

Endolysosomal remodeling and acidity dictates ferroptosis sensitivity to iron oxide nanoparticles

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ABSTRACT

Iron oxide nanoparticles (IONPs) exhibit remarkable anti-tumoral activity, largely mediated by the generation of reactive oxygen species (ROS). Although certain IONPs can induce ferroptosis, the determinants underlying cell-type-specific sensitivity remain poorly understood. Here, we provide novel mechanistic insight by demonstrating that lysosomal acidity and plasticity critically regulate iron mobilization, ROS compartmentalization, and the ferroptotic response triggered by dimercaptosuccinic acid-coated IONPs (DMSA-IONPs) in MDA-MB-231 breast cancer and U87MG glioma cells. DMSA-IONPs generate ROS in both cell models, however, their subcellular localization markedly differed. In MDA-MB-231 cells, highly acidic lysosomes retained redox-active iron (Fe^{2+}), leading to localized ROS accumulation, lysosomal enlargement and pronounced lipid peroxidation, ultimately inducing ferroptosis, which was reverted by the anti-ferroptosis drug Ferrostatin-1. In contrast, the less acidic lysosomes of U87MG cells released iron into the cytosol and mitochondria, resulting in diffuse ROS production without lipid peroxidation and conferring resistance to ferroptosis despite higher nanoparticle uptake. While IONP-mediated ROS generation via iron mobilization and Fenton-like reactions is well established, our findings identify lysosomes as critical determinants of cellular responses to IONPs exposure. Specifically, lysosomal acidity and endolysosomal trafficking govern ferroptotic sensitivity to IONPs by controlling the site of ROS accumulation. Our results demonstrate that IONP-induced ferroptosis depends not only on total ROS levels but also on ROS subcellular distribution, with ROS accumulated in lysosomes triggering lipid peroxidation of these organelles. These insights highlight the importance of evaluating lysosomal physiology across tumor type to optimize nanoparticle-based ferroptosis therapies, particularly for tumors resistant to apoptosis.

1. Introduction

Despite significant progress in cancer therapy, the development of resistance to conventional treatments remains a major obstacle in many malignancies [1]. Consequently, considerable effort has been devoted to identifying alternative cell death mechanisms that can be therapeutically exploited. Because cancer cells often exhibit elevated oxidative stress and altered iron metabolism, they may be particularly vulnerable

to therapies that further increase reactive oxygen species (ROS) production and lipid oxidative damage [2,3]. One emerging anti-cancer strategy involves increasing ROS production beyond the threshold tolerated by cancer cells, thereby promoting oxidative damage and non-apoptotic cell death pathways such as ferroptosis, an iron-dependent form of regulated cell death [4]. One such pro-oxidant strategy to induce ROS production involves the use of metal-containing nanoparticles (NPs), as transition metal ions like

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Cu^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} and Fe^{3+} can catalyze the oxidation of H_2O_2 and generate ROS, especially hydroxyl radicals ($\cdot\text{OH}$), through the so-called Fenton and Haber-Weiss reactions [5,6]. These $\cdot\text{OH}$ radicals can oxidize certain amino acids in proteins, nucleotides in DNA and polyunsaturated fatty acid (PUFA) chains in lipids [7], affecting the structure and function of these biomolecules. Among the metal-containing NPs, iron oxide NPs (IONPs) are those preferentially used in biomedical applications due to their safety, biocompatibility and magnetic properties [8], as well as their intrinsic oxidizing capacity given the presence of Fe^{2+} and Fe^{3+} ions in their structure. IONPs have proven to be powerful tools, their biocompatibility residing in the body's molecules to sequester iron (hemoglobin, ferritin and myoglobin) and its organs capable of storing, metabolizing and eliminating iron (liver and spleen) [9,10]. Indeed, iron is an essential element and it is needed for DNA and RNA synthesis, oxygen transport, cell respiration, heme synthesis, enzyme synthesis (especially those containing Fe-S clusters), immune function and metabolism [11].

Iron metabolism is closely related to the development of tumors as iron homeostasis is abnormal in cancer cells, reflecting their increased iron demands due to their enhanced metabolism and higher rate of proliferation [12]. Therefore, the affinity, uptake and utilization of iron by cancer cells is increased. Since iron uptake by cancer cells is greatly enhanced, their intracellular iron storage and trafficking is altered in order to cope with the increased risk of iron-related oxidative stress, an important adaptation as iron overload can contribute to the formation of damaging ROS through the Fenton reaction [13]. The reduction of ferric iron (Fe^{3+}) to bioactive ferrous iron (Fe^{2+}) occurs in endosomal compartments and lysosomes through the activity of STEAP reductases, enzymes that are strongly overexpressed in some types of cancer [14]. This step is necessary to export iron into the cytosol via the DMT1 transporter [15]. In the cytosol bioactive ferrous iron forms the labile iron pool (LIP), which can be stored in the form of ferritin or mobilized and transferred to mitochondria where it can be used to synthesize iron-based co-factors that mediate specific cellular processes [16]. Lysosomes play a central role in iron trafficking and in the crosstalk between organelles, as they are the first organelles to receive and reduce iron prior to its translocation to different intracellular locations [17]. Moreover, through autophagy lysosomes are responsible for the degradation of iron-containing organelles like mitochondria or of iron-containing molecules such as ferritin [17].

The fact that lysosomes are important organelles for iron storage makes them important organelles for redox homeostasis, as excessive iron accumulation can drive ROS formation and render lysosomes susceptible to oxidative damage. Excess iron is reduced to the redox-active ion (Fe^{2+}) in an acidic and reducing environment, which can in turn react with H_2O_2 to generate the reactive $\cdot\text{OH}$ radical. These $\cdot\text{OH}$ radicals are highly reactive and can peroxidate membrane lipids by extracting electrons from their unsaturated fatty acids, thereby creating an unstable lipid radical and triggering a self-propagating chain reaction of lipid peroxidation [18]. The continued peroxidation of lysosomal membrane lipids can trigger lysosomal membrane permeabilization (LMP) and consequently, a loss of lysosomal membrane integrity that results in cathepsin and hydrolase release into the cytosol [19]. Persistent lysosomal damage and rupture can eventually lead to cell death via different mechanisms, with cathepsins representing the main executioners of lysosomal-dependent cell death [20]. The degree of lysosomal damage influences the induction of different cell death mechanisms, such as apoptosis, necroptosis, pyroptosis and ferroptosis [21]. Cancer cells generally have more lysosomes to support their enhanced metabolism, proliferation and adaptation to stressful environments [20]. However, the enhanced lysosomal activity may also render cancer cells more susceptible to LMP, providing an alternative strategy to induce cancer cell death [22,23]. Therefore, lysosomes emerge as potential targets for cancer therapy.

Through their involvement in iron and redox homeostasis, lysosomes have been associated with non-apoptotic cell death mediated by iron,

known as ferroptosis. Ferroptosis is characterized by the availability of redox-active iron, the iron-dependent peroxidation of membrane phospholipids and alterations to the cellular antioxidant system [17,24]. Cells have developed antioxidant machinery to prevent oxidative damage to phospholipids, which is mainly regulated by the glutathione (GSH) system and it is based primarily on the activity of glutathione peroxidase 4 (GPX4), and of amino-acid transporters that regulate the availability of glutamate and cysteine in the cell [25,26]. Therefore, compromising the GSH antioxidant system can sensitize cells to ferroptosis.

As cancer cells become resistant to apoptosis, inducing ferroptosis has emerged as a promising therapeutic strategy. As such, studies have focused on the induction of this type of cell death by accumulating ROS, iron or PUFA-containing phospholipids, or by depleting endogenous inhibitors of ferroptosis like GSH, NADPH or GPX4 [27]. Several small molecules have been reported to induce ferroptosis by targeting different key regulators [28]. However, their poor solubility, toxicity and bioavailability hinder their clinical application, making metal-containing NPs an increasingly attractive therapeutic strategy [29]. Nearly a decade has passed since the first NP capable of inducing ferroptosis was reported: ultra-small poly(ethylene glycol)-coated silica NPs that induced ferroptosis in amino acid starved cancer cells, thereby inhibiting cancer growth [30]. Given the close relationship between iron metabolism and ferroptosis, IONPs stand out as particularly promising agents due to their ability to release iron and modulate the induction of ferroptosis [31]. Indeed, gallic acid and polyacrylic acid-coated IONPs have recently been shown to induce cell death via ferroptosis [32]. Although IONPs and ferroptosis induction has previously been linked, the precise mechanism that dictates how IONPs induce ferroptosis in different cancer cell line has not yet been described.

We previously demonstrated that dimercaptosuccinic acid-coated IONPs (DMSA-IONPs) induce robust intracellular ROS production in cancer cells, resulting in altered mitochondrial metabolism [33]. However, ferroptosis induction and cell type-specific sensitivity were not further investigated. Among the IONP formulations tested [33], DMSA-IONPs possess a particularly strong pro-oxidant capacity and, therefore, represented the most suitable IONP formulation for investigating the relationship between ROS generation and ferroptosis induction. Here, we investigated how DMSA-IONPs modulate lysosomal function, iron mobilization and oxidative stress in two different cancer cell lines, the MDA-MB-231 breast cancer (BC) cell line and the U87MG glioma cell line. By comparing their endolysosomal remodeling, acidification dynamics and capacity to sequester or redistribute ferrous ions, we aimed to identify the cell-intrinsic determinants that dictate ROS production and the sensitivity or resistance to DMSA-IONP-induced ferroptosis. While it has previously been established that iron ions mobilize into the cytosol upon IONP treatment, inducing ROS and cell death, we propose a novel mechanistic insight in which the subcellular localization of iron and ROS dynamics critically determine ferroptosis sensitivity. Our findings reveal that retention of iron ions within lysosomes leads to an even greater increase in ROS production and drives ferroptotic cell death. DMSA-IONPs have been considered promising tools *in vivo* due to their limited toxicity and long-term bioavailability, [10] suggesting that they may be suitable for anti-cancer therapies *in vivo*. Our results revealed that lysosomal plasticity constitutes a critical decision point controlling the onset of ferroptosis and highlight the need of considering lysosomal physiology when designing nanoparticle-based pro-oxidant therapies.

2. Materials and methods

2.1. Synthesis and characterization of DMSA-IONPs

DMSA-IONPs were synthesized following Massart's co-precipitation protocol as described previously [34]. Following the synthesis of the iron cores, they were coated with DMSA using previously described

protocols [35]. The size, shape and distribution of the DMSA-IONPs formed was determined by transmission electron microscopy (TEM), obtaining the images with a Gatan ES1000Ww camera connected to a JEOL JEM 1011 transmission electron microscope. The size of the DMSA-IONPs in suspension was assessed by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS system (Malvern). The presence and nature of the coating was determined from the Fourier-transform infrared spectroscopy (FTIR) spectra recorded between 4000 and 250 cm^{-1} on a Bruker Vertex 70 V spectrometer with 2 cm^{-1} resolution.

2.2. Cell cultures

The human MDA-MB-231 BC cell line (ATCC_HTB-26) and the U87MG glioma cell line (ATCC_HTB-14) were purchased from the ATCC (American Type Culture Collection). Both cell lines were cultured in DMEM (Dublecco's modified Eagle's medium) supplemented with 10% FBS (Fetal Bovine Serum), 1% non-essential amino acids, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 100 IU/ml Penicillin and 100 mg/ml streptomycin. The cell cultures were maintained at $37\text{ }^{\circ}\text{C}$ in an atmosphere of 5% CO_2 and at 90% relative humidity.

2.3. Cellular localization of DMSA-IONPs by TEM

MDA-MB-231 and U87MG cell lines were seeded in petri dishes and cultured for 24 h, after which they were exposed to DMSA-IONPs ($125\text{ }\mu\text{gFe/ml}$) for 24 h. The cells were then washed three times with phosphate buffer saline (PBS) to remove the non-internalized DMSA-IONPs and fixed for 2 h at room temperature (RT) with 2% glutaraldehyde and 1% tannic acid diluted in 0.4 M HEPES buffer (pH 7.2). The cells were recovered by gentle scraping and resuspended in HEPES, post-fixed for 30 min at $4\text{ }^{\circ}\text{C}$ with 1% osmium tetroxide and 2% uranyl acetate, dehydrated in a series of acetone solutions and gradually infiltrated with Epon resin. The resin was allowed to polymerize ($60\text{ }^{\circ}\text{C}$, 48 h) and ultrathin sections ($60\text{--}70\text{ nm}$) were obtained with a diamond knife mounted on a Leica EM UC6 ultramicrotome. These sections were then attached to a formvar/carbon-coated gold grid and visualized on a JEOL-1400 flash 100 KV transmission electron microscope, acquiring images at different magnifications with a Gatan ONE VIEW camera.

2.4. Quantification of intracellular iron after IONP treatment

Cells were seeded in 12-well plates and cultured for 24 h prior to their exposure to different concentrations of DMSA-IONPs (125 , 250 or $500\text{ }\mu\text{gFe/ml}$) for 24, 48 or 72 h. After treatment, the cells were rinsed three times with PBS to remove any IONPs not internalized, recovered using trypsin-EDTA and counted in a Neubauer chamber. The cells were recovered by centrifugation (1200 rpm, 5 min) and digested for 1 h at $90\text{ }^{\circ}\text{C}$ and overnight (ON) at RT with 1 ml of HNO_3 (63%). After digestion, the volume was brought up to 10 ml with distilled water and the iron content per cell was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES: Perkin Elmer-2400).

2.5. Immunofluorescence analysis of IONP localization and oxidative stress in lysosomes

MDA-MB-231 and U87MG cells were seeded on coverslips in a 24-well plate and treated with working concentrations of DMSA-IONPs for 24 h. To assess ROS production, cells were incubated for 30 min with the Dihydrorhodamine (DHR) probe (Molecular probes, Carlsbad, CA, USA) diluted 1:500 in cell culture medium. The cells were then washed and fixed for 15 min with 4% paraformaldehyde (PFA), and after again washing the coverslips with PBS, they were counterstained with DAPI diluted 1:500 in PBS for 10 min at RT and mounted with Fluoromount-G (0100-01, Southern Biotech). To assess the localization of DMSA-IONPs and ROS to lysosomes, after fixation the coverslips were permeabilized for 10 min with 0.1% Triton X-100 at RT prior to

blocking with the DAKO Antibody Diluent with Background Reducing Components (S3022 Agilent Technologies). After blocking, the cells were probed ON at $4\text{ }^{\circ}\text{C}$ with the primary antibodies diluted in DAKO Antibody Diluent in a humidified chamber. The following day, the coverslips were washed and probed for 1 h at RT with the relevant secondary antibody also diluted in DAKO Antibody Diluent. Lysosomes were labeled with the Lamp2 primary antibody (1:200, sc-18822: Santa Cruz). After staining, the coverslips were washed with PBS, counterstained for 10 min at RT with DAPI diluted 1:500 in PBS and mounted in Fluoromount-G (0100-01, Southern Biotech). The cells were visualized on a multispectral confocal Leica Stellaris 5 microscope using 63X/1.40 oil objective with a zoom factor of 3X or 4X and the images obtained were analyzed with Image J Software. To study the changes in lysosomal number, diameter and shape, at least 20 cells per condition were analyzed.

To visualize IONPs by confocal microscopy, the confocal reflectance microscopy (CRM) technique was used to visualize IONPs. For this technique, the signal is generated by elastically backscattered light from the sample, and the detector is aligned to collect backscattered light at the same wavelength as the incident laser. Moreover, the detector is specifically configured to capture backscattered light allowing label-free imaging of IONPs within cells. Reflection confocal microscopy was used to visualize the intracellular location and spatial distribution of DMSA-IONPs; however, it but was not intended for the quantitative assessment of nanoparticle uptake.

2.6. Flow cytometry analysis of the oxidative stress induced by exposure to DMSA-IONPs

For flow cytometry analyses, MDA-MB-231 and U87MG cells were cultured in 12-well plates and treated for 3 or 24 h with working concentrations of DMSA-IONPs. After washing, the cells were then incubated with DHR (diluted 1:20,000 in medium) for 30 min under cell culture conditions, and subsequently recovered by trypsinization and centrifugation. For flow cytometry analysis, the cells were resuspended in PBS, propidium iodide (PI) was added to each sample, and DHR fluorescence was analysed using FL1 (ex/em = $488/525\text{ nm}$) and PI using FL3 (ex/em = $488/620$) on a Cytomics FC500 cytometer. For each sample, 50,000 events were acquired and the DHR fluorescence/ROS production of live cells was quantified with the FlowJo Software.

2.7. Immunofluorescences to analyze labile iron pool and lipid peroxidation

The LIP and lipid peroxidation was studied *in vivo* by dark-field confocal microscopy using a Leica TCS SP5 and Leica TCS SP8 multi-spectral confocal system, respectively. Cells were cultured in μ -Slide 8 well ibiTreat plates (Ibidi) and exposed to working concentrations of IONPs for 3 or 24 h, after which they were washed with Hank's balanced salt solution containing calcium and magnesium (HBSS/Ca/Mg: Gibco 14025-092). For LIP detection, cells were stained for 30 min with a working solution of FerroOrange ($1\text{ }\mu\text{mol/l}$: FerroOrange 36104, Cell Signaling) under cell culture conditions. The cells were then washed twice with HBSS and counterstained for 10 min with a 1:200 Hoechst solution in the same buffer. Finally, the cells were washed and visualized on a Leica TCS SP5 confocal microscope, measuring the FerroOrange intensity using ImageJ Software in at least 200 cells per condition. To detect lipid peroxidation, cells were stained for 30 min with the BODIPY 581/591 C11 probe ($4\text{ }\mu\text{M}$) in cell culture medium, and then washed and counterstained with Hoechst as above. The cells were visualized on a Leica TCS SP8 multispectral confocal system, measuring the Bodipy red and green intensity of at least 200 cells per condition using ImageJ Software.

