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Cytotoxic and dysmetabolic impact of polystyrene nanoplastics, a new potential atherosclerotic cardiovascular risk factor, on a steatosis model of HepG2 cells

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ABSTRACT

The widespread presence of nanoplastics (NPs) in the environment has recently raised concerns regarding the human health. More specifically, adverse effects related to NP exposure and potentially associated with the occurrence and progression of cardiometabolic diseases, including atherosclerotic cardiovascular disease (ASCVD) and metabolic dysfunction-associated steatotic liver disease (MASLD), are currently under investigation. To understand the toxic and dysmetabolic effects induced by NPs in the liver, a major player in cardiometabolic health, we aimed at characterizing the cytotoxic effects induced by polystyrene NPs (PS-NPs) of 500 nm in human hepatocarcinoma HepG2 cells. PS-NPs tested at concentrations of 10, 100, and 200 μg/mL reduced HepG2 cell viability. Intracellular PS-NP content increased according to exposure time and concentration. Moreover, exposure to 500 nm PS-NPs altered glucose uptake after 24 hour-NP exposure (200 μg/mL). This study may contribute to unveil the PS-NP involvement in the pathological mechanisms associated with liver diseases, including MASLD.

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Keywords
Polystyrene nanoplastics; MASLD; ASCVD; cytotoxicity; glucose uptake

Impact of nanoplastics on human health

The intensive industrial development of the last decades brought important changes such as the use of plastic products in our every-day lives [1]. Nowadays, the production of plastics is still increasing [2], as well as the inappropriate recycling and littering of plastic products into the environment [3]. This has led to their degradation under the action of atmospheric agents and the consequent releasing of micro- and nano-plastic fragments throughout the environment and its living organisms [4]. Thereby, humans are subject to microplastics (MPs) and nanoplastics (NPs) exposure both directly and indirectly [5]. Particularly, the constant uncontrolled exposure to NPs has recently raised concerns about the environmental, animal, and human health undermining the One Health approach [6].

A growing body of evidence have shown the presence of MPs and NPs in different human-derived biological matrices, such as blood [7], urine [8] and breast milk [9], as well as in human tissues and organs like testicles [10], ovaries [11], placenta [12], lungs [13] and liver [14].

Moreover, many studies have suggested a role for MPs and NPs in hemolysis and impairment of the coagulation system [15], reproductive function [16] and cardiovascular system [17]. Plastic particles have also been found in human-derived surgically excised carotid artery plaques and correlated with higher risk of myocardial infarction, stroke and death [18]. Indeed, polystyrene-derived NPs (PS-NPs) were also found in the aorta of ApoE $^{-/-}$ mice where they exacerbate the stiffness of the artery and facilitate the formation of the atherosclerotic plaques [19]. Moreover, it was observed that PS-NPs can induce metabolic alterations in macrophages leading them to turn into foam cells, as also usually detected into atherosclerotic plaques [20].

In addition, several studies have investigated the wide spectrum of toxic effects using *in vivo* and *in vitro* animal models [5, 21], and human-derived cell lines [22]. These studies highlighted events like accumulation of plastic particles with different sizes in liver, kidneys and gut, cytotoxicity, DNA damage, increased oxidative stress, inflammation and immune response, neurotoxicity, energy and metabolic disruption. Interestingly, some studies have also reported metabolic dysfunction related to liver toxicity induced by NPs both in aquatic models and mam-

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mals [23]. Since the liver plays a crucial role in the clearance of xenobiotics and its functions support the cardiometabolic health, NPs were proposed as a new potential risk factor involved in liver diseases such as metabolic dysfunction-associated steatotic liver disease (MASLD) [24].

Here we aimed at characterizing the potential cytotoxicity of PS-NPs (500 nm) carrying a negatively charged surface, functionalized with both carboxylic (COO^-) and sulphate (SO_4^-) ions. Our selected NPs were tested in *vitro* in a model of hepatocarcinoma, namely the HepG2 cell line. Cells were treated with PS-NPs for 24 and 48 hours to assess both their viability and NP uptake after exposure. Moreover, we have investigated the ability of HepG2 to uptake glucose (6-NBDG) following NP treatment.

