmente é de extrema importância para os estudos toxicológicos, uma vez que ainda é muito difícil de obter amostras de NMPs provenientes da natureza com qualidade ou até mesmo reprodutibilidade. Vale destacar a importância de se avaliar doses mais relevantes aquelas da natureza, mesmo que não se possa identificar os efeitos biológicos, permitindo um debate na comunidade científica sobre o real risco da exposição causado pelos NMPs.

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