To assess the localization of iron or lipid peroxidation inside lysosomes or mitochondria, after FerroOrange or BODIPY staining, the cell's lysosomes were stained for 1.5 h under cell culture conditions with a

SiR-lysosome probe (1 μ M: SC012, Spirochrome) or their mitochondria were stained for 30 min with MitoTracker Green FM (200 nM: M7514 - Invitrogen) prior to counterstaining with Hoechst.

2.8. Assessment of Glutathione (GSH)

Cells were seeded in petri dishes and cultured for 24 h before they were exposed to different concentrations of DMSA-IONPs for 24 h, or left untreated. After washing with PBS, the cells were recovered by scraping, centrifuged and resuspended in 100 μ l of PBS. GSH was quantified with a colorimetric kit (GSH Assay kit, ab239727: Abcam) according to the manufacturer's instructions and measured at 450 nm using a spectrophotometer Multiskan FC (Thermo scientific). The absorbance is proportional to the amount of GSH and the GSH concentration was calculated relative to the GSH standard in the kit, expressed relative to the total amount of protein in the samples determined previously with a BCA assay (PierceTM Rapid Gold BCA Protein Assay Kit: Thermo Scientific). The values are expressed as the absolute values in pg of protein and as a ratio normalized to the amount in untreated cells.

2.9. Assessment of lysosomal damage using Acridine Orange

For flow cytometry analyses, MDA-MB-231 and U87MG cells were cultured in 12-well plates and treated for 24 h with the working concentrations of DMSA-IONPs. The cells were then washed and incubated with Acridine Orange (AO: 0.1 μ g/ml) for 15 min under cell culture conditions. After staining, the cells were recovered by trypsinization, centrifuged and resuspended in PBS for flow cytometry. Green AO fluorescence was analysed using FL1 (ex/em = 488/525 nm), while red AO emission using FL3 (ex/em = 488/620) on a Cytomics FC500 cytometer. For each sample, 50,000 events were acquired and the DHR fluorescence/ROS production of live cells was quantified with FlowJo Software. Lysosomal damage was represented as the decrease on red emission.

2.10. Spheroid formation and tumor cell growth

MDA-MB-231 and U87MG spheroids were prepared by the hanging droplet method [36]. As such, 20 μ l droplets of culture medium containing 1000 cells/droplet were deposited on the lid of a petri dish. The lid was inverted and placed on the top of the petri dish filled with PBS to obtain a high-humidity atmosphere, obtaining U87MG spheroids after 72 h and MDA-MB-231 spheroids after 96 h. The spheroids formed were recovered and incubated for 24, 48 and 72 h with different DMSA-IONP concentrations (125, 250 and 500 μ gFe/ml). Spheroid growth was evaluated by taking pictures of the spheroids over time using a Zeiss Axiovert 40 CFL microscope coupled to a LEICA DFC300 FX camera and measuring the X-diameter, Y-diameter and the outline of the spheroids.

2.11. Long term proliferation and cell death

To analyze the long-term proliferation of MDA-MB-231 and U87MG cells, cells were seeded in 12-well plates and allowed to attach for 6–8 h. Subsequently, the cells were cultured in low serum starvation medium with (0.5% FBS) to synchronize their growth. The cells were then treated with distinct concentrations of DMSA-IONPs (125, 250 and 500 μ gFe/ml) for different times (24, 48 and 72 h), washed twice with cold HBSS and then fixed with cold methanol for 10 min at RT before the wells were left to dry. The cells were stained for 10 min with 0.5% (W/V) Crystal Violet solution (V5265, Sigma), rinsed carefully with water and once they were dry, images of the cells were acquired with a Zeiss Axiovert 40 CFL microscope coupled to a LEICA DFC300 FX camera using a 10X objective. To quantify cell viability, the cells were incubated with 10% (V/V) acetic acid for 10 min at RT in a rocker to dissolve the crystal violet and once it was fully dissolved, the absorbance of the samples was

read at 570 nm on a Multiskan FC spectrophotometer. The values of the samples at each time point and dose of DMSA-IONPs were normalized to the untreated cells to define the proliferation index.

To analyze the induction of cell death over time by flow cytometry, an Annexin V-FITC kit (10010-02, SouthernBiotech) was used. Cells were seeded in 12-well plates and after 24 h, the cells were incubated with different concentrations of DMSA-IONPs (125, 250 and 500 μ gFe/ml) for different times (24, 48 and 72 h). The cells were then washed with PBS, and recovered by trypsin digestion and centrifugation, resuspending the pellet obtained in 100 μ l binding buffer. The cells were incubated with 5 μ l Annexin V-FITC for 10 min on ice and subsequently, 250 μ l of binding buffer and 3 μ l of PI was added to each sample. Annexin V-FITC fluorescence was analysed using FL1 (ex/em = 488/525 nm) and PI using FL3 (ex/em = 488/620) on a Cytomics FC500 cytometer, and for each sample 50,000 events were acquired, and the live/dead population established with FlowJo Software.

2.12. The mRNA expression of genes involved in iron and glutathione metabolism

To analyze the effect of DMSA-IONPs on genes related to iron and glutathione metabolism, MDA-MB-231 and U87MG cells were first treated for 24 h with different concentrations of DMSA-IONPs (125, 250 and 500 μ gFe/ml). The total RNA from untreated and treated cells was extracted using the High Pure RNA Isolation Kit (Roche), quantified with a NanoDrop 1000 device (Thermo scientific), and the RNA (2 μ g) was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, ThermoFisher) and random primers. The cDNA was used to perform qRT-PCRs with a Power SYBR Green PCR mix (Applied Biosystems, ThermoFisher) and specific primers (Sigma-Aldrich) to amplify transcripts for *GPX4* (encoding glutathione peroxidase 4), *SLC7A11* (encoding the cystine transporter xCT), *SLC11A2* (encoding divalent metal transporter 1, DMT1), Transferrin Receptor (TFRC), *SLC48A1* (encoding heme transporter 1, HRG1), lipocalin 2 (*LCN2*, encoding neutrophil gelatinase-associated lipocalin-NGAL), iron responsive element binding protein 2 (*IREB2*) and *SLC40A1* (encoding ferroportin, FPN1). The qRT-PCR data was analysed by the threshold cycle ($2^{-\Delta\Delta CT}$) method [37] and normalized to β -actin expression.

2.13. Effect of the antioxidant Ferrostatin-1 and the iron chelator Deferoxamine

To assess Ferrostatin-1 (SML0583: Sigma) toxicity on MDA-MB-231 and U87MG cells, cells were left untreated or treated with 1, 2.5 or 5 μ M of Ferrostatin-1 in medium for 24 h, and their viability was then assessed by flow cytometry after Annexin-V-PI staining. To assess the effect of Ferrostatin-1 on DMSA-IONP internalization, the neutralization of lipid peroxidation, ROS production and cell death induction, cells were treated for 24 h with increasing doses of DMSA-IONPs alone, or after first exposing them for 1 h to Ferrostatin-1 (1, 2.5 or 5 μ M), followed by a 24 h incubation of Ferrostatin-1 together with the DMSA-IONPs. The cells were then recovered and ICP-OES was used to evaluate DMSA-IONP internalization, while the Bodipy C-11 probe was used to determine the effect of Ferrostatin-1 on lipid peroxidation, analysing Bodipy green fluorescence using FL1 (ex/em = 488/525 nm) and PI using FL3 (ex/em = 488/620) on a Cytomics FC500 cytometer. Erastin-treated cells (10 μ M) were used as a positive control for ferroptosis. For each sample, 20,000 events were acquired and analysed with FlowJo Software. To determine the effect of Ferrostatin-1 on ROS production DHR probe was used and analysed by flow cytometry as previously detailed. To determine the effect of Ferrostatin-1 on cell death, Annexin-V-PI staining was used, analysing Annexin V-FITC fluorescence using FL1 (ex/em = 488/525 nm) and PI fluorescence using FL3 (ex/em = 488/620) on a Cytomics FC500 cytometer. For each sample, 50,000 events were acquired and the live/dead population was assessed with

FlowJo Software.

To determine whether iron chelation could attenuate DMSA-IONP-induced oxidative stress, rescue experiments were performed using deferoxamine (DFO). Cells were left untreated (control), treated with DMSA-IONPs alone for 3, 6, or 24 h, or co-treated with DMSA-IONPs and DFO (10 μ M) for 3 h. Additional rescue experiments were performed in which cells were first incubated with DMSA-IONPs for 3 h, followed by the addition of DFO (10 μ M), and incubation was continued until the 6- or 24-h time points. Intracellular ROS levels and lipid peroxidation were subsequently quantified by flow cytometry using a Cytomics FC500 cytometer, as previously described.

2.14. Western blotting

Cells were lysed in a buffer containing 1% Triton X-100 and the protease inhibitors leupeptin (10 μ g/ml), Aprotinin (10 μ g/ml), NaF (5 mM), Sodium Orthovanadate (1 mM), PMSF (1 mM), EDTA (0.5 mM) and okadaic acid (0.2 μ M). Equal amounts of protein (40 μ g/well) were loaded onto gels and resolved by SDS-PAGE, and the proteins were transferred to 0.2 μ m PVDF membranes and blocked for 1 h at RT with 10% milk Tris-buffered saline (TBS)/1% Tween 20. The membranes were probed ON at 4°C with primary antibodies against Ferritin (1:500, ab75973: Abcam), caspase 3 (1:1000, 9662S: Cell Signaling Technology), cleaved caspase 3 (1:500, 9661 L: Cell Signaling Technology) and β -actin (1:1000, A5441: Sigma) diluted in TBST/1% non-fat milk. Antibody binding was detected for 1 h at RT using secondary-HRP conjugated anti-rabbit (P0448: Dako) and anti-mouse (P0447: Dako) antibodies diluted 1:1000 in TBST/1% milk, which were visualized with the SuperSignal™ West Femto Maximum Sensitivity Substrate (34094: ThermoScientific).

2.15. Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$ m) was assessed using the TMRE Mitochondrial Membrane Potential Assay Kit (ab113852, Abcam) according to the manufacturer's instructions. Cells were treated with DMSA-IONPs at the indicated concentration and for the specified time points. Following treatment, cells were washed with PBS and incubated with 200 nM tetramethylrhodamine ethyl ester (TMRE) in complete culture medium for 20 min at 37 °C. After incubation, cells were washed to remove excess dye and prepared for flow cytometric analysis. TMRE fluorescence was measured in the FL2 channel following excitation with a 488 nm laser using a Cytomics FC500 flow cytometer (Beckman Coulter). A total of 10,000 events were acquired per sample, and the median fluorescence intensity (MFI) was quantified using FlowJo software.

2.16. Lysosomal acidity functional assays

To study lysosomal pH, LysoTracker Green DND-26 and LysoSensor Yellow/Blue dextran (L22460, ThermoFisher) were used. For LysoTracker Green DND-26 staining and flow cytometric analysis, MDA-MB-231 cells were seeded in 12-well plates and cultured for 24 h. Cells were then left untreated or treated with DMSA-IONPs for 24 h. Subsequently the cells were incubated with LysoTracker Green DND-26 diluted 1:2000 in culture medium for 2 h. After staining, cells were harvested and analyzed using a Cytomics FC500 flow cytometer. Lysosomal acidification was evaluated by measuring LysoTracker Green DND-26 fluorescence intensity in the FL1 channel (Ex/Em = 488/525 nm), and mean fluorescence intensity values were quantified using FlowJo software. For LysoSensor Yellow/Blue dextran staining and confocal microscopy analysis, cells were incubated with the LysoSensor probe for 1 h at 37 °C. Images were acquired using a dark-field Leica TCS SP8 confocal microscope equipped with a 63X/1.4NA oil-immersion objective. Fluorescence intensity was quantified using Image J software.

To determine whether lysosomal alkalization modulates DMSA-

IONP-induced ROS production and lipid peroxidation, functional validation experiments were performed using with the V-ATPase inhibitor bafilomycin A. MDA-MB-231 and U87MG were left untreated or treated with DMSA-IONPs (125 μ g/ml) for 3 or 24 h, either alone or co-incubated with bafilomycin A following a 1 h pretreatment with bafilomycin A (35 nM). ROS production and lipid peroxidation were subsequently assessed by flow cytometry as described above.

To investigate whether disruption of lysosomal integrity influences DMSA-IONP-induced ROS production and lipid peroxidation, cells were treated with the lysosomotropic agent chloroquine (CQ). As a weak base, chloroquine accumulates within acidic lysosomes, where it becomes protonated, leading to osmotic swelling, lysosomal destabilization, and ultimately lysosomal membrane permeabilization (LMP). MDA-MB-231 and U87MG cells were left untreated or treated with DMSA-IONPs (125 μ g/ml) alone for 24 h. For lysosomal perturbation experiments, cells were pretreated with chloroquine (10 μ M) for 1 h, followed by co-incubation with chloroquine and DMSA-IONPs for an additional 24 h. Following treatment, intracellular ROS production and lipid peroxidation were quantified by flow cytometry as described above.

2.17. Statistical analysis

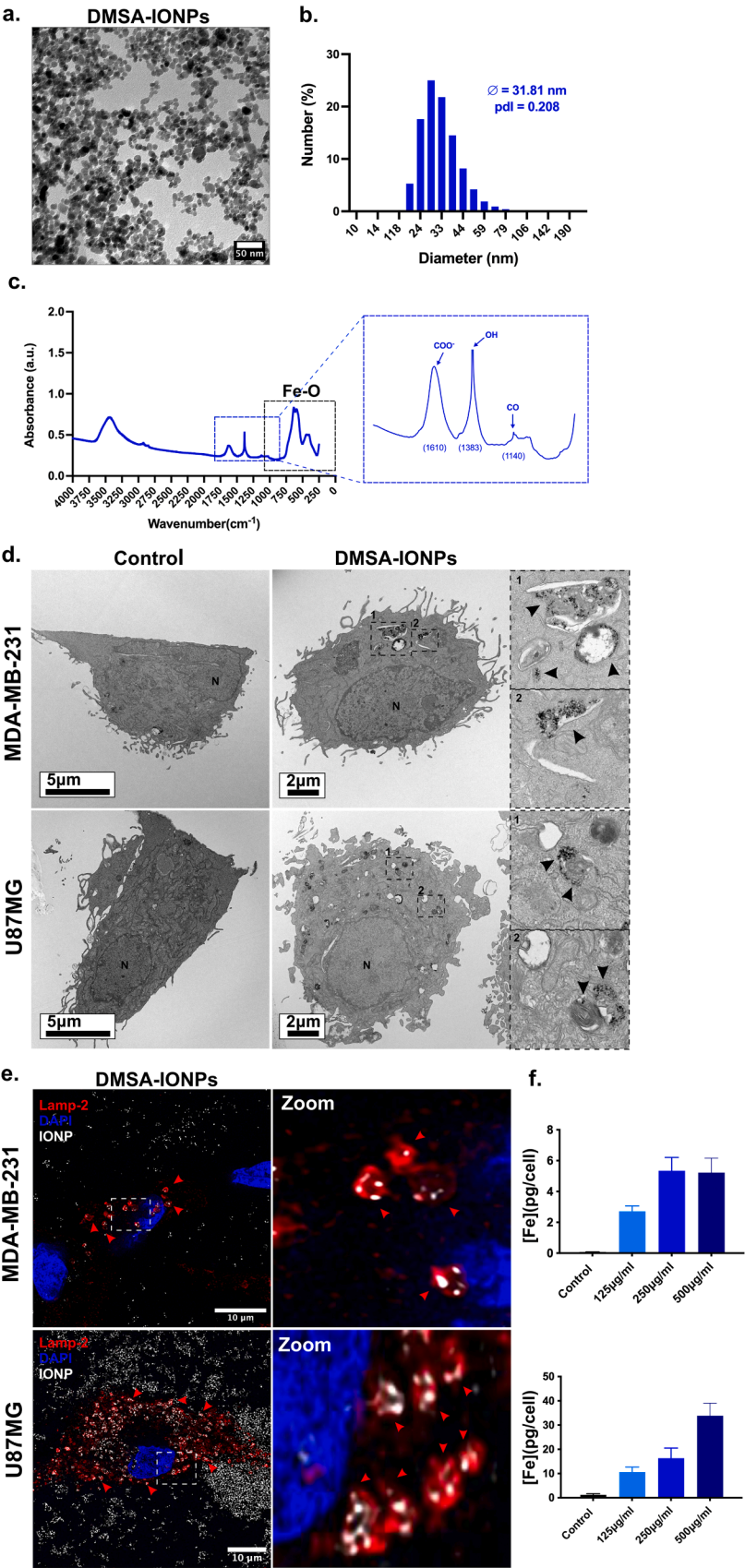
All the data are presented as the mean $\pm$ standard deviation (SD) and analyzed by a one-way and two-way analysis of variance (ANOVA). A Dunnett's or Tukey's multiple comparisons test were applied to calculate the differences between the distinct values. Values of $p < 0.05$ were considered statistically significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. GraphPad Prims version 10.1.1 was used for all the statistical analyses.

3. Results

3.1. DMSA-IONP characterization and internalization by tumor cells

NP based pro-oxidant therapy requires the internalization and degradation of IONPs in lysosomes to produce iron ions that may react with oxidants like H_2O_2 , and generate ROS. To investigate the cell-intrinsic characteristics that determine sensitivity to ferroptosis, DMSA-IONPs were characterized, and their internalization and subcellular localization were analyzed in MDA-MB-231 BC and U87MG glioma cell lines. DMSA-IONPs have been characterized extensively, [33, 38] but a new batch was synthesized and characterized for this study (Fig. 1). These DMSA-IONPs were spherical, with a core diameter of 10 nm (Fig. 1a) and a hydrodynamic size of 31.81 nm, and they were homogeneously dispersed with a polydispersity index (PDI) of 0.208 (Fig. 1b). The characteristic infrared bands detected through FTIR spectroscopy demonstrated that the IONPs were coated with the corresponding organic compound (Fig. 1c), showing peaks at 1625 and 1383 cm^{-1} for DMSA and Fe-O bond-specific IR bands between 400 and 850 cm^{-1} .