Materials and Methods

Chemicals

MEM with Earle's Salts, Dulbecco's Phosphate Buffered Saline (DPBS), L-Glutamine 100X (200 mM), Minimum Essential Medium Non-Essential Amino Acids Solution (100X), Penicillin/Streptomicin 100X, Sodium Pyruvate 100 mM, and Fetal Bovine Serum (FBS) were purchased from EuroClone S.p.A. (Milan, Italy). Green, red and blue, fluorescent polystyrene nanoparticles (Fluoro-Max Dyed Aqueous Fluorescent Particles) with 500 nm diameter were purchased from Fisher Scientific (20053 Rodano, Italy) as aqueous suspensions at 1% solids by weight and used without any further modification. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose (6-NBDG) were obtained from Invitrogen (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased by Thermo Fisher.

Cell culture

The human hepatocarcinoma-derived HepG2 cell line was obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA) and cultured in MEM previously reconstituted with 10.000 U/mL penicillin, 10 mg/mL streptomycin, non-essential amino acids (0.1 mM), sodium pyruvate (100 mM) and L-glutamine (200 mM) and further supplemented with 10% heat-inactivated FBS. HepG2 were seeded in cell culture petri dishes at a density of $1,5 \times 10^6$ and kept at 37°C with 5% CO_2 for one week. Prior to experiments HepG2 cells were detached with trypsin-EDTA (1X) and seeded into multi-well plates at a different density as follows: 1×10^4 cells/well (MW 96); 1×10^5 cells/well (MW 24).

Succinate dehydrogenase activity (MTT assay)

The MTT assay was performed to assess HepG2 viability after NP treatment. HepG2 were plated in MW96 and incubated at 37°C with 5% CO_2 for 7 days and then treated with polystyrene nanoplastics (PS-NPs) 500 nm at different concentrations (10, 100 and 200 µg/mL) for 24 and 48 hours, respectively. HepG2 were thereby exposed to MTT for 4 hours. MTT-formazan crystals were dissolved in DMSO while keeping the cell culture plates on an orbital shaker (Hosmotic s.r.l., Vico Equense, NA) for 20 minutes. Each plate was read at 570 nm with the EnSpire Multimodel Plate Reader 23001049 (PerkinElmer, MA, USA).

Cellular internalization of 500 nm PS-NPs

To explore the internalization of PS-NPs (500 nm), HepG2 cells were cultured in 24 multi-well plates and exposed to 10, 100 and 200 µg/mL PS-NPs for 24 and 48 hours, respectively. Then, HepG2 cells were washed with DPBS 1X, detached with trypsin-EDTA 1X and re-suspended in DPBS after centrifugation (5 minutes, 2500 rpm). Cytofluorimetric analysis was run with NovoCyte 3000 Flow Cytometer System (Agilent Technologies, CA, USA).

6-NBDG uptake analysis following 500 nm PS-NPs exposure

Prior to 6-NBDG treatment, HepG2 cells were cultured in 24 multi-well plates and exposed to 10, 100 and 200 µg/mL PS-NPs for 24 and 48 hours, respectively. The cell culture medium was thereby replaced by 6-NBDG solution (20 µM) prepared in DPBS 1X, after washing. Cells were incubated for 30 minutes at 37°C with 5% CO_2. After incubation, cells were washed and collected in suitable tubes for cytofluorimetric analysis run by Flow cytometer NovoCyte3000.

Statistical analysis

Statistical analysis was performed using one-way ANOVA and Dunnett multiple test adjustment corrections for normally distributed data. Normality of data distribution was checked by Shapiro-Wilk normality test. P values ≤0.05 were considered significant. Data were shown as mean value ± standard deviation (SD). Statistics was generated using the GraphPad Prism 9.0 software.

Results

500 nm PS-NPs slightly reduced HepG2 cell viability after 24 hours at the highest concentration (200 µg/mL) tested and after 48 hours in all the experimental groups

To evaluate cytotoxic effects induced by 500 nm PS-NPs, MTT assay was performed after exposing HepG2 cells to 500 nm PS-NPs for 24 and 48 hours (**Figure 1**). 500 nm PS-NPs significantly reduced HepG2 cell viability at the highest concentration (200 µg/mL) following 24 hours of treatment. HepG2 cell viability was significantly reduced at all tested concentrations after 48 hours. Moreover, at the highest concentration (200 µg/mL), after 48 hour-treatment, HepG2 cell viability resulted higher compared to the other tested concentrations although statistically lower than the control group. This latter could be potentially related to the exit of PS-NPs from HepG2 cells as time of exposure to 500 nm PS-Ns increases. The reduction of HepG2 cell viability was maintained around the 80% threshold (dashed line, highlighted in red), considered the actual threshold below which cell viability is substantially impaired.