DMSA-IONP uptake and localization in these cancer cells was assessed following exposure to 125 μ g/ml DMSA-IONPs for 24 h, studying their internalization and localization by TEM and confocal microscopy. DMSA-IONPs were internalized by both tumor cell lines and localized in endosome-like vesicles (Fig. 1d) that corresponded to lysosomes, as suggested by lamp-2 staining (Fig. 1e). Moreover, the internalization of DMSA-IONPs at different concentrations (125, 250 and 500 μ g/ml) was assessed after 24 h, quantifying iron content by ICP-OES. There was a dose dependent internalization of DMSA-IONPs by both cell lines (Fig. 1f), although the highest dose of 500 μ g/ml of DMSA-IONPs did not enhance their uptake in MDA-MB-231 cells. Both cell lines exhibited localization of DMSA-IONPs within lysosomes, however, U87MG cells internalized substantially more DMSA-IONPs than MDA-MB-231 cells. Specifically, MDA-MB-231 cells reached an iron content of 2.71, 5.4 and 5.3 pgFe/cell (125, 250 and 500 μ g/ml, respectively), whereas U87MG cells showed markedly higher iron contents of 10.6,



(caption on next page)

Fig. 1. DMSA-IONP characterization and internalization by MDA-MB–231 and U87MG tumor cells. **a.** TEM micrograph of DMSA-IONPs. Scale bar: 50 nm. **b.** DMSA-IONP hydrodynamic size in water. **c.** FTIR spectra of DMSA-IONPs. Characteristic IR iron-oxygen bands were detected at $850\text{--}400\text{ cm}^{-1}$. Characteristic IR 1625 , 1383 and 1140 cm^{-1} bands were detected, corresponding to the COO^- , OH and CO groups as indicated in the insert on the right. **d.** TEM images of DMSA-IONP internalization and localization in MDA-MB–231 (upper panel) and U87MG (lower panel) tumor cells: N, nucleus. Dashed squares (1 and 2) show the presence of DMSA-IONPs in endosome-like vesicles (black arrowheads), amplified to the right of the panel. Scale bar: $5\text{ }\mu\text{m}$ for control condition and $2\text{ }\mu\text{m}$ for DMSA-IONP treated cells. **e.** Confocal images of DMSA-IONP treated MDA-MB–231 (upper panel) and U87MG (lower panel) tumor cells showing DMSA-IONPs in lysosomes (red arrowheads): Lysosomes marked with Lamp–2 antibody (red); nucleus marked with DAPI (blue); DMSA-IONPs visualized by reflection (grey/white). The dashed inserts are amplified on the right of the panel. Images were acquired on a Leica STELLARIS 5 multispectral confocal system using an oil-immersion $63\times$ objective and a zoom factor of 3 or $4\times$. Scale bar: $10\text{ }\mu\text{m}$. **f.** ICP-OES quantification of DMSA-IONP internalization by MDA-MB–231 (upper graph) and U87MG (lower graph) tumor cells after 24 h in the presence of increasing doses of DMSA-IONPs ($125\text{ }\mu\text{g/ml}$, $250\text{ }\mu\text{g/ml}$ and $500\text{ }\mu\text{g/ml}$).

16.4 and 33.9 pgFe/cell at the same doses.

3.2. DMSA-IONPs induce a clear cell-dependent distribution of ROS and lipid peroxidation across tumor cell lines

Ferroptosis is a type of ROS- and iron-dependent cell death, characterized by excess iron accumulation, oxidative stress and lipid peroxidation [39]. To study the different cell-specific sensitivity to IONP-induced ferroptosis, first ROS production and lipid peroxidation induction were assessed in both cell lines.

As DMSA-IONPs exhibit dose-dependent internalization, ROS production was also evaluated following exposure to increasing concentrations of DMSA-IONPs (125 , 250 and $500\text{ }\mu\text{g/ml}$). Cells were exposed to DMSA-IONPs for 24 h and ROS production was visualized by confocal imaging of DHR staining (Figs. 2a and 2b). DMSA-IONPs induced ROS production in both cell lines but to a different extent. Interestingly, ROS production by MDA-MB–231 cells was specifically induced in large round or punctate structures (red arrowheads) and in the cytoplasm, as witnessed by the diffuse staining at higher doses of DMSA ($250\text{ }\mu\text{g/ml}$ and $500\text{ }\mu\text{g/ml}$; Fig. 2a). Moreover, DMSA-IONPs (white arrowheads) were present in these punctate structures where ROS accumulated. By contrast, ROS production in U87MG cells was more diffuse throughout the cytoplasm (Fig. 2b) but also co-localized with DHR staining (white arrowheads).

To quantify ROS production over time, cells were incubated with increasing doses of DMSA-IONPs (125 , 250 and $500\text{ }\mu\text{g/ml}$) for 3 and 24 h, quantifying ROS production with DHR staining by flow cytometry (Fig. 2c-d and Figure S1). DMSA-IONPs induced time and dose-dependent ROS production in both cell lines, with higher induction on the breast cancer MDA-MB–231 cell line compared to the glioma U87MG cell line. For example, at the highest dose ($500\text{ }\mu\text{g/ml}$) MDA-MB–231 experienced a 10-fold increase in ROS production after a short exposure (3 h) (Fig. 2c) and it shifted to a 13-fold change after a 24 h incubation (Fig. 2c). Meanwhile, in U87MG ROS production shifted from a 2- to 4-fold increase relative to the controls at 3 h (Fig. 2d) to a 5- to 15-fold change at 24 h (Fig. 2d) with increasing doses of DMSA-IONPs.

The accumulation and mobilization of iron can provoke excess ROS production, which in turn may trigger lipid peroxidation [24]. Lipid peroxidation results from the oxidation of PUFAs in membrane lipids [40]. In relation to this, ferroptosis is the iron-dependent damage to lipids that ends up with lipid oxidation and the lethal accumulation of lipid hydroperoxide [41]. Once ROS production was confirmed, lipid peroxidation was measured in MDA-MB–231 and U87MG cells after a 24 h exposure to increasing doses of DMSA-IONPs, as evaluated with the C11-Bodipy lipophilic ROS sensor that shifts from red to green emission when oxidized (visualized by confocal microscopy: Figs. 2e and 2g). DMSA-IONP treatment increased lipid peroxidation in both tumor cell lines but to a different extent (Fig. 2e-h). Lipid peroxidation in MDA-MB–231 cells was apparent as large puncta and spherical structures (green arrowheads, Fig. 2e), similar to those observed with DHR staining (Fig. 2a). In terms of Bodipy green fluorescence, MDA-MB–231 cells shifted from mean values of 5000 a.u. in untreated cells towards mean values from 7400 to 8000 a.u. after exposure to DMSA-IONPs (Fig. 2f). Lipid peroxidation in U87MG cells adopted a more diffuse

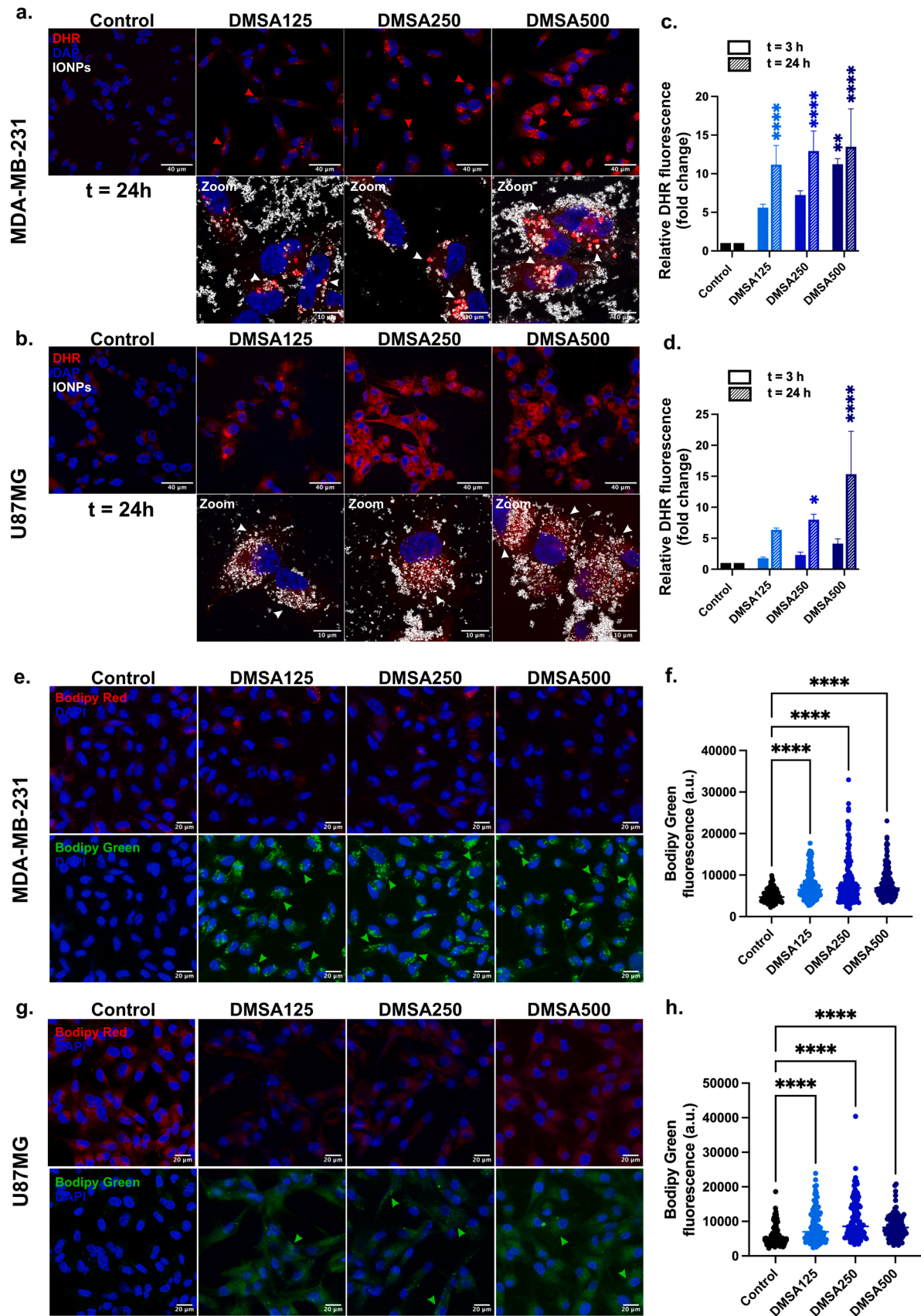
pattern but with the presence of some small punctate structures (green arrowheads, Fig. 2g). This diffuse staining resembles the diffuse production of ROS (Fig. 2b) in U87MG cells. The Bodipy green fluorescence intensity in these cells shifted from mean values of 4300 a.u. when untreated towards mean values of $8500\text{--}10,000\text{ a.u.}$ following exposure to DMSA-IONPs (Fig. 2h).

While these results demonstrate that DMSA-IONPs induce ROS in both types of tumor cells in a dose- and time-dependent manner, ROS levels were markedly higher in MDA-MB–231 than in U87MG cells, despite the former internalizing fewer DMSA-IONPs. Moreover, lipid peroxidation was also more strongly induced in MDA-MB–231 compared to U87MG. Moreover, MDA-MB–231 presented ROS and lipid peroxides accumulation in large round or punctate structures, while U87MG cell lines showed a diffused pattern. Therefore, although DMSA-IONPs can induce ROS and lipid peroxidation in both cell lines, it clearly demonstrates a cell-specific disposition.

3.3. DMSA-IONP treatment sensitizes MDA-MB–231 cell line to ferroptosis, marked by elevated ROS and lipid peroxidation-driven cell death

After correlating the increase in ROS production with the induction of lipid peroxidation (Fig. 2), we evaluated whether long-term exposure to DMSA-IONPs induced tumor cell death by ferroptosis. Hence, MDA-MB–231 and U87MG cells were treated for 24, 48 and 72 h with increasing doses of DMSA-IONPs, and cell death was assessed at different times (Fig. 3).

Cell death was assessed by Annexin V-PI staining and flow cytometry, quantifying the proportion of total dead and alive cells after exposure to DMSA-IONPs (Figs. 3a and 3b). The medium and highest dose of DMSA-IONPs (250 and $500\text{ }\mu\text{g/ml}$) induced significant death of MDA-MB–231 cells in a time and dose-dependent manner (Fig. 3a), whereas the only dose that induced significant U87MG cell death was the highest dose of DMSA-IONPs ($500\text{ }\mu\text{g/ml}$) after a 72 h incubation (Fig. 3b). The mechanism underlying this cell death was studied through Annexin V-PI staining (Figs. 3c and 3d), distinguishing early apoptotic (only Annexin V staining), late apoptotic (both Annexin V and PI staining) and necrotic (only PI staining) cell death. DMSA-IONPs increased both early and late apoptosis in MDA-MB–231 cells, whereas late apoptosis and necrosis was induced after 48 and 72 h treatments with the highest doses of DMSA-IONPs ($500\text{ }\mu\text{g/ml}$) in U87MG cells. Ferroptosis is a non-apoptotic mechanism of cell death [42] and Annexin-V staining suggests apoptotic cell death as opposed to ferroptosis. Although Annexin V labelling is conventionally associated with phosphatidylserine externalization during apoptosis, it has also been linked to necrosis [43]. Furthermore, while ferroptosis has been described as a regulated-necrosis, it has also been linked to Annexin-V staining due to the induction of lipid peroxidation [43,44]. Therefore, the death of MDA-MB–231 cells after DMSA-IONP exposure could be due to ferroptosis even though these cells are Annexin-V stained. To determine whether cell death might be due to apoptosis, caspase–3 protein levels and activation were assessed in response to a 24 h exposure to DMSA-IONPs (Figure S2). As a positive control for the cleavage of caspase–3, cells were treated overnight with $1\text{ }\mu\text{M}$ staurosporine (STS). Overnight treatment with DMSA-IONPs did not induce caspase–3



(caption on next page)

Fig. 2. DMSA-IONPs induce time and dose-dependent ROS production and lipid peroxidation in MDA-MB–231 and U87MG cells with a distinctive accumulation. a and b. Confocal microscopy visualization of ROS production using the DHR probe in MDA-MB–231 (a) and U87MG (b) cells treated for 24 h with DMSA-IONPs or untreated. Red arrowheads indicate the induction of ROS production in punctate/round structures: ROS production, DHR staining (red); DAPI stained nucleus (blue); DMSA-IONPs visualized by reflection (grey/white). The lower panel indicates the presence of DMSA-IONPs in the punctate/round structures where ROS are produced (white arrowheads). Images were acquired on a Leica STELLARIS 5 multispectral confocal system using an oil-immersion 63X objective and a zoom factor of 3 or 4X. Scale bar: 40 μ m. **c.** Mean fluorescence intensity of ROS production in MDA-MB–231 cells. **d.** Mean fluorescence intensity of ROS production in U87MG cells. **e.** Lipid peroxidation in MDA-MB–231 cells assessed in confocal images of the BODIPY probe after a 24 h treatment with DMSA-IONPs: upper panel, red BODIPY fluorescence; lower panel green BODIPY fluorescence; nuclei stained with DAPI in blue; green arrowheads indicate lipid peroxidation in large round punctate structures. Scale bar: 20 μ m. **f.** Lipid peroxidation in MDA-MB–231 cells quantifying the green BODIPY fluorescence after DMSA-IONP treatment: each dot represents the fluorescence of one cell. **g.** Lipid peroxidation in U87MG cells assessed in confocal images of the BODIPY probe after a 24 h treatment with DMSA-IONPs: upper panel, red BODIPY fluorescence; lower panel green BODIPY fluorescence; nuclei stained with DAPI in blue. Scale bar: 20 μ m. **h.** Lipid peroxidation in U87MG cells quantifying the BODIPY green fluorescence after DMSA-IONP treatment: each dot represents the fluorescence of one cell. To analyze the differences of ROS production and lipid peroxidation between untreated and DMSA-IONP treated cells, a two-way ANOVA and a one-way ANOVA with Dunnett's multiple comparison test were performed, respectively: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

cleavage, whereas overnight treatment with STS did (Figure S2). These results indicate that the cell death observed after 24 h of treatment is not due to apoptosis.

To confirm that cell death induction was ROS- and lipid peroxidation-dependent, first IONP internalization and ROS production over time were assessed (Figure S3). In both cell lines, DMSA-IONPs were internalized in a dose and time-dependent manner (Figure S3a and S3b). Next, to evaluate if the induction of ROS provoked by DMSA-IONPs at longer incubations is maintained or altered, DHR staining was evaluated by flow cytometry (Figure S3c and S3d). ROS production induced by increasing doses of DMSA-IONPs remained similar over time in MDA-MB–231 cells, although a significant increase in ROS production occurred over time from 8-fold to an 11-fold increase in DHR fluorescence (Figure S3c). ROS production by U87MG cells also increased significantly in a dose and time-dependent manner, shifting from a 1.5- to 2-fold increase in DHR fluorescence after a 24 h towards a 3.5-, 5.8- and 10-fold increase in DHR fluorescence after a 72 h incubation at these concentrations of DMSA-IONPs (Figure S3d).

Next, to determine whether cell death was to any extent dependent on lipid peroxidation and ferroptosis, cell death was assessed in cells treated with DMSA-IONPs in the presence of a Ferroptosis inhibitor, Ferrostatin–1. Ferrostatin–1 is an antioxidant that inhibits lipid peroxidation by scavenging ferrous iron and alkoxyl radicals produced during lipid peroxidation [45]. The toxicity of ferrostatin–1 (1, 2.5 and 5 μ M, 24 h) was first studied by flow cytometry in both cell lines by staining them with Annexin-V-PI (Figure S4a). Ferrostatin–1 was not toxic to these cell lines at any of the doses tested (Figure S4a), as no cell death was induced. The effect of Ferrostatin–1 on DMSA-IONP internalization was assessed (Figure S4b), exposing the cells to ferrostatin–1 for 1 h and then to DMSA-IONPs (500 μ g/ml) in the presence of ferrostatin–1 for 24 h. When the iron content inside the cell after treatment was quantified using ICP-OES (Figure S4b), ferrostatin–1 clearly had no effect on DMSA-IONP internalization in both cell lines (Figure S4b). Next, the effect of ferrostatin–1 on the lipid oxidation induced by DMSA-IONPs was also studied with the Bodipy C11 probe by flow cytometry in cells treated in a similar manner (Fig. 3e and Figure S4c). In the presence of ferrostatin–1 there was less lipid peroxidation in MDA-MB–231 cells than when they are exposed to DMSA-IONPs alone, an effect that was less marked in U87MG cells where lipid peroxidation was previously shown more diffused (Fig. 2). Interestingly, MDA-MB–231 cells exhibited a pronounced increase in lipid peroxidation upon erastin treatment, whereas U87MG displayed a more modest increase in lipid peroxidation (1.2-fold increase, Fig. 3e), further confirming the higher susceptibility to ferroptosis for MDA-MB–231 cells.

Since ferrostatin–1 reduced the lipid peroxidation induced by DMSA-IONPs, we assessed if it also affected the cell death induced by DMSA-IONPs. Cells were treated in a similar manner as above and the cell death induced was assessed by flow cytometry after Annexin-V-PI staining (Fig. 3f). Exposure to the lower doses of DMSA-IONPs (125 and 250 μ g/ml) in the presence of ferrostatin–1 dampened the cell

death induced in MDA-MB–231 cell line (Fig. 3f), indicating that cell death was due to lipid peroxidation. Moreover, this reduction in cell death occurred in the absence of caspase–3 cleavage (Figure S2). By contrast, at higher DMSA-IONP dose (500 μ g/ml) the presence of ferrostatin–1 did not significantly diminish cell death (Fig. 3f), suggesting that cell death at higher doses of DMSA-IONPs was not due to ferroptosis. Conversely, U87MG did not experience any significant changes on cell death, not even at the highest dose of 500 μ g/ml. Moreover, in the positive control group, erastin treatment induced a marked increase in cell death in MDA-MB–231 cells, whereas only a limited increase was observed in U87MG cells (Fig. 3f), further supporting the lower sensitivity of U87MG cells to ferroptotic stimuli.

Next, ROS production in the dead cell population was assessed following exposure to DMSA-IONPs (Figure S4d–e). In MDA-MB–231 cells, two distinct populations with different levels of ROS production were observed: one exhibiting low ROS levels comparable to controls; and another displaying markedly higher ROS production than that in the live cell population (a 20–30-fold change relative to the controls: Figure S4d). By contrast, analysis of ROS production in dead U87MG cells (Figure S4e) revealed a heterogeneous distribution rather than a single uniform peak, with an average DHR intensity 7- to 8-fold higher than that of the controls, values similar to those observed in the live cell population, but including a subpopulation with higher DHR intensities, reaching 12- to 14-fold above control levels (Figure S4e expressed as high). In addition, ROS production was evaluated in the presence and absence of ferrostatin–1 treatment together with DMSA-IONPs (Fig. 3g and S4f). Ferrostatin–1 significantly reduced ROS production at all tested doses (Fig. 3g), demonstrating that cell death induction at the lower DMSA-IONPs concentration (125 and 250 μ g/ml) was primarily due to ferroptosis. In contrast, cell death induction at the higher dose (500 μ g/ml) appeared to be independent of ROS production.

Altogether these results highlight that MDA-MB–231 is sensitive to ferroptosis cell death, whereas, while ROS and lipid peroxidation are induced in U87MG cell line, no cell death induction was observed. The distinct ROS production pattern and cell death responses pointed to a cell line-specific behavior upon DMSA-IONPs treatment in both cell lines. Besides, it clearly states that overloading cells with DMSA-IONPs can induce cell death but in a non-ROS dependent manner, probably due to the excessive accumulation of DMSA-IONPs in lysosomes which may activate alternative lysosome-dependent cell death pathways. All in all, the distinct ROS profiles and cell death responses observed between the two cell lines suggested differential cellular behavior.

3.4. DMSA-IONPs limit tumor cell proliferation in both 2D and 3D models

High levels of ROS could interfere with tumor cell proliferation by affecting cell cycle progression, [46] although sustained ROS production could also trigger cell death. Although no cell death induction was observed in U87MG, we wondered whether long-term incubation with increasing concentrations of DMSA-IONP could limit cell proliferation in

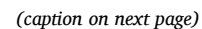


Fig. 3. DMSA-IONP treatment sensitizes MDA-MB–231 cell line to ferroptosis, marked by elevated ROS and lipid peroxidation-driven cell death. a–b. The induction of MDA-MB–231 (a) and U87MG (b) cell death after a 24, 48 and 72 h treatment with increasing doses of DMSA-IONPs assessed by flow cytometry following Annexin/PI staining: living cells, grey; dead cells, pink. **c–d.** Population of MDA-MB–231 (c) and U87MG (d) cells that underwent cell death after a 24, 48 and 72 h treatment with increasing doses of DMSA-IONPs. Light pink, Annexin-V⁺/PI⁻; red, Annexin-V⁺/PI⁺; dark red: Annexin-V⁺/PI⁺. **e.** The effect of DMSA-IONPs on lipid peroxidation in the presence of ferrostatin–1 assessed through the green fluorescence intensity of the Bodipy C–11 probe by flow cytometry. Untreated cells (–) or those treated with 1, 2.5 or 5 μ M ferrostatin–1, alone or in conjunction with DMSA-IONPs for 24 h. Positive control: Erastin (10 μ M)-treated cells. Green Bodipy fluorescence was normalized to the untreated cells. **f.** The effect of DMSA-IONPs in the presence of ferrostatin–1 on cell death assessed by flow cytometry of Annexin-V-PI-stained cells: Grey, living cells; pink, dead cells. Positive control: Erastin (10 μ M)-treated cells. **g.** The effect of DMSA-IONPs in the presence of ferrostatin–1 on ROS production by flow cytometry of DHR stained cells. The differences in cell viability, cell death populations, Bodipy green fluorescence and DHR staining between untreated and DMSA-IONP treated cells were evaluated by two-way ANOVA with a Dunnett's multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

2D and 3D culture models.

First, to evaluate the effect of DMSA-IONPs on long-term proliferation, cells were stained with crystal violet after an exposure of 24, 48 and 72 h and visualized by optical microscopy (Figs. 4a and 4c). Cell confluence was affected by the highest dose of DMSA-IONPs (500 μ g/ml) at each time point, and all doses of the DMSA-IONPs used affected cell confluence at 72 h. The absorbance of crystal violet was used to quantify cell proliferation at every time point and each concentration of DMSA-IONPs (Figs. 4b and 4d). All doses of DMSA-IONPs reduced the viability of both cell lines after a 72 h exposure, with 79%, 68% and 41% viability of MDA-MB–231 cells exposed to DMSA-IONPs at 125, 250 and 500 μ g/ml, respectively (Fig. 4b) and 80%, 68% and 55% viability of U87MG cells exposed to DMSA-IONPs at 125, 250 and 500 μ g/ml, respectively (Fig. 4d). In both cell lines, cell viability was only reduced after the shortest incubation (24 h) at the highest dose of DMSA-IONPs 500 μ g/ml, with 61% viability of MDA-MB–231 cells and 90% viability of U87MG cells. After a 48 h exposure, only U87MG proliferation was significantly affected (Fig. 4d) by all concentrations of DMSA-IONPs, whereas no significant differences were observed for MDA-MB–231 proliferation at doses of 125 and 250 μ g/ml (Fig. 4b). We previously found that long-term incubation with DMSA-IONPs (125 μ g/ml) could affect tumor cell proliferation by increasing the proportion of cells arrested in the G0/G1 phase of the cell cycle [33]. Here, we corroborated that higher doses of DMSA-IONPs also affect the proliferation of MDA-MB–231 and U87MG tumor cells.

Considering that tumors do not maintain a 2D structure in the body, 3D cell cultures help to reproduce the shape, polarization and heterogeneity of cancer cells *in vivo* [47]. As DMSA-IONPs affect cancer cell proliferation and death through the production of ROS over time, we studied their influence in a 3D cancer cell models to better understand their potential to produce these effects *in vivo*. As such, tumor spheroids were obtained by the hanging-drop method, obtaining U87MG cell spheroids after 72 h and MDA-MB–231 cell spheroids after 96 h. These tumor spheroids were then incubated for up to 72 h with increasing doses of DMSA-IONPs, monitoring their size (Fig. 4e–h). While U87MG spheroids had a diameter of approximately 300 μ m (Fig. 4h), MDA-MB–231 spheroids were less uniform and had diameters of approximately 350 μ m (Fig. 4f). While untreated MDA-MB–231 spheroids increased in size to 370 μ m after 72 h, DMSA-IONP treatment of MDA-MB–231 spheroids decreased their size, recording diameters of 270, 290 and 285 μ m after a 72 h exposure to concentrations of 125, 250 and 500 μ g/ml, respectively (Fig. 4f). Similarly, untreated U87MG spheroids increased size over time from 300 up to 450 μ m of diameter in 72 h, while those exposed to DMSA-IONPs achieved diameters of 390, 370 and 360 μ m at concentrations of 125, 250 and 500 μ g/ml, respectively (Fig. 4h), showing a clear restriction of spheroid growth.

3.5. DMSA-IONPs affects the GSH antioxidant system in cancer cells

As different cell-specific sensitivity upon ferroptosis was observed, we wondered if cell lines presented different antioxidant mechanisms to fight for lipid peroxidation. In addition to lipid peroxidation, ferroptosis is characterized by altered GSH consumption and altered GPX4 concentrations or activity [39]. Therefore, the induction of ferroptosis and

antioxidant defenses were further assessed by measuring the GPX4 and SLC7A11 mRNA expression and the GSH concentration in untreated and DMSA-IONP treated cells (Fig. 5a–d). GPX4 mRNA expression by MDA-MB–231 cells was not modified on exposure of DMSA-IONPs, although a significant increase in these transcripts was induced in U87MG cells by exposure to the lowest and highest doses (125 and 500 μ g/ml; Fig. 5a). By contrast, a 24 h exposure of MDA-MB–231 cells to DMSA-IONPs reduced the GSH concentration by 50%, as seen through both the absolute GSH concentrations (Fig. 5b) and the ratio normalized to untreated cells (Fig. 5c). DMSA-IONP treatment also reduced the GSH concentration in U87MG cells, but only at the lower doses (125 and 250 μ g/ml; Fig. 5b–c). SLC7A11 (encoding the xCT transporter) mRNA expression was upregulated in both cell lines, with a greater fold increase observed in U87MG cells than in MDA-MB–231 cells (Fig. 5d). The stronger induction of SLC7A11 in U87MG correlated with the increased of GPX4 expression observed in this cell line, consistent with the role of xCT-mediated cystine uptake in supporting GSH synthesis and antioxidant response [48].

3.6. DMSA-IONPs induce a different disposition of the total labile iron pool in BC cell line compared to the U87MG glioma cell line

The distinct ROS production pattern and cell death responses pointed to a cell line-specific behavior upon DMSA-IONPs treatment which was not mainly done by different antioxidant defenses induction. As ferroptosis is a type of ROS- and iron-dependent cell death, we wondered if the distinct cell death behavior was due to cell-specific sensitivity to IONP-induced ferroptosis. The main characteristics of ferroptosis are excess iron accumulation, oxidative stress and lipid peroxidation [39]. One of the biochemical features of ferroptosis is the accumulation of excess iron in the form of ferrous ions (Fe^{2+}), which can directly generate ROS through the Fenton reaction. Hence, an increase in the cellular LIP is usually observed during ferroptosis [49] and thus, different regulators of iron metabolism affect the cell's sensitivity to ferroptosis.

To assess if exposure to DMSA-IONPs modified the cellular LIP distinctly on cells, cells treated with DMSA-IONPs for 3 and 24 h were stained with the iron-selective FerroOrange probe and compared to untreated cells (Figs. 6a and 6c). FerroOrange specifically detects free iron in living cells (mostly Fe^{2+}), which can then be related to cell damage. The treatment of tumor cells with DMSA-IONPs augmented the LIP, as witnessed by an increase in FerroOrange fluorescence after shorter incubations ($t = 3$ h), whereas the FerroOrange intensity decreased after a 24 h exposure. Moreover, the pattern of FerroOrange fluorescence was quite different in each cell line. In MDA-MB–231 cells the LIP accumulated in large punctate structures (white arrowheads), both in untreated and DMSA-IONP treated cells (Fig. 6a), whereas U87MG cells there was a more diffuse accumulation of the LIP extending throughout the cytoplasm (Fig. 6c). Quantification of FerroOrange fluorescence intensity (Figure S5) revealed higher overall fluorescence levels in U87MG cells than in MDA-MB–231 cells. The changes in FerroOrange fluorescence also differed between cell lines (Figs. 6b and 6d). In MDA-MB–231 cells, DMSA-IONP treatment significantly increased the total intracellular LIP after a 3 h treatment but resulted in a decrease

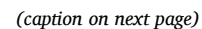


Fig. 4. Proliferation of MDA-MB–231 and U87MG cells in 2D and 3D cell culture after exposure to DMSA-IONPs. a. Representative images of the long-term proliferation of crystal violet stained MDA-MB–231 cells after treatment with increasing doses of DMSA-IONPs for 24, 48 and 72 h. b. Quantification of crystal violet absorbance in MDA-MB–231 cells after treatment with increasing doses of DMSA-IONPs for 24, 48 and 72 h. c. Long-term proliferation of crystal violet stained U87MG cells after treatment with increasing doses of DMSA-IONPs for 24, 48 and 72 h. d. Quantification of crystal violet absorbance in U87MG cells after treatment with increasing doses of DMSA-IONPs for 24, 48 and 72 h. e. Representative images of untreated MDA-MB–231 spheroids and those exposed to DMSA-IONPs for 24, 48 and 72 h. Scale bar: 200 μ m. f. MDA-MB–231 spheroid diameter over time. g. Representative images of untreated U87MG spheroids and of those exposed to DMSA-IONPs for 24, 48 and 72 h. Scale bar: 200 μ m. h. U87MG spheroid diameter over time. The differences on cell growth between untreated and DMSA-IONP treated cells were evaluated by two-way ANOVA with a Dunnett's multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

after 24 h compared with untreated controls (Fig. 6b). Conversely, U87MG cells showed an increase in the LIP only after 3 h of treatment, whereas FerroOrange fluorescence returned to levels comparable to those of control cells after 24 h of exposure (Fig. 6d).

Iron mobilization is regulated by multiple of proteins [15] and given the nature of DMSA-IONPs, and that they modify the total intracellular LIP, the changes in genes and proteins involved in iron metabolism, iron uptake, storage, utilization and efflux were evaluated (Fig. 6e–g). DMSA-IONP treatment induced an increase in the *IREB2*, *LCN2* and *SLC48A1* gene transcripts expressed by MDA-MB–231 cells, genes encoding the IRP2, Lipocalin and HRG1 proteins, respectively, and implicated in the mobilization of free iron (Fig. 6e). Similarly, DMSA-IONP treatment induced an increase in *IREB2* and *SLC48A1* mRNA transcripts in U87MG cells, in addition to an increase in *SLC11A2* transcripts that encode the DMT1 transporter responsible for the export of iron from lysosomes to the cytosol (Fig. 6e). The ferritin protein responsible for iron storage was also assessed (Fig. 6f) and DMSA-IONPs induced an increase in ferritin (Fig. 6f) in both cell lines, thereby limiting the free iron in the cytoplasm. As such, ferritin accumulation is a cellular mechanism to avoid iron overload and the increase in ferritin could explain the decrease in LIP at 24 h. Hence, both cell lines experienced changes on the total LIP and iron regulation genes and proteins, but MDA-MB–231 experienced the accumulation of iron in large punctate structures, whereas U87MG exposed a diffused iron pattern throughout the whole cytoplasm.

3.7. Accumulation of iron ions inside lysosomes triggers confinement of ROS and lipid peroxides within these organelles sensitizing MDA-MB–231 cells towards ferroptosis

The treatment of MDA-MB–231 and U87MG cells with DMSA-IONP altered the distribution of iron and lipid peroxidation, and while MDA-MB–231 cells accumulated ROS, iron and lipid peroxides in punctate structures, in U87MG cells they were accumulated diffusely throughout the cell. These results suggest that the different cell death induction, might be somehow dependent on the localization of iron ions and ROS, as MDA-MB–231 cells experienced cell death induction, while U87MG barely did.

Lysosomes are storage compartments for intracellular iron due to autophagic breakdown of iron-containing proteins or cellular debris, [50] although labile iron can be also imported into mitochondria to perform certain cellular functions [51]. To examine if iron accumulates in the lysosomes, mitochondria or cytosol, cells exposed to DMSA-IONPs for 24 h were stained with the FerroOrange iron-selective probe together with the SiR-lysosome probe (to detect lysosomes) or the MitoTracker probe (to identify mitochondria: Fig. 7a and Figure S6). In MDA-MB–231 cells, FerroOrange accumulates in puncta that correspond to lysosomes and that become larger following DMSA-IONP treatment (Fig. 7a, left). By contrast, FerroOrange staining is diffuse in U87MG cells and spread all over the cytosol, yet also co-localizing to some extent with lysosomes albeit not as clearly as in MDA-MB–231 cells (Fig. 7a, right). When assessing the intracellular iron in mitochondria it is clear that the punctate accumulations of iron in MDA-MB–231 cells do not correspond to mitochondria (Figure S6a). By contrast, the FerroOrange staining in U87MG cells did appear to co-localize with the mitochondrial marker and its diffuse distribution

appears to adopt a long and thin form similar to mitochondrial structures (Figure S6b). Thus, DMSA-IONPs appear to enhance iron retention in the lysosomes of MDA-MB–231 cells, organelles responsible for degradation and recycling but that also fulfil a role in iron homeostasis [17]. Accordingly, MDA-MB–231 cells respond to DMSA-IONPs by retaining iron in lysosomes and limit its export into the cytosol, thereby avoiding an increase in the total LIP. By contrast, DMSA-IONPs seem to induce the release of iron from lysosomes into the cytosol of U87MG cells from where it is imported into mitochondria. These results are consistent with the DMT1 transporter overexpression by U87MG cells, as DMT1 is responsible for exporting iron from endosomal compartments into the cytosol [15].