500 nm PS-NPs are taken up in a dose-response fashion by HepG2 cells after 24 and 48 hours

To evaluate the cellular internalization of 500 nm PS-NPs, we have performed a cytofluorimetric analysis by exploiting the fluorophore coated to PS-NPs (**Figure 2**). HepG2 cells were exposed to fluorescent 500 nm PS-NPs for 24 and 48 hours and the fluorescent signal was detected by cytofluorometric analysis. 500 nm PS-NPs were taken up in a dose-response manner both after 24 and 48 hours of treatment. More specifically, it was observed an increase in the uptake of 500 nm PS-NPs after 24 hour-exposure in HepG2 cells of +1190%, +8543% and +9991% for each concentration (10, 100 and 200 µg/mL, respectively) compared to the control group. Conversely, after 48 hours of exposure, 500 nm PS-NPs uptake in HepG2 cells increased of +1356%, +5882% and +8014% for each concentration (10, 100 and 200 µg/mL, respectively) compared to the control group. The uptake appeared lower after 48 hour-treatment at the middle and highest concentrations (i.e., 100 and 200 µg/mL) with respect to the 24 hour-treatment. Possibly, this could be explained by the potential exit of 500 nm PS-NPs from HepG2 cells.

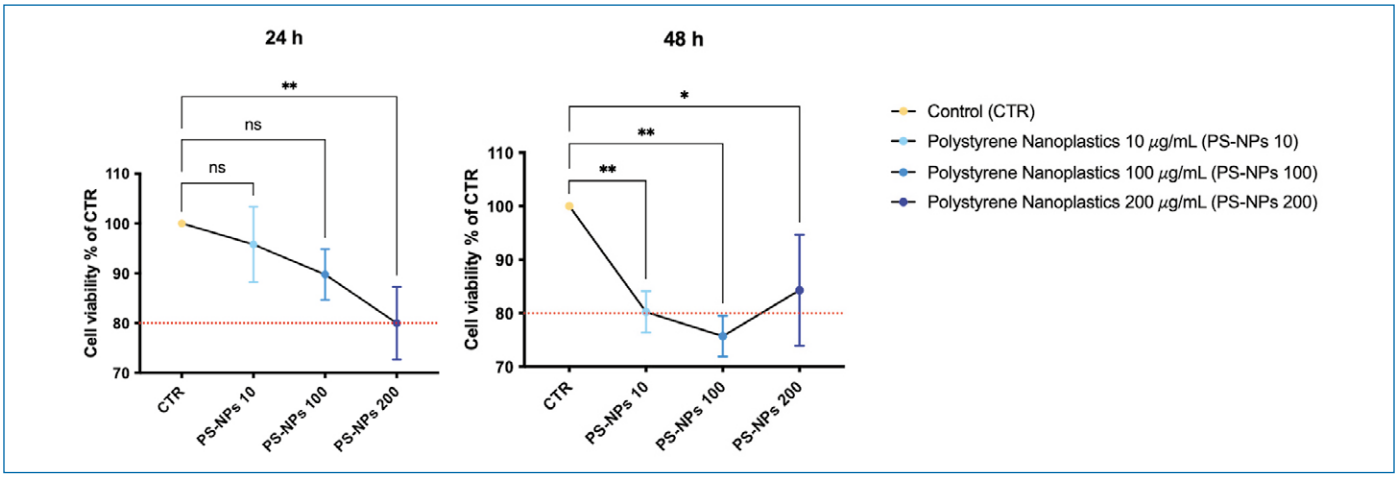

Figure 1 | Effects of PS-NPs on cell viability (MTT Assay). Each point of the linear regression curve reports mean value ± SD obtained from 3 independent experiments, each run in quintuplicates. CTR=100%. Viability threshold: 80%. *p<0.05, **p<0.01 (One-way ANOVA followed by Dunnett's multiple comparison correction).