To determine whether the localization of iron ions within mitochondria could affect mitochondrial function, mitochondrial membrane potential ($\Delta\Psi$ m) was evaluated after 24, 48, and 72 h of treatment with increasing concentrations of DMSA-IONPs (125, 250, and 500 μ g/ml) (Figure S7). MDA-MB–231 cells did not exhibit significant changes in mitochondrial membrane potential under any of the conditions tested (Figure S7a). In U87MG cells, a modest decrease in mitochondrial membrane potential was observed after 24 h of treatment with 125 μ g/ml DMSA-IONPs. However, this effect was transient, and $\Delta\Psi$ m returned to basal levels at later time points (Figure S7b).

The presence of free iron in lysosomes of MDA-MB–231 cells could induce a Fenton reaction and the production of ROS inside lysosomes, whereas the release of iron from lysosomes into the cytosol and its uptake by mitochondria in U87MG cells could induce general ROS production in this cell line. Having observed ROS in large punctate structures in MDA-MB–231 cells (Fig. 2a) and a diffuse distribution throughout the cytoplasm in U87MG cells ROS (Fig. 2b), the production of ROS inside lysosomes due to oxidative stress was assessed by staining MDA-MB–231 and U87MG cells treated with DMSA-IONPs for DHR and with a Lamp–2 antibody (Fig. 7b). After exposure to DMSA-IONPs, oxidative stress was evident in lysosomes of MDA-MB–231 cells through their co-staining with these markers (green arrowheads, Fig. 7b upper panels) but a more diffuse pattern of ROS in U87MG cells was observed, with ROS mainly present in the cytosol outside the lysosomes (Fig. 7b lower panels).

Moreover, to determine the role of iron ions in DMSA-IONP-induced ferroptosis and to discriminate ferroptotic events from non-specific lysosomal oxidative damage, ROS generation, and lipid peroxidation were evaluated in cells treated with DMSA-IONPs in the presence of the iron chelator deferoxamine (DFO) (Figure S8). Rescue experiments were performed by adding DFO after 3 h of DMSA-IONP treatment, and the effects were evaluated at 3 h, 6 h and 24 h. DFO attenuated both ROS production (Figure S8a and S8c) and lipid peroxidation (Figure S8b and S8d), while having no effect on DMSA-IONP uptake (Figure S8e–f). These findings further demonstrate the pivotal role of iron ions in the induction of DMSA-IONP-mediated ferroptosis.

Although ferroptosis have always been described as lipid peroxidation mainly taking place at the plasma membrane, the iron present in organelles like lysosomes and mitochondria could trigger lipid peroxidation in these structures [52]. Although U87MG lipid peroxidation was clearly more diffuse than in MDA-MB–231 cells, lipid peroxidation in the latter was evident in large round structures resembling intracellular organelles. As oxidative stress and FerroOrange staining followed a similar pattern, and DMSA-IONPs apparently accumulated in lysosomes,

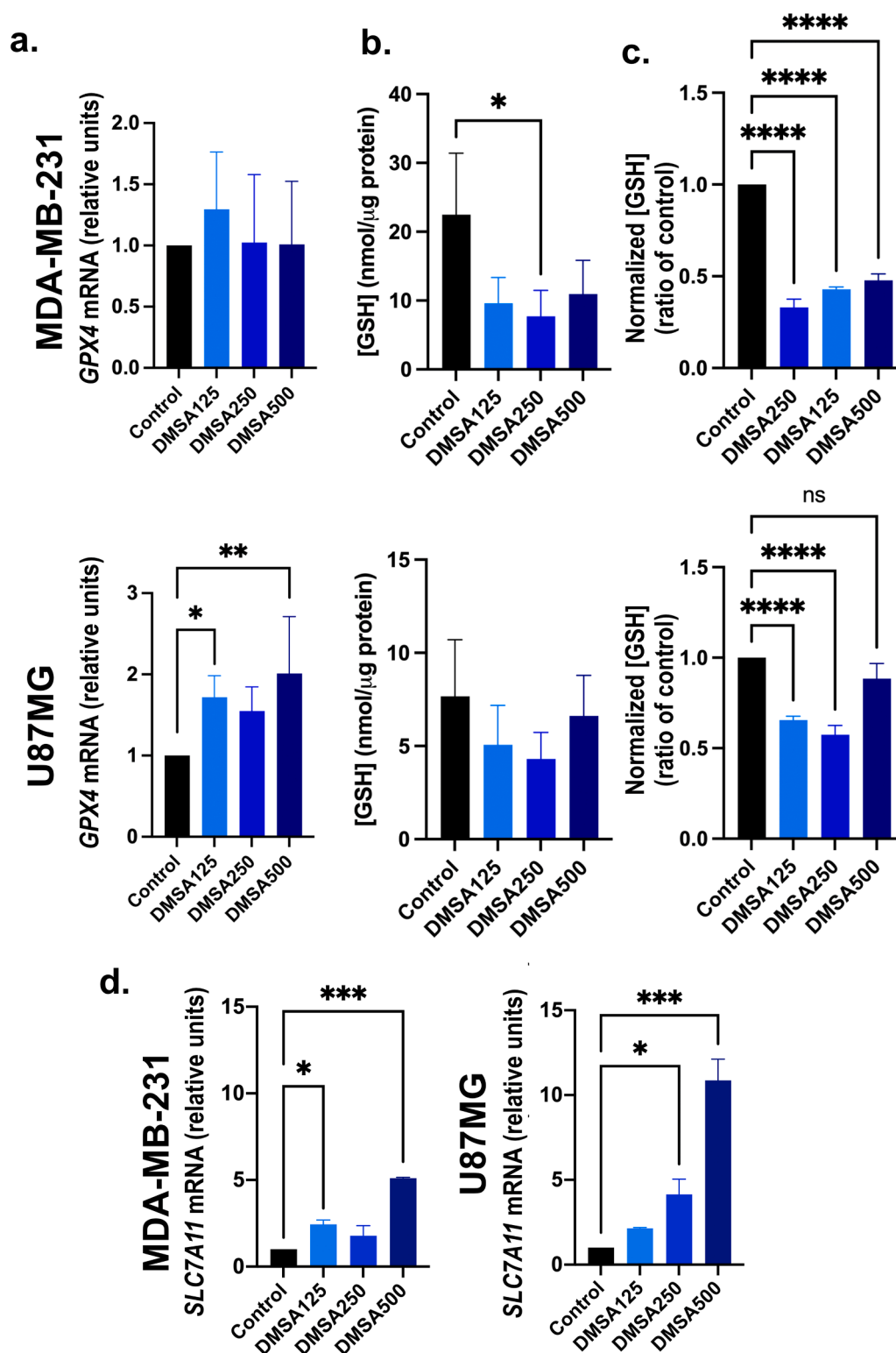
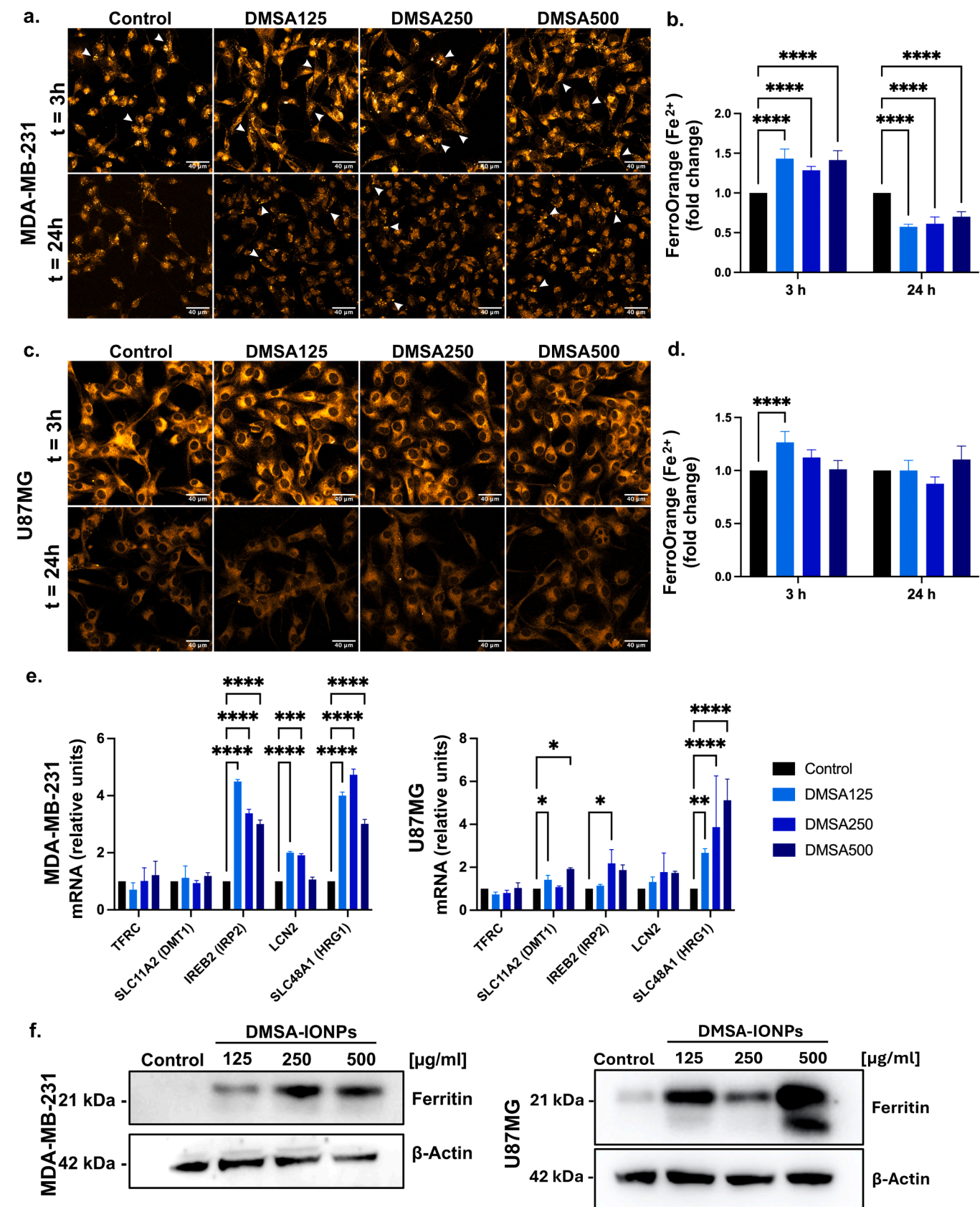


Fig. 5. Glutathione-dependent antioxidant responses following DMSA-IONP treatment. **a.** Relative GPX4 mRNA expression in MDA-MB-231 and U87MG cells after a 24 h treatment with increasing concentrations of DMSA-IONPs, assessed by RT-qPCR. **b and c.** Intracellular GSH levels in MDA-MB-231 and U87MG cells after 24 h of treatment with increasing concentrations of DMSA-IONPs. Data are presented as absolute GSH concentration (nmol GSH/ μ g protein) (**b**) and as values normalized to untreated controls (**c**). Results represent the mean values ($\pm$ SD) of three independent experiments. **d.** Relative SLC7A11 (xCT) mRNA expression in MDA-MB-231 and U87MG cells after a 24 h treatment with increasing concentrations of DMSA-IONP, assessed by RT-qPCR. Data represent the mean values ($\pm$ SD) of three independent experiments. A one-way ANOVA with Dunnett's multiple comparison test was used to analyze the differences in GSH concentration and GPX4 and SLC7A11 mRNA level between untreated and DMSA-IONP-treated cells: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.



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Fig. 6. Changes to the LIP in MDA-MB–231 and U87MG cells after exposure to DMSA-IONPs. **a.** The LIP in MDA-MB–231 cells studied with the FerroOrange probe in confocal images after a 3 and 24 h treatment with different doses of DMSA-IONPs. White arrowheads indicate the presence of FerroOrange stain in a round punctate structures. Images were acquired on a Leica SP5 multispectral confocal system with a 63X oil-immersion objective. Scale bar: 40 μm . **b.** Quantification of fold change on FerroOrange fluorescence in MDA-MB–231 cells after a 3 and 24 h exposure to DMSA-IONPs. **c.** The LIP in U87MG cells studied with the FerroOrange probe in confocal images after a 3 and 24 h exposure to different doses of DMSA-IONPs. Images were acquired on a Leica SP5 multispectral confocal system with a 63X oil-immersion objective. Scale bar: 40 μm . **d.** Quantification of fold change on FerroOrange fluorescence intensity in U87MG cells after a 3 and 24 h exposure to DMSA-IONPs. **e.** The expression of genes involved in iron metabolism by MDA-MB–231 and U87MG cells after exposure to increasing doses of DMSA-IONPs. **f.** The iron metabolism protein Ferritin in MDA-MB–231 and U87MG cells after treatment with increasing doses of DMSA-IONPs. The differences between untreated and DMSA-IONP treated cells were analyzed with a one-way or two-way ANOVA and a Dunnett's multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

the increase in free lysosomal iron would appear to induce ROS production and lead to oxidation at lysosomal membranes that would compromise their integrity. Thus, the presence of lipid peroxidation in lysosomes was further assessed in MDA-MB–231 cells treated with DMSA-IONPs by staining with the Bodipy and SiR-lysosome probe (Fig. 7c), producing clear dual staining of lysosomes in MDA-MB–231 cells exposed to DMSA-IONPs (magenta arrowheads, Fig. 7c left). Lipid peroxidation in lysosomes was also assessed in U87MG cells (Fig. 7c right) but a more diffuse pattern of lipid peroxidation was observed and hence, the correlation was less clear. Together, these results demonstrate that DMSA-IONPs induce lipid peroxidation but not at the plasma membrane but rather in lysosomes of MDA-MB–231 cells, and to a lesser extent of U87MG cells. This lipid peroxidation is driven by the accumulation of DMSA-IONPs and free Fe^{2+} in lysosomes, and could compromise their function and/or stability.

Lipid peroxides and ROS distribution was also studied over time. To evaluate if long term incubation with DMSA-IONPs altered plasma membrane oxidation, lipid peroxidation was assessed following a 48 h and 72 h exposure to DMSA-IONPs (Figure S9a–d). Although increased plasma membrane lipid peroxidation and cell rounding has been linked to subsequent membrane collapse and ferroptosis, [53] no plasma membrane peroxidation was observed in either cell line after a 48 or 72 h exposure to DMSA-IONPs (Figure S9a–d). Moreover, lipid peroxidation in U87MG cells was induced in punctate accumulations resembling lysosomes after longer exposures (72 h: Figure S9d). When ROS distribution after longer exposures to increasing doses of DMSA-IONPs was studied by confocal microscopy (48 h and 72 h exposures: Figure S9e–f), ROS were produced and accumulated in punctate structures in MDA-MB–231 cells with some spreading into the cytosol at higher doses (Figure S9e). In U87MG cells, ROS mainly accumulated in the cytosol but also in a punctate pattern (Figure S9f) after longer exposures. This result is consistent with the accumulation of lipid peroxides after a 72 h exposure to DMSA-IONPs.

These data indicates that, upon lysosomal internalization of DMSA-IONP, MDA-MB–231 cells exhibit strong iron retention and lipid peroxidation within these organelles, whereas DMSA-IONP internalization by U87MG cells leads to iron diffusion outside lysosomes. At longer times points, U87MG cells appear to accumulate ROS and lipid peroxides in the same round structures, lysosomes, which is consistent with the cell death induction observed after 72 h treatment.

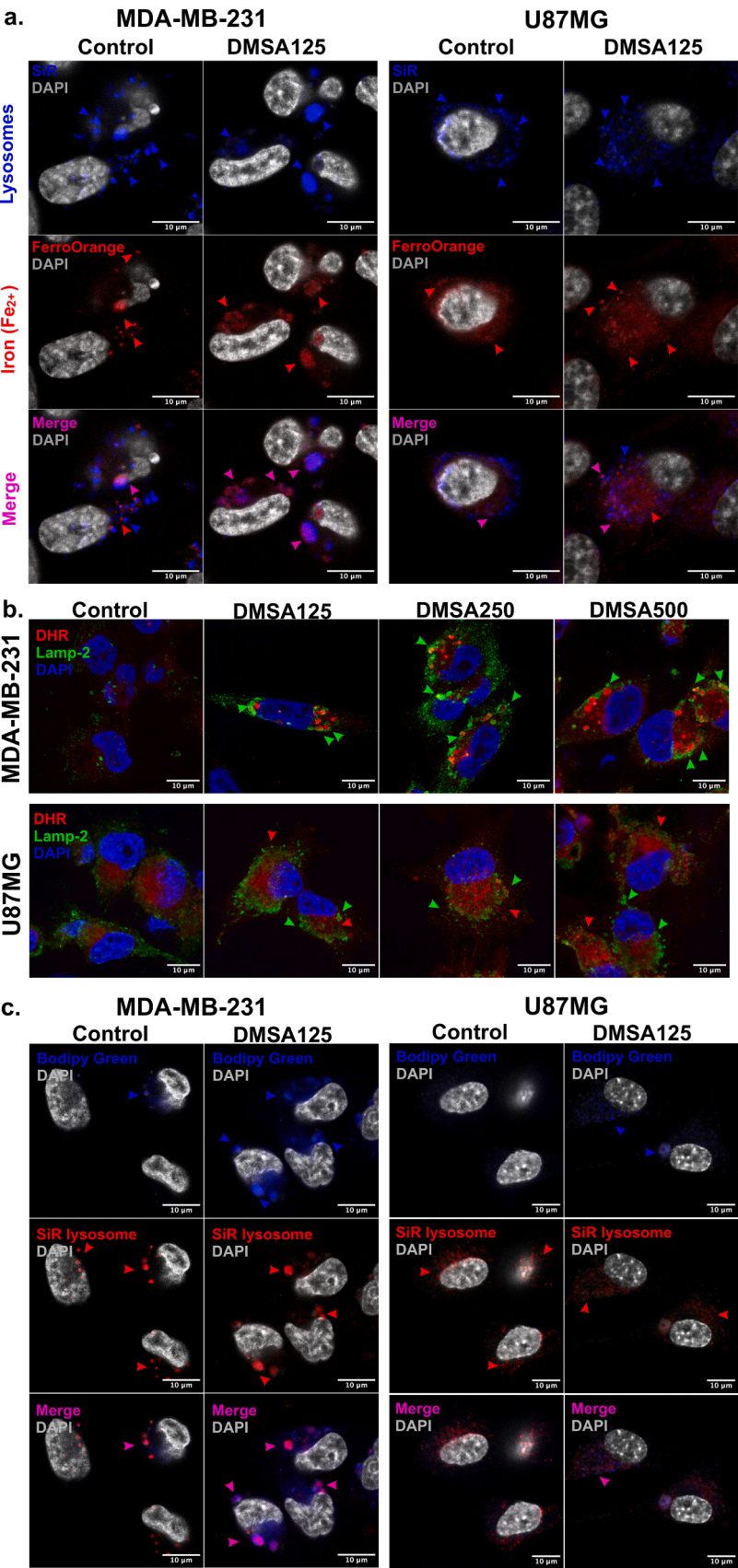
3.8. The production of ROS and lipid oxidation in lysosomes alters their integrity in MDA-MB–231 cells

Both tumor cell lines exhibited distinct patterns of iron, ROS and lipid peroxide accumulation, reflecting differences in lysosomal behavior or fitness. Lysosomes are not static but rather, they change their number, size and position in response to intracellular stress, which effects lysosomal activity and cellular homeostasis. [54] The size of lysosomes in MDA-MB–231 cells appears to increase after DMSA-IONPs treatment as the SiR-lysosome stain labels larger punctate structures than in U87MG cells (Fig. 7c). To confirm that DMSA-IONPs affect lysosomal stability and function through peroxidation of their membranes, lysosomal number, size and circularity were studied in confocal images of cells exposed to DMSA-IONPs (Fig. 8a–d). DMSA-IONPs did

not significantly affect lysosomal number in MDA-MB–231 cells (Fig. 8b) but they did affect lysosomal size (Fig. 8c) and circularity (Fig. 8d). The lysosomes in DMSA-IONP treated MDA-MB–231 cells were bigger, shifting from an average area of $0.45 \mu\text{m}^2$ in untreated cells to an area of 0.85, 1.2 and $0.81 \mu\text{m}^2$ when exposed to 125, 250 and 500 $\mu\text{g}/\text{ml}$ of DMSA-IONPs, respectively (Fig. 8c). This increase in lysosomal size was also corroborated by TEM (Figure S10). By contrast, lysosomal shape was not dramatically affected by the DMSA-IONPs as circularity remained between 0.8 and 1 a.u., although a small increase in lysosomal circularity was observed in cells exposed to 250 $\mu\text{g}/\text{ml}$ of DMSA-IONPs (Fig. 8d). U87MG cells clearly have more (Figs. 8a and 8b) and smaller lysosomes (Fig. 8c) than MDA-MB–231 cells, and they are less concentrated in the perinuclear area with a more diffuse distribution throughout the cytoplasm (Fig. 8a). DMSA-IONP treatment increased the number of lysosomes in U87MG cells, increasing from around 70 lysosomes per cell (untreated) to 100–120 lysosomes per cell exposed to DMSA-IONPs (Fig. 8b).

Lysosomes of DMSA-IONP treated U87MG cells were similar in size to those in untreated cells, ranging from an average lysosomal area of $0.52 \mu\text{m}^2$ in untreated cells to an area of 0.53, 0.57 and $0.60 \mu\text{m}^2$ cells exposed to 125, 250 and 500 $\mu\text{g}/\text{ml}$ of DMSA-IONPs, respectively (Fig. 8c). Again, lysosomal shape was not dramatically affected as their circularity remained between 0.8 and 1 a.u., although the smaller lysosomal area observed after exposure to 125 $\mu\text{g}/\text{ml}$ DMSA-IONPs corresponded with a mild increase in lysosomal circularity (Fig. 8d). Moreover, the small increase in lysosomal size when U87MG cells were exposed to 500 $\mu\text{g}/\text{ml}$ DMSA-IONPs was associated with a mild decrease in lysosomal circularity (Fig. 8d).

As lysosomes are one of the main storage sites for iron, their fitness has been linked to ferroptosis. Inhibition of the autophagy-lysosome pathway appears to slow ferroptosis [55] and inhibiting lysosomal function by dampening V-ATPase activity also seems to dampen ferroptosis, [56] such that lysosomal activity is essential for ferroptosis. We assessed if lysosomal pH was somehow affected by DMSA-IONPs, measuring LysoTracker green DND–26 intensity in MDA-MB–231 and U87MG cells exposed to DMSA-IONPs by flow cytometry (Fig. 8e–h). Lysosomal pH increased in both cell lines after DMSA-IONP treatment, with a stronger increase in MDA-MB–231 cells (4-fold increase: Figs. 8e and 8f) than in U87MG cells (2-fold increase: Figs. 8g and 8h). When the mean fluorescence intensity of LysoTracker green was compared between MDA-MB–231 (Fig. 8e) and U87MG cells (Fig. 8g) this was 3 times more intense in MDA-MB–231 than in U87MG cells. LysoTracker green DND–26 specifically stains acidic compartments in cells and thus, U87MG cell lysosomes have a higher, less acidic pH than those in MDA-MB–231 cells, which have more lysosomes per cell but weaker LysoTracker green staining. The more acidic pH of MDA-MB–231 lysosomes may reflect the stronger ROS production in these organelles, whereas in the glioma cell line the lower pH takes longer to accumulate ROS inside these organelles and much of the ROS produced is exported to the cytosol. Lysosomal pH was further characterized using LysoSensor Yellow/Blue dextran probe (Figure S11), yielding results consistent with those obtained using LysoTracker green. MDA-MB–231 cells displayed a punctate LysoSensor staining pattern (Figure S11a), resembling the lysosomal distribution previously observed with the SiR-lysosome probe (Fig. 7). In contrast, U87MG cells exhibited a more diffused LysoSensor



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Fig. 7. Ferrous iron, ROS and lipid peroxidation in lysosomes. **a.** The presence of labile iron inside lysosomes of MDA-MB–231 and U87MG cells was evaluated using the FerroOrange and SiR-lysosome probes: lysosomes, blue; FerroOrange, red; merged images in magenta. The images were acquired on a Leica TCS SP8 multispectral confocal system using a 63X water-immersion objective and a zoom factor of 5X. **b.** ROS production inside lysosomes of MDA-MB–231 and U87MG cells: ROS stained with the DHR probe, red; lysosomes stained with Lamp–2 antibody, green; nuclei stained with DAPI, blue. Green arrowheads indicate the presence of DHR positive punctate structures surrounded by Lamp–2 staining. Scale bar: 10 μ m. **c.** The induction of lipid peroxidation in lysosomes of MDA-MB–231 and U87MG cells as assessed through the colocalization of the SiR-lysosome and BODIPY probe by confocal microscopy: Lipid peroxidation visualized through Bodipy Green fluorescence, blue; lysosomes stained with SiR-lysosome, red; DAPI stained nuclei, grey; colocalization in merged images, magenta. Scale bar: 10 μ m.

staining pattern throughout the cytoplasm (Figure S11b). Under basal conditions, MDA-MB–231 exhibited significantly more acidic lysosomes, as indicated by a LysoSensor yellow fluorescence intensity approximately 10-fold higher than that observed in U87MG cells (Figure S11c). Following DMSA-IONP treatment, both cell lines showed increased LysoSensor yellow fluorescence; however, fluorescence intensity remained approximately 10-fold higher in MDA-MB–231 cells than in U87MG cells (Figure S11c). Taken together, the results obtained with LysoTracker green, LysoSensor, and the quantification of total lysosome number by confocal microscopy indicate that U87MG cells possess a greater number of smaller lysosomes, with a less acidic luminal pH. By contrast, MDA-MB–231 cells exhibit fewer but larger lysosomes, characterized by stronger LysoTracker and LysoSensor staining, indicative of a more acidic lysosomal environment.

These differences in lysosomal properties may contribute to the distinct ferroptotic responses observed in the two cell lines. Indeed, U87MG cells displayed delayed and attenuated lysosomal lipid peroxidation and reduced cell death following DMSA-IONP treatment. Collectively, these findings suggest that lysosomal integrity and acidity are important determinants of cellular sensitivity to DMSA-IONP-induced ferroptosis.

To further evaluate the status of lysosomes, LMP was assessed by flow cytometry of (AO) stained cells after a 24 h treatment with increasing doses of DMSA-IONPs (Figure S12). Despite the ROS and lipid peroxidation in MDA-MB–231 lysosomes, only a mild and non-significant decrease in AO red fluorescence intensity was observed in these cells, indicating LMP was not affected (Figure S12a). By contrast, lower doses of DMSA-IONPs (125 and 250 μ g/ml) induced a decrease in red AO fluorescence in U87MG cells corresponded to the induction of LMP (Figure S12b). These results demonstrate that MDA-MB–231 cells can accumulate free Fe^{2+} inside lysosomes, which triggers ROS production and lipid peroxidation but remains limited to the inside of lysosomes, without affecting membrane integrity. This protection may be due to lysosome fusion in an attempt to repair membrane damage, reflected by the increased lysosome size upon DMSA-IONP treatment (Figs. 8a and 8c). Hence, in these cells ROS production is only observed inside lysosomes and only the highest dose (500 μ g/ml) is mild cytosolic accumulation of ROS observed. Conversely, U87MG cells have smaller lysosomes (Figs. 8a and 8c) that accumulate higher amounts of DMSA-IONPs (Fig. 1), altering the lysosomal membrane and releasing lysosomal components into the cytosol, as witnessed by the diffuse iron and ROS labelling throughout the cell.

To determine whether the differences in endosomal compartment characteristics, including integrity, vesicle size, maturation, and acidification, were associated with distinct cellular internalization pathways, the mechanism involved in DMSA-IONP uptake were investigated in both cell lines [57] (Figure S13). Nanoparticle internalization was quantified in the presence or absence of inhibitors of macropinocytosis (amiloride), clathrin-mediated endocytosis (chlorpromazine), and caveolin-mediated endocytosis (methyl- β -cyclodextrin). As previously reported, DMSA-IONPs uptake in MDA-MB–231 cells was significantly reduced following inhibition of clathrin- and caveolin-mediated endocytosis, indicating the involvement of both pathways [57]. In contrast, U87MG cells exhibited not only clathrin- and caveolin-dependent uptake, but also a significant contribution of macropinocytosis to DMSA-IONP internalization (Figure S13).

3.9. Effect of lysosomotropic agents on ROS and lipid peroxidation following DMSA-IONP treatment

As lysosomal acidification and integrity are key determinants of ROS generation and lipid peroxidation within lysosomes, additional functional validation experiments were performed to assess the contribution of lysosomal acidification to ferroptosis sensitivity. Lysosomal pH was pharmacologically increased using Bafilomycin A prior to DMSA-IONP treatment. After 3 and 24 h of co-treatment of DMSA-IONPs with Bafilomycin A, ROS production and lipid peroxidation were evaluated (Fig. 9a–d). Disruption of lysosomal acidification significantly modulated both ROS (Figs. 9a and 9c) and lipid peroxidation (Figs. 9b and 9d) in both cell lines, although DMSA-IONPs uptake was not affected (Figure S14 a). These findings support a central role of the acidic lysosomal environment in DMSA-IONP-induced ROS generation and lipid peroxidation.

Several studies have proposed that the induction of lysosomal membrane permeabilization (LMP) by lysosomotropic agents can enhance ferroptosis susceptibility [58]. Consistent with this model, we hypothesize that LMP could promote the release of lysosomal iron and hydrolytic enzymes into the cytosol, thereby amplifying oxidative damage and promoting ferroptotic cell death through a positive feedback loop. Notably, in our study, DMSA-IONPs induced lysosomal iron accumulation, ROS generation, and lipid peroxidation in MDA-MB–231 cells without causing detectable LMP at 24 h post-treatment. To further evaluate the role of lysosomal membrane integrity, we performed additional experiments combining the DMSA-IONPs with chloroquine (CQ), a well-established inducer of lysosomal membrane destabilization and permeabilization (Fig. 9e–j). Interestingly, CQ co-treatment with DMSA-IONPs lowered ROS production in MDA-MB–231 cells (Fig. 9e), without affecting DMSA-IONP uptake (Figure S14b), further supporting the notion that ROS generation in this cell line largely depends on maintenance of an acidic lysosomal environment. In contrast CQ did not significantly alter ROS production in U87MG cells (Fig. 9h). Moreover, co-treatment of CQ with DMSA-IONPs increased lipid peroxidation (Figs. 9f and 9i) and cell death (Figs. 9g and 9j) in both cell lines indicating that the induction of lipid peroxidation in lysosomes together with the induction of LMP sensitizes cells to ferroptosis. These findings indicate that disruption of lysosomal membrane stability and integrity markedly increases ferroptosis susceptibility and further support the notion that lysosomes play a central role in controlling the accumulation of iron, ROS and lipid peroxides. Therefore, lysosomal membrane permeabilization appears to function as an amplifying event that enhances ferroptotic signaling once oxidative damage has been established within the lysosomal compartment.

Overall, our findings highlight the critical importance of lysosome fitness in determining susceptibility to lysosome-driven ferroptosis. The confinement of iron, ROS and lipid peroxides within lysosomes appear to be the key factor promoting ferroptotic cell death. This confinement is facilitated to acidic nature of lysosomes, whereas less acidic lysosomes tend to favor iron ions export to the cytosol, resulting in a more diffuse pattern of ROS generation that is insufficient to trigger extensive cell death.

4. Discussion

NP based pro-oxidant therapy relies on their capacity to push ROS levels above the ROS survival threshold in cancer cells, thereby inducing