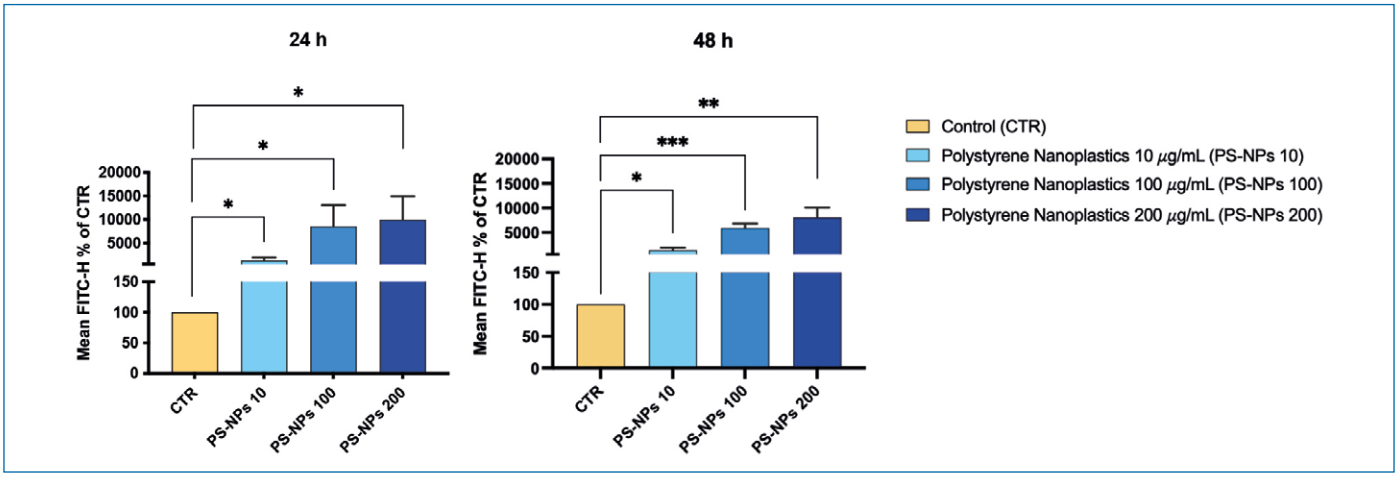

Figure 2 | PS-NPs internalization by HepG2 cells (cytofluorimetry). MFI = mean fluorescence intensity. Each bar reports mean value ± SD obtained from 3 independent experiments each run in triplicates. CTR=100%. *p<0.05, ** p<0.01, ***p<0.001 (Unpaired t-test).