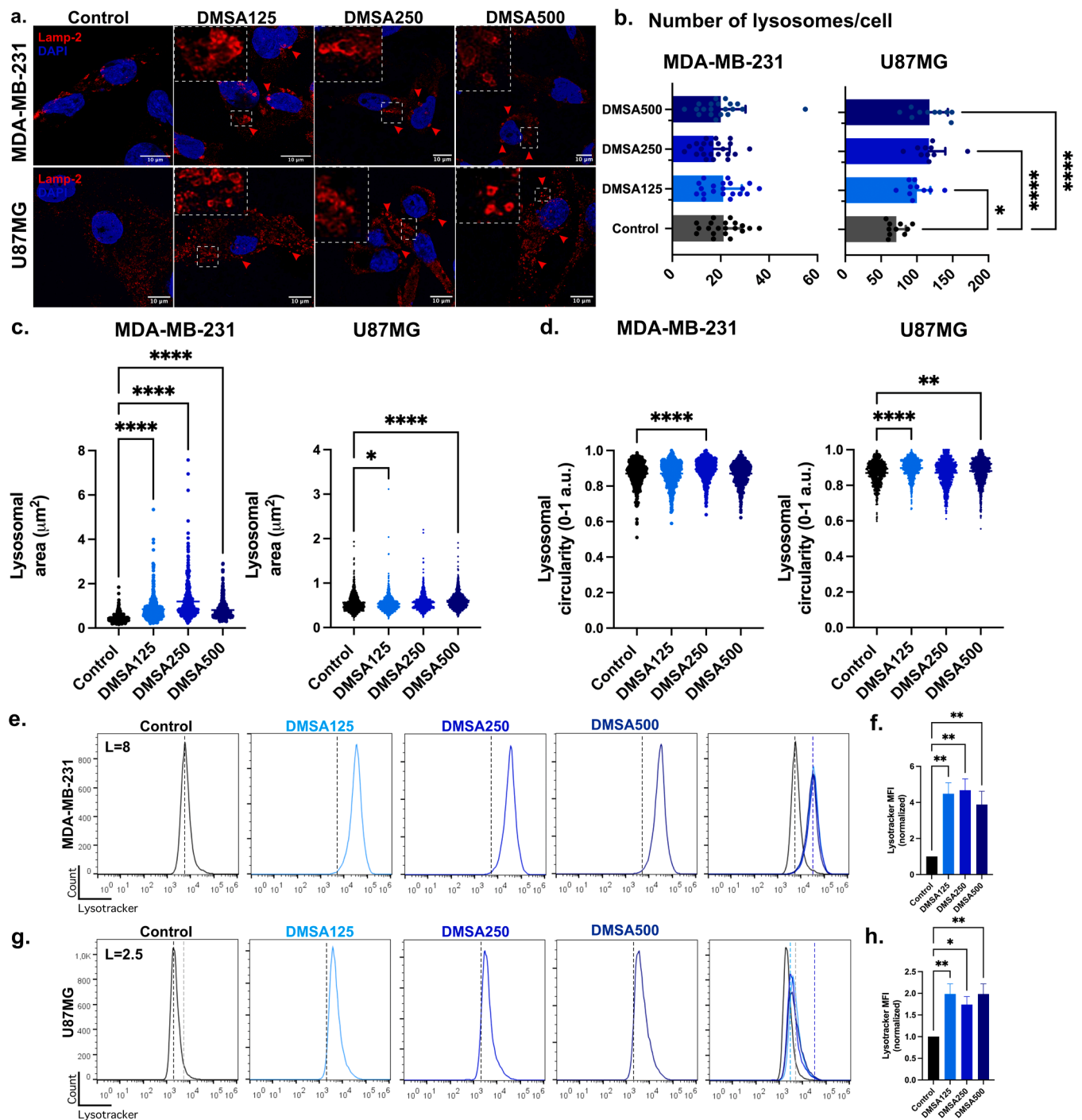
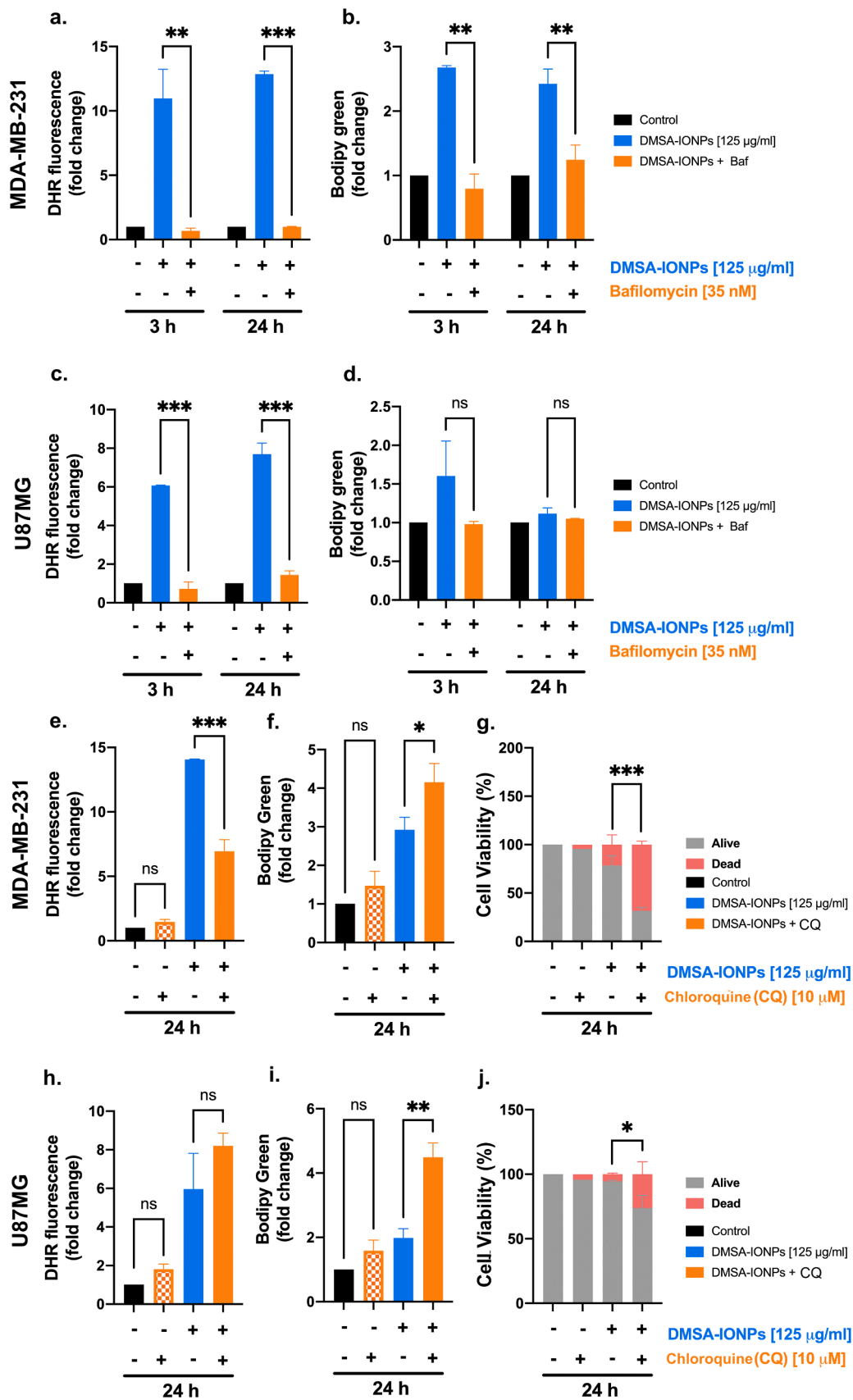


Fig. 8. Changes in lysosomal shape, number, size and pH after DMSA-IONP treatment. **a.** Confocal images of lysosomes in MDA-MB-231 (upper panel) and U87MG cells (lower panel) treated with increasing doses of DMSA-IONPs for 24 h: lysosomes stained with Lamp-2 antibody, red; DAPI stained nuclei, blue; DMSA-IONPs visualized by reflection, white/grey. Scale bar: 10 μ m. **b.** Number of lysosomes per cell after treatment with increasing doses of DMSA-IONPs for 24 h ($n = 20$ MDA-MB-231 cells per condition and $n = 10$ U87MG cells). **c.** Lysosome size assessed as lysosome area (μ m²) per cell after treatment with increasing doses of DMSA-IONPs for 24 h ($n = 20$ MDA-MB-231 cells per condition and $n = 10$ U87MG cells). **d.** Lysosome circularity (0-1 a.u.) per cell after treatment with increasing doses of DMSA-IONPs for 24 h ($n = 20$ MDA-MB-231 cells per condition and $n = 10$ U87MG cells). **e-h.** Lysosomal green DND-26 mean fluorescence intensity (MFI) in untreated (control) and DMSA-IONP treated cells (24 h at different doses of 125, 250 and 500 μ g/ml). **e.** LysoTracker histogram from MDA-MB-231 cells: the dashed black line represents the MFI of untreated (control) cells; the blue dashed line represents the MFI of DMSA-IONP treated cells. **f** and **h.** Quantification of LysoTracker MFI after DMSA-IONP treatment of MDA-MB-231 cells (**f**) and U87MG cells (**h**). **f.** LysoTracker histogram from U87MG cell line: the dashed black line represents the MFI of untreated (control) cells; the dashed grey line represents the MFI of MDA-MB-231 untreated cells; the light-blue dashed line represents the MFI of DMSA-IONP treated U87MG cells; the dark-blue dashed line represents the MFI of MDA-MB-231 treated cells. The differences between different doses of DMSA-IONPs on lysosome number, area and circularity, and on LysoTracker intensity were assessed with a one-way ANOVA using Tukey's and Dunnett's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.



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Fig. 9. Lysosomotropic agents affect lysosomal function and regulates DMSA-IONP-induced ROS production and lipid peroxidation. a – d. Lysosomal alkalization by Bafilomycin attenuates ROS production and lipid peroxidation in MDA-MB–231 and U87MG cell line. ROS production (a and c) and lipid peroxidation (b and d) were assessed in cells treated with DMSA-IONPs in the presence of Bafilomycin (35 nM). e – j. Lysosomal destabilization and permeabilization induced by Chloroquine enhance ferroptosis susceptibility by increasing lipid peroxidation and cell death following DMSA-IONP treatment. ROS production (e and h), lipid peroxidation (f and i) and cell death (g and j) were evaluated in cells treated with DMSA-IONP in the presence of Chloroquine (10 μ M). Statistical differences in ROS, lipid peroxidation and cell death upon co-treatment of DMSA-IONPs with the lysosomotropic agents were analyzed using one-way ANOVA followed with Sidak's multiple comparison test and a two-way ANOVA with a Dunnett's multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

cell death. IONP-based ROS production is due to the accumulation of IONPs in acidic compartments, which drives the degradation of their coating and releases iron into the cytosol. This increase in total iron triggers ROS production, which ultimately induces the death of cancer cells. In this work, we highlight the critical role of lysosomes in determining cellular responses to IONP exposure. We show that ferroptosis induced by IONPs is governed not only by the total amount of ROS or the extent of IONP uptake, but also by their site of accumulation. In particular, lysosomal accumulation renders cells more sensitive to ferroptosis induction, as it can trigger lipid peroxidation within these organelles. Here, we demonstrate that DMSA-IONPs induce significant oxidative stress in MDA-MB–231 BC cells and in U87MG glioma cells, as witnessed by their enhanced ROS levels. However, these two cell lines behave distinctly, with MDA-MB–231 cells mainly accumulating ROS in lysosomes, whereas the ROS produced by U87MG cells on exposure to DMSA-IONPs not only localized to lysosomes but also, it was more generally spread throughout the cytosol. In addition, the sustained ROS production in response to long-term incubation with DMSA-IONPs affected the proliferation of both cancer cell types in 2D and 3D cell cultures. Although cell proliferation was affected in both cell lines, cell death was only induced in U87MG cells after a 72 h exposure to DMSA-IONPs, whereas MDA-MB–231 BC cell death was induced at all the doses and times tested. Together these results demonstrate that long term treatment with DMSA-IONPs induces an increase in ROS production in tumor cells, affecting their proliferation and enhancing their susceptibility to cell death.

Studies with other types of NPs have linked cytotoxicity and tumor cell death to oxidative stress. Studies with cuprous oxide NPs showed that their cytotoxicity in bladder cancer cell lines (T24, J82, 5637 and UMUC3) was dependent on oxidative stress and activation of the ERK signaling pathway [59]. Elsewhere, chitosan-coated gold NPs induced ROS dependent cell death of HeLa and MCF–7 [60] tumor cells, and of CEM and K562 [61] leukemia cell lines. When different sizes of gold NPs were assessed, the lymphoma U937 cell line produced ROS and underwent cell death when exposed to small gold NPs [62]. In another study, cadmium sulphide NPs were seen to induce cell death dependent on ROS production in the A549 cell line [63]. Thus, there is extensive evidence of the potential of NPs to induce oxidative stress dependent cell death.

The different patterns of ROS accumulation and of viability might indicate that DMSA-IONPs have different effects depending on the cell line, a behavior that dictates the predisposition to cell death. Hence, we evaluated how DMSA-IONPs affect the levels of iron ions, iron metabolism, lipid peroxidation and lysosome stability in the two cell lines studied. The FerroOrange probe demonstrated that DMSA-IONPs increase the free Ferrous ion (Fe^{2+}) mobilization in both cell lines after short-term incubations, which decreased after exposure beyond 24 h. The decrease in free iron corresponded to ferritin accumulation in both cell lines after a 24 h exposure to DMSA-IONPs (Fig. 6f), as cells use ferritin to limit the free iron that accumulates to avoid cytotoxicity [64]. In addition, cells induce the expression of the *LCN2* gene (Fig. 6e) that encodes the lipocalin protein, which plays a central role in iron transport, and in immune system and inflammatory responses [65,66]. Thus, this increase in lipocalin suggests the cells are trying to buffer the increase in free iron induced by DMSA-IONPs. In addition, the HRG1 protein encoded by the *SLC48A1* gene is responsible for regulating intracellular heme availability by controlling heme-transport [67] but also, its activity has been associated with that of the proton ATPase

found in endosomal compartments that promotes endosomal acidity [68]. Thus, the increase in *SLC48A1* gene expression could be related to an increase in endosomal acidity, with LysoTracker indicating a stronger increase in lysosomal acidity in MDA-MB–231 cells than in U87MG glioma cells. In addition, we highlighted how the two different cancer cell lines mobilize iron distinctly, towards lysosomes, mitochondria or spread throughout the cytosol. The MDA-MB–231 BC cells retain iron inside lysosomes, whereas the U87MG glioma cells accumulate ferrous iron in lysosomes, mitochondria and in the cytosol. Although U87MG cells exhibited a higher overall labile iron signal, this parameter alone did not correlate with ferroptosis sensitivity. Instead, the preferential localization of Fe^{2+} within lysosomes, together with lysosomal ROS production and lipid peroxidation in MDA-MB–231 cells, suggests that the intracellular compartmentalization of redox-active iron is a more important determinant of ferroptosis susceptibility than the absolute size of the labile iron pool.

STEAP reductases are strongly overexpressed in triple-negative BC patients and STEAP3 overexpression is associated with the overall survival of BC patients [69]. Indeed, it has been proposed that STEAP4 overexpression may serve as a biomarker for BC and a potential pharmacological target [70]. The overexpression of these reductases by BC cells could contribute to the formation and accumulation of bioactive ferrous iron in lysosomes. Conversely, the mobilization of iron ions from lysosomes to the cytosol in U87MG glioma cells is concomitant with an increase in *SLC11A2* gene expression, which encodes the DMT1 transporter. Hence, these cells might attempt to overcome the iron overload in lysosomes by exporting this towards the cytosol, a role known to be fulfilled by the DMT1 transporter [15]. Moreover, DMT1 overexpression may also enhance mitochondrial iron uptake, [71,72] as witnessed by the localization of labile iron in mitochondria of untreated and DMSA-IONP treated U87MG cells. DMT1 has been shown to regulate vesicular iron trafficking and intracellular iron distribution in MDA-MB–231 cells [72], where modulation of DMT1 profoundly alters the labile iron pool and mitochondrial iron translocation. In that study, silencing of DMT1 transporter shifted iron localization from a diffuse cytosolic distribution to an iron-retention phenotype characterized by large punctate structures [72], resembling those observed in the present work. DMT1 has also been implicated in the regulation of ferroptosis susceptibility in U87MG cells [73]. Specifically, nutrient starvation induced lysosomal dysfunction and altered the expression of iron-related proteins, leading to DMT1 dysregulation. Under these conditions, cell death occurred through a ferroptotic mechanism associated with iron accumulation and impaired DMT1 function [73]. Furthermore, a previous study using a combined treatment of 2-deoxy-D-glucose and chloramphenicol in the same cell line highlighted the close relationship between iron dynamics and ferroptosis induction [74], further supporting the importance of intracellular iron trafficking in regulating ferroptotic cell death. Additional studies have linked DMT1-mediated iron transport to cell death in other cancer types. In both cancer stem cells [75] and in head and neck cancer cells [76], pharmacological inhibition of DMT1 resulted in iron accumulation within lysosomes, enhanced ROS production, and increased cell death. In the present study, we observed distinct lysosomal or endosomal iron export and retention between MDA-MB–231 and U87MG, although the molecular mechanism governing these differences were not investigated in detail. Interestingly, in addition to DMT1, the lysosomal ion channel TRPML1 functions as a Fe^{2+} -permeable transporter in late endosomes

and lysosomes [77] and has been identified as a negative regulator of ferroptosis. Both pharmacological inhibition and genetic depletion of TRPML1 have been shown to promote ferroptosis in breast cancer stem cells [78] and in AKT-hyperactivated cancer models [79]. These findings suggest that ferroptosis susceptibility depends not only on the total intracellular iron pool but also on the subcellular compartmentalization of redox-active iron within the endolysosomal system. Therefore, differences in lysosomal iron handling, potentially involving DMT1 and/or TRPML1, may contribute to the distinct ferroptosis sensitivities observed between MDA-MB-231 and U87MG cells in our study. Although these mechanisms were not directly investigated here, the available literature supports the notion that iron localization within lysosomal compartments is an important determinant of ferroptosis susceptibility and represents a plausible factor underlying distinct cellular responses to IONP-mediated iron release and oxidative stress.

Given the nature of IONPs, it is no surprise that they somehow modify genes and proteins related to iron metabolism [80]. Polyethylenimine-coated IONPs increase ferritin light chain and ferretin mRNA expression in macrophages, [80] and in a pancreatic cancer cell line [81]. Moreover, Citrate and Dextran-coated IONPs also affect the genes and proteins involved in iron metabolism to a different extent in three different macrophage cell lines (NCTC, THP1-M1 and THP1-M2 cells), inducing a stronger increase in the former liver-derived NCTC macrophages [82]. In addition, we showed that DMSA-IONPs can interfere with iron metabolism in respiratory epithelial cells affecting SARS-CoV-2 and IAV infection by lowering viral titers [83,84]. Therefore, the ability of IONPs to modulate iron responsive genes and proteins can affect cell behavior, modulating bioactive iron mobilization and ROS production. This may explain why ROS production is restricted to lysosomes in MDA-MB-231 BC cells, whereas it spreads throughout the cytosol in U87MG cells.

Our previous physicochemical characterization of these DMSA-IONPs demonstrated that they undergo degradation under lysosomal-mimicking conditions, releasing iron ions and exhibiting changes in hydrodynamic diameter consistent with acidic-driven dissolution [33]. In addition, we previously characterized the intrinsic oxidative catalytic activity of DMSA-IONPs [33], which can be enhanced under acidic conditions [85]. Beyond the release of soluble iron species, IONPs can directly catalyze Fenton and Fenton-like reactions at their surface through redox cycling of surface $\text{Fe}^{2+}/\text{Fe}^{3+}$ sites [86,87]. Under lysosomal conditions, progressive nanoparticle degradation exposes $\text{Fe}^{2+}/\text{Fe}^{3+}$ sites of the nanoparticle core facilitating the heterogeneous Fenton and Fenton-like reactions at the solid-liquid interface, providing an additional source of redox-active iron that can amplify intracellular ROS production. Therefore, both the intrinsic surface catalytic activity of the nanoparticles and lysosomal iron release are expected to contribute to ROS generation. The enhancement of catalytic activity under acidic conditions [85] may explain why the BC cell line, which display a more acidic and functionally lysosomal compartment, exhibited greater accumulation of ROS and lipid peroxidation products within lysosomes than the glioma cell line. Taken together, our previous observations regarding lysosomal degradation and iron release from DMSA-IONPs, together with the present findings of Fe^{2+} accumulation, ROS generation, and lipid peroxidation within lysosomes, support the hypothesis that lysosomal processing of DMSA-IONPs increases intracellular iron bioavailability and thereby contributes to ferroptotic cell death.

Iron ions exhibited distinct intracellular accumulation patterns depending on the cell line. In the BC cell line, iron accumulation was mainly localized within lysosomes, whereas in the glioma cell line iron was localized in mitochondria and cytosol. Mitochondrial iron accumulation may influence mitochondrial metabolism, as iron is required for the synthesis of iron-containing cofactors involved in multiple cellular processes [16]. Analysis of mitochondrial membrane potential (Figure S7) revealed no significant changes in MDA-MB-231 cells, consistent with the absence of detectable mitochondrial iron

accumulation. In contrast, U87MG cells exhibited a slight decrease in mitochondrial membrane potential after 24 h of treatment; however, this effect was transient and recovered at later points. These results suggest that sustained mitochondrial dysfunction is unlikely to be the primary factor underlying the differential cellular responses observed in this study and further support the relevance of lysosomal regulation as a key determinant of ferroptosis sensitivity. Furthermore, in a previous study we investigated the impact of oxidative stress induced by differently coated IONPs on mitochondrial metabolism in cancer cells [33]. Both MDA-MB-231 and U87MG cells showed reduced OXPHOS activity following DMSA-IONP treatment at 24 h. However, despite a reduction in mitochondrial ROS production in MDA-MB-231 cells, no significant alterations in mitochondrial membrane potential were observed in either cell line, although U87MG cells exhibited a non-significant decrease.

The different pattern on iron mobilization and ROS production defined the cell's susceptibility to lipid peroxidation. MDA-MB-231 cells accumulated lipid peroxides inside lysosomes, indicating that ROS and iron accumulation inside these organelles triggered the oxidation of lipids affecting lysosomal stability. Lysosomal damage can induce lysosomal enzyme release into the cytosol, initiating programmes of cell death [88]. Therefore, lysosomes try to repair membrane damage through the activity of the endosomal sorting complex required for transport (ESCRT), [89] or there is an attempt to sequester damaged lysosomes by promoting their fusion with functional lysosomes with a view to restore the lysosomal pH [90] or to redistribute lytic enzymes between these lysosomes [91]. Therefore, the lysosomal damage observed in MDA-MB-231 cells is concomitant with the appearance of enlarged lysosomes, probably due to the fusion of lysosomes to restore lysosomal function. This behaviour is consistent with that of hepatic cells exposed to IONPs, which produced an increase in lysosomal size, altered lysosomal circularity, and a change in lysosomal function and stability due to the induction of LMP [92]. Enlarged lysosomes have been related to lysosomal dysfunction [54,93] and thus, DMSA-IONPs would appear to affect lysosomal function in MDA-MB-231 BC cells. The localization of lysosomes in the cell can influence their pH, such that lysosomes closer to the nucleus have a more acidic pH whereas those at the periphery often have a higher pH [94]. Moreover, the location of lysosomes also determines their susceptibility to damage, with those in the perinuclear area apparently more susceptible to lysosomal membrane destabilization and lysosome-induced cell death [20]. Therefore, the perinuclear lysosomes seen in MDA-MB-231 cells are likely to be more acidic and more susceptible to damage. The higher pH lysosomes in U87MG cells are less likely to degrade and this may also explain why the ROS production inside these lysosomes does not induce so much lipid peroxidation but rather, ROS is more evenly spread throughout the cell.

Previous studies have demonstrated that nanocarrier trafficking differs between MDA-MB-231 and U87MG cells, leading to distinct degrees of endosomal retention and lysosomal colocalization depending on the cellular context [95]. These intrinsic differences in intracellular trafficking may influence the rate and extent of lysosomal acidification, thereby affecting iron release from DMSA-IONPs, ROS generation, and ultimately cellular susceptibility to ferroptosis. Both tumor types appear to rely heavily on lysosomal function. MDA-MB-231 cells have been shown to dependence on TFEB-regulated lysosomal programs [96] and lysosomal iron trafficking pathways [79], whereas glioblastoma cells exhibit extensive remodeling of lysosomal and endolysosomal compartments, including enhanced dependence on lysosomal acidification and v-ATPase activity [97]. These observations raise the possibility that cell-type-specific differences in lysosomal function may contribute to the differential responses observed in our study. However, this mechanism was not directly investigated and therefore remains speculative.

Importantly, several studies have highlighted the critical role of lysosomes in the initiation and execution of ferroptosis. For example, the ionophore salinomycin has been reported to induce iron accumulation

within lysosomes, leading to ROS generation, lysosomal membrane permeabilization (LMP), and ultimately ferroptotic cell death, thereby identifying lysosomal iron accumulation as an initiating event in salinomycin-induced ferroptosis [98]. Furthermore, two recent studies have also suggested that lysosomes serve as central hubs for ferroptosis initiation, either by propagating lipid peroxidation to neighboring organelles through radical chain reactions [99] or through LPO-mediated LMP, which facilitates the dissemination of LPO throughout the cell [58].

Consistent with this model, we hypothesize that lysosomal membrane permeabilization (LMP) could subsequently release lysosomal iron and hydrolytic enzymes into the cytosol, further amplifying oxidative damage and promoting ferroptotic cell death through a positive feedback loop. Notably, in our study, DMSA-IONPs induced lysosomal iron accumulation, ROS generation, and lipid peroxidation in MDA-MB-231 cells without causing detectable LMP at 24 h after treatment. To further evaluate the role of lysosomal membrane integrity, we performed additional experiments combining the DMSA-IONPs with chloroquine (CQ), a well-known inducer of lysosomal membrane destabilization and permeabilization. Co-treatment of DMSA-IONPs and CQ significantly enhanced both lipid peroxidation and cell death in MDA-MB-231 cells, which already displayed a ferroptotic phenotype following nanoparticle exposure. Importantly, CQ co-treatment also sensitized U87MG cells, which did not initially exhibit clear ferroptotic features, resulting in increased lipid peroxidation and cell death. These findings indicate that disruption of lysosomal membrane stability and integrity markedly increases ferroptosis susceptibility and further support the notion that lysosomes play a central role in controlling the accumulation of iron, ROS and lipid peroxides. Therefore, lysosomal membrane permeabilization appears to function as an amplifying event that enhances ferroptotic signaling once oxidative damage has been established within the lysosomal compartment.

Collectively, our results support a model in which lysosomal iron sequestration is an early event that drives ROS generation and lipid peroxidation within lysosomes. Subsequent lysosomal membrane permeabilization further amplifies oxidative stress and promotes ferroptotic cell death. Although lysosomal dysfunction may also arise as a downstream consequence of ferroptosis, our findings suggest that, in the context of DMSA-IONPs treatment, lysosomal iron accumulation and ROS generation act predominantly as initiating events that determine ferroptosis susceptibility, likely in a manner influenced by cell-specific differences in endosomal trafficking and lysosomal fitness. These observations suggest that the capacity of cells to accumulate iron-derived ROS within lysosomes, together with their susceptibility to lysosomal membrane destabilization, may be important determinants of ferroptosis sensitivity. Although the present study was not specifically designed to validate lysosomal ROS trapping as a predictive biomarker, our data support the hypothesis that this phenotype could serve as a functional indicator of responsiveness to lysosome-targeted, iron-based nanotherapies that rely on ROS generation and ferroptosis induction. In this context, lysosomal ROS accumulation may reflect the ability of cells to effectively process iron-containing nanoparticles and generate the oxidative stress required to initiate ferroptotic signaling.

The accumulation of iron and ROS in lysosomes of the BC cells, and the associated lipid peroxidation, was related to cell death. Treatment with the ferroptosis inhibitor ferrostatin-1 dampened the death of MDA-MB-231 cells induced by the lower doses of DMSA-IONPs, indicating that limiting the iron and ROS accumulation in lysosomes to some extent promoted the lipid peroxidation that triggers cell death. Besides, Ferrostatin-1 localizes in lysosomes [100] and hence, this antioxidant reduces the oxidation of lipids in lysosomes, particularly in MDA-MB-231 cells. Treating cancer stem cells with Salinomycin, an antibacterial ionophore, was seen to trigger iron accumulation in lysosomes, thereby inducing cancer cell death [98]. Moreover, ferritin endocytosis induced the lysosomal accumulation of redox-active iron, promoting ferroptosis of hepatocytes via lysosomal membrane lipid

peroxidation and the ensuing LMP [101]. Previous studies with gallic acid and polyacrylic acid-coated IONPs also showed that IONPs can induce ROS accumulation and lipid peroxidation, leading to ferroptosis in different cancer cell lines [32]. Similarly, oleic acid-coated IONPs induced glioma cancer cell death by ferroptosis in an autophagy-dependent manner [102]. The exact mechanism driving ferroptosis is not yet known, although the induction of ferroptosis is related to the dysfunction of peroxide removal, the presence of redox-active iron and the oxidation of PUFAs [42]. PUFA oxidation can affect the fluidity and structure of the cell membrane, increasing permeability and leading to a loss of membrane integrity [103] or the formation of membrane pores [53]. The accumulation of lipid peroxides may be toxic to cancer cells due to their transformation into active molecules that can consume amino acids, nucleic acids and proteins, [52] as well as into aldehydes that can crosslink with proteins involved in primordial cellular processes [104]. Therefore, lipid peroxide accumulation in MDA-MB-231 cells triggers cell death, indicating that ferroptosis is driven by lysosome-dependent lipid peroxidation resulting from ROS production. In contrast, in the glioma U87MG cell line, which contains less acidic lysosomes, ROS productions and lipid peroxidation within lysosomes are only markedly observed after 72 h exposure to DMSA-IONPs, coinciding with increased cell death. Moreover, lysosomal overload with DMSA-IONPs may induce mechanical stress in these organelles, potentially activating alternative lysosome-dependent cell death pathways. Our findings indicate that perturbations in intracellular iron handling and trafficking constitute a central event following DMSA-IONP exposure, with ferroptosis representing one downstream consequence rather than the sole outcome of these alterations. We previously demonstrated that DMSA-IONP treatment induces mitochondrial dysfunction and reduces oxidative phosphorylation in MDA-MB-231 cells [33]. In the present study, we show that these cells accumulate and retain iron within lysosomal compartments. This observation suggests that intracellular iron trafficking may be impaired, limiting the efficient redistribution of iron to other intracellular compartments, including mitochondria. Consequently, the lysosomal iron retention observed here may provide a mechanistic explanation for the mitochondrial metabolic impairment reported previously. This interpretation is supported by recent studies demonstrating that genetic ablation of DMT1 alters intracellular iron trafficking, increases labile iron accumulation, decreases mitochondrial iron delivery, and induces mitochondrial dysfunction [72]. Together these findings support the concept that proper intracellular iron trafficking is required for maintenance of mitochondrial function and are consistent with our previous observations in MDA-MB-231 cells. In contrast, U87MG cells displayed considerably lower lysosomal iron retention. This finding suggests that iron redistribution from endolysosomal compartments may occur more efficiently in these cells, potentially preserving mitochondrial iron homeostasis. Consistent with this hypothesis, our previous studies did not reveal significant mitochondrial dysfunction in U87MG cells following DMSA-IONP treatment [33]. Thus, differences in intracellular iron trafficking may underlie the distinct metabolic responses observed between the two cell lines. Collectively, these results suggest that the cellular capacity to process and redistribute nanoparticle-derived iron is a critical determinant of both metabolic adaptation and susceptibility to ferroptotic cell death.

Also, DMSA-IONPs can not only induce lipid peroxidation but they can also affect the antioxidant system responsible for counterbalancing lipid peroxidation. GSH is the main antioxidant associated with ROS scavenging and GPX4 is the main enzyme responsible for the reversion of peroxidized lipids [105]. DMSA-IONPs alter the total GSH in both cell lines, also inducing GPX4 overexpression in U87MG cells. Class I ferroptosis inducers are characterized by consuming GSH [28] and therefore, DMSA-IONPs could be considered as type 1 inducers. The iron chelating properties of GSH mean it can bind directly to iron and as such, it can prevent non-specific oxidation of proteins due to the Fenton Reaction [106,107]. Hence, treating cancer cells with DMSA-IONPs

directly affects the antioxidant status of cells. Class II and III ferroptosis inducers alter the activity and/or expression of GPX4 [28] and in that context, DMSA-IONPs do not negatively affect GPX4 expression but in U87MG cells, they even induce an increase in its expression. This increase in GPX4 expression in U87MG cells could be responsible for the weaker lipid peroxidation in these cells given its role in reversing these events [105]. Furthermore, both cell lines exhibited upregulation of the cystine transporter xCT, (SLC7A11), although the increase was more pronounced in U87MG cells. Notably, the stronger induction of SLC7A11 correlated with the increased expression of GPX4 in this cell line. Upregulation of SLC7A11 enhances cystine uptake, providing the substrate required synthesis of GSH [48], which in turn support GPX4-dependent detoxification of lipid peroxides through its phospholipid hydroperoxidase activity [108]. Therefore, the coordinated upregulation of SLC7A11 and GPX4 may represent an adaptive antioxidant response that protects U87MG cells from excessive lipid peroxidation and ferroptotic cell death. Interestingly, SLC7A11 overexpression has been also associated with disulfide stress, which can disrupt F-actin organization cytoskeletal dynamics [108]. In this regard, we previously demonstrated that DMSA-IONPs alter F-actin cytoskeleton in MDA-MB–231 cell, leading to impaired cell migration [57]. Although the relationship between iron trafficking, redox imbalance, and cytoskeletal remodeling requires further investigation, these observations suggest that the effects of DMSA-IONPs extend beyond ferroptosis and involve broader alterations in cellular homeostasis.

Taken together, our findings support a model in which disruption of intracellular iron trafficking, rather than ferroptosis alone, represents the primary cellular consequence of DMSA-IONP exposure. Altered iron compartmentalization influences multiple interconnected processes, including oxidative stress responses, lipid peroxidation, mitochondrial metabolism, antioxidant adaptation, cytoskeletal organization, and ultimately susceptibility to ferroptotic cell death.

Here, ferroptosis is mainly due to oxidative stress and lipid peroxidation in lysosomes, and it depends on the functionality and size of lysosomes in the different cancer cell lines. Larger and more acidic lysosomes in MDA-MB–231 cells induced stronger ROS production, which was retained inside lysosomes and was correlated with more intense lipid peroxidation. The ROS produced in lysosomes affected their size and stability as they were bigger than those in U87MG cells. By contrast, the less acidic lysosomes in U87MG cells induce milder ROS production, which is not retained in the lysosomes but rather extends throughout the cytosol. This diffuse ROS production induces only a mild increase in lipid peroxidation that does not lead to ferroptosis at least at shorter incubation times. Therefore, the presence of more acidic and larger lysosomes reflects susceptibility to induce ferroptosis in tumor cell lines, as glioma cells exhibited reduced sensitivity to ferroptosis perhaps due to the export of lysosomal components into the cytosol that induces more extended ROS production throughout the cell. This reduced sensitivity may be due to the astrocyte nature of U87MG, as astrocytes are dedicated to maintaining homeostasis and neurotoxicity, and thus they can neutralize the effects of endocytosing cellular debris. U87MG cells have previously been seen to export intracellular material through vesicles, [109] and the presence of lysosomes near the plasma membrane is consistent with this. Therefore, although these cells internalize more DMSA-IONPs and produce more ROS, there is nominal lipid peroxidation in lysosomes and the cells can neutralize cytosolic ROS to overcome cell death.

5. Conclusions

The antitumoral pro-oxidant potential of DMSA-IONPs was assessed in two different cancer cell lines, the MDA-MB–231 BC cell line and the U87MG glioma cell line. Our findings reveal that their downstream consequences diverge sharply due to intrinsic differences in lysosomal physiology and iron-handling capacities. We demonstrate that ferroptosis induced by IONPs is not determined by the amount of ROS

generated, but by where ROS and iron ions accumulate. Lysosomal fitness acts as the critical checkpoint that determines whether oxidative stress remains sublethal or progresses into ferroptosis.

MDA-MB–231 cells exhibit pronounced lysosomal acidification upon nanoparticle uptake, accumulating both ferrous ions and ROS within enlarged lysosomes. This confinement promotes intense lipid peroxidation, compromises lysosomal integrity and ultimately triggers ferroptosis. Conversely, U87MG glioma cells display a markedly different response with less acidification of lysosomes, active export of ferrous ions to the cytosol and mitochondria, and dissipation of ROS across multiple compartments. This redistribution reduces localized lipid peroxidation and protects these cells from undergoing ferroptosis despite similar initial oxidative stress.

These contrasting behaviors indicate that ferroptotic sensitivity is not dictated solely by the amount of ROS generated but rather by the ability of cells to maintain or alter the acidic, iron-rich environment within lysosomes. Lysosomal plasticity, defined by acidity, buffering capacity and the balance between iron sequestration or export, emerges as a critical determinant of whether DMSA-IONPs initiate ferroptosis. Moreover, overloading both cell lines with nanoparticles caused generalized oxidative stress promoting ROS-independent cell death mechanisms likely associated with lysosomal instability.

Together, these results highlight the importance of lysosomal acidity, stability and buffering capacity for ferroptosis induction. They underscore that achieving effective ferroptosis requires more than delivering redox-active iron: it requires understanding how each cancer cell type regulates lysosome plasticity. This insight is crucial for designing nanoparticle formulations, as ferroptosis-based nanotherapies must take into account for the intrinsic endolysosomal behaviour of each tumor type.

CRedit authorship contribution statement

Neus Daviu: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alberto Irigaray-Moreno:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Carla Graciano-Casero:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Domingo F. Barber:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The authors declare that the research does not include experimentation with animals or human subjects, so approval by an ethics committee was not necessary.

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thesis student of the Biomolecules and Cell Dynamics Masters course at the Universidad Autónoma de Madrid, and he was then awarded a JAE-Intro ICU scholarship (JAEICU_24.00026) by the Nanomedicine CSIC HUB. C. Graciano-Casero was a master's thesis student on the Clinical Biotechnology Master's Degree of the European University of Madrid. This research work was performed in the framework of the Nanomedicine CSIC HUB and the Cancer CSIC HUB. The Centro Nacional de Biotecnología (CNB-CSIC) is a Severo Ochoa Center of Excellence (CEX2023-001386-S) funded by MICIU/AEI /10.13039/501100011033.

Declaration of Competing Interest

The authors have no competing financial interests to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2026.119875](https://doi.org/10.1016/j.biopha.2026.119875).

Data Availability

Data will be made available on request.

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