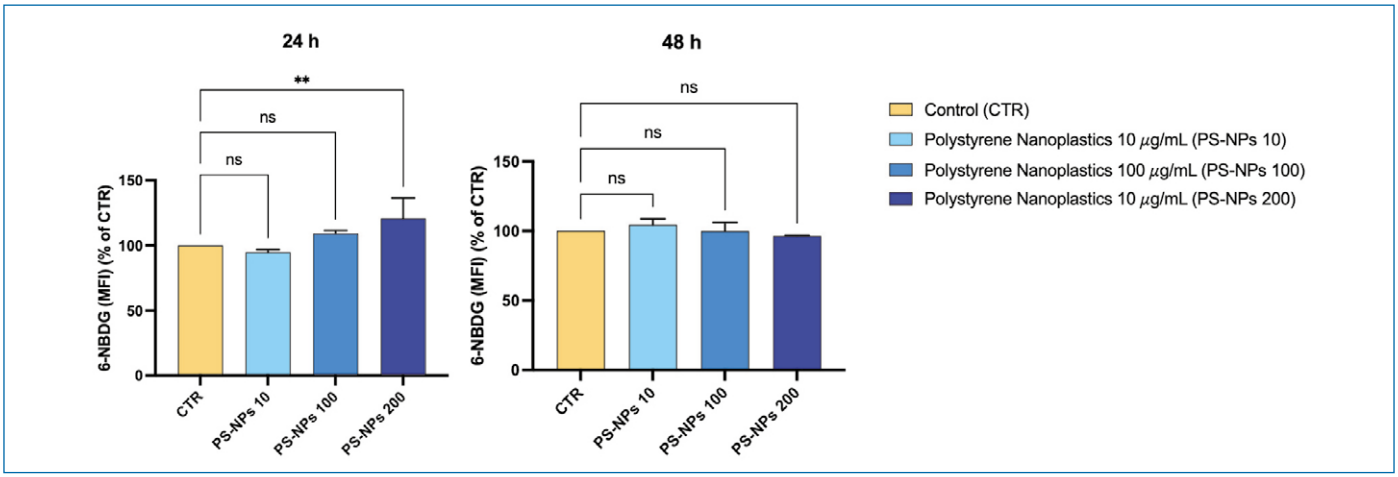

Figure 3 | Effects of PS-NPs on 6-NBDG uptake by HepG2 cells (cytofluorimetry). MFI = mean fluorescence intensity. 6-NBDG: 20 μM (30 minutes). Each bar reports mean value ± SD obtained from 3 independent experiments each run in triplicate. "ns": non-significant; **p<0.01 (24 h: One-way ANOVA followed by Dunnett's multiple comparison correction; 48 h: Kruskal-Wallis followed by Dunnett's multiple comparison correction).

6-NBDG uptake in HepG2 cells was not affected after 24 and 48 hours of 500 nm PS-NPs exposure

The effect of exposure to 500 nm PS-NPs on 6-NBDG uptake by HepG2 cells was evaluated (**Figure 3**). No significant effects on 6-NBDG uptake were observed after 24/48 hours of treatment, except for the highest concentration (200 µg/mL) after 24 hour-treatment. However, after 24 hour-treatment with 500 nm PS-NPs, it was also observed a trend in increasing uptake of 6-NBDG uptake by HepG2 cells in a dose-response fashion. The increase of glucose uptake in HepG2 could be due to the stress response induced by exposure to 500 nm PS-NPs in the acute phase of treatment. Conversely, the same trend in increasing glucose uptake was not observed after 48 hour-treatment with 500 nm PS-NPs. In this latter, glucose uptake in HepG2 cells appeared to decrease in a dose-response manner compared to 24 hours of exposure to 500 nm PS-NPs. Possibly, this could suggest that glucose uptake in HepG2 cells may decrease in a time-dependent manner, as the exposure time to 500 nm PS-NPs is associated with increased metabolic impairment.

Discussion

In light of the potential cytotoxic effects of PS-NPs, specifically to the liver, we started to address this issue by exposing human HepG2 cells to 500 nm PS-NPs and found effects on cell viability and accumulation of PS-NPs, while glucose uptake appeared only slightly affected. The observed reduction of cell viability did not exceed 20%. Nevertheless, it is known that NPs affect cell viability differently according to cell types, plastic polymers, sizes or functional surface groups characterizing the plastic particles [25-27]. Compared to MPs, NPs are characterized by a greater surface-to-volume ratio and surface properties allowing them to carry environmental pollutants and microorganisms, thus exerting a "Trojan horse" effect [28]. Herein we observed a reduced intracellular content of 500 nm PS-NPs in HepG2 especially after 48 hour-exposure, possibly due to passive release of PS-NPs. Accordingly, Liu et al. have shown that 500 nm PS-NPs are taken up by rat basophilic leukemia cells mainly through micropinocytosis and excreted using both passive penetration and active lysosomal exocytosis [29]. In a different study, based on mouse embryonic fibroblasts, PS-NPs clearance occurred mainly via exocytosis when the retrograde intracellular transport was inhibited [30]. To our knowledge, the mechanisms modulating PS-NPs dynamics in HepG2 cells were not yet investigated. PS-NPs were also found to induce metabolic dysfunctions linked to both glucose and lipid metabolisms, as observed in animal models [31]. Here, we speculated that potential metabolic alterations associated with impairment of the cellular glucose uptake were not observed considering the acute exposure time to 500 nm PS-NPs. Hence, time of treatment or dosages should be increased to detect relevant metabolic impairments in HepG2 cells, as it was seen in animal models. Recent studies based on animal models have also described how PS-NPs can contribute to adipose tissue dysfunctions [32], atherosclerosis [19], vascular disease [33] and MASLD [23]. However, the pathological mechanisms linking PS-NP-induced hepatic dysfunctions to ASCVD remain to be elucidated. To this purpose, future studies will investigate PS-NP effects in a steatotic-like model of HepG2 cells recapitulating key features of MASLD.

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Author Contributions

Conceptualization, C.G., L.D.D., P.M.; methodology, C.G., L.C., F.T.K., L.D. and, P.M.; validation, C.G L.C. and P.M.; investigation, C.G.; data curation, C.G; writing-original draft preparation, C.G; writing-review and editing, C.G., L.C., and P.M.; supervision, P.M.; project administration, P.M.; funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

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Competing interests

All the authors have nothing to disclose.

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