Differences in toxicity, mitochondrial function and miRNome in human cells exposed in vitro to Cd as CdS quantum dots or ionic Cd

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Abstract: Cadmium is toxic to humans, although Cd-based quantum dots exerts less toxicity. Human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1) were exposed to ionic Cd, Cd(II), and cadmium sulfide quantum dots (CdS QDs), and cell viability, cell integrity, Cd accumulation, mitochondrial function and miRNome profile were evaluated. Cell-type and Cd form-specific responses were found: CdS QDs affected cell viability more in HepG2 than in THP-1; respective IC20 values were ~3 and ~50 µgml-1. In both cell types, Cd(II) exerted greater effects on viability.
Mitochondrial membrane function in HepG2 cells was reduced 70% with 40 µgml-1 CdS QDs but was totally inhibited by Cd(II) at corresponding amounts. In THP-1 cells, CdS QDs has less effect on mitochondrial function; 50 µgml-1 CdS QDs or equivalent Cd(II) caused 30% reduction or total inhibition, respectively. The different in vitro effects of CdS QDs were unrelated to Cd uptake, which was greater in THP-1 cells.
For both cell types, changes in the expression of miRNAs (miR-222, miR-181a, miR-142-3p, miR-15) were found with CdS QDs, which may be used as biomarkers of hazard nanomaterial exposure. The cell-specific miRNome profiles were indicative of a more conservative autophagic response in THP-1 and as apoptosis as in HepG2.

**Cover Letter**

Dear Editor,

we wish to thank you and the Reviewer #1 for the helpful suggestions.

We have prepared accordingly a modified version of the paper '*Differences in toxicity, mitochondrial function and miRNome in human cells exposed in vitro to Cd as CdS quantum dots or ionic Cd'*, that we hope it is now suitable with the requests and publishable on *Journal of Hazardous Materials*. Please also found enclosed separately a 'Response to Reviewer' for your considerations.

Thank you again because we are certain the procedure has enriched our paper.

With best regards

Nelson Marmiroli

*Director of CINSA*
*Emeritus Professor*
*University of Parma*

# Response to Reviewer

## Reviewer #1

| | *Response* |
|---|---|
| **1.** The abstract needs work and inclusion of the objectives and specific results. | The authors accept the reviewer suggestion.<br>Therefore, the abstract has been modified:<br>[*Cadmium is toxic to humans, although Cd-based quantum dots exerts less toxicity. Human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1) were exposed to ionic Cd, Cd(II), and cadmium sulfide quantum dots (CdS QDs), and cell viability, cell integrity, Cd accumulation, mitochondrial function and miRNome profile were evaluated.*<br>*Cell-type and Cd form-specific responses were found: CdS QDs affected cell viability more in HepG2 than in THP-1; respective $IC_{20}$ values were ~ 3 and ~ 50 µg $ml^{-1}$. In both cell types, Cd(II) exerted greater effects on viability.*<br>*Mitochondrial membrane function in HepG2 cells was reduced 70% with 40 µg $ml^{-1}$ CdS QDs but was totally inhibited by Cd(II) at corresponding amounts. In THP-1 cells, CdS QDs has less effect on mitochondrial function; 50 µg $ml^{-1}$ CdS QDs or equivalent Cd(II) caused 30% reduction or total inhibition, respectively. The different in vitro effects of CdS QDs were unrelated to Cd uptake, which was greater in THP-1 cells.*<br>*For both cell types, changes in the expression of miRNAs (miR-222, miR-181a, miR-142-3p, miR-15) were found with CdS QDs, which may be used as biomarkers of hazard nanomaterial exposure. The cell-specific miRNome profiles were indicative of a more conservative autophagic response in THP-1 and as apoptosis as in HepG2.*] |
| **2.** I cannot find the data to support that the NPs aggregate/agglomerate size were characterized in cell media. | Details on the characterization in cell media are now reported in Paragraph 2.1, **lines 124 - 131** (pages 6) [*… Average particle size (dh) of the aggregates and zeta potential in deionized water were estimated 178.7 nm and +15.0 mV, respectively. The zeta potential of CdS QDs were comparable in water and in the culture medium used: QDs have approximately neutral charge. The hydrodynamic diameters of CdS QDs were comparable in water; the difference observed in the experimental systems is due to the presence of divalent cations and serum protein that characterizes the culture medium...*] and in Appendix A. Comparison of data in water and in culture medium are reported in Table A.9. |
| **3.** Line 25 abstract: two human cell lines | The change was not made because the abstract was modified as suggested by the reviewer. |
| **4.** Line 37 abstract: changes in the expression of miRNAs | Change made.<br>Line **39** (page 2): [*…For both cell types, changes in the expression of miRNAs…*]. |

| | |
|---|---|
| **5.** Line 70: damaging? Nucleic acid membranes | Change made.<br>Line **71** (page 3): [*…indirectly affecting integrity of proteins, nucleic acid and membranes…*]. |
| **6.** Line 75: allowed for the identification | Change made.<br>Line **76** (page 4): [*…has allowed for the identification…*]. |
| **7.** Line 75-79: The transcriptomic approach has allowed for the identification of molecular mechanisms of CdS QDs exposure, highlighting potential candidates for exposure biomarkers. This paper describes the miRNA profiles as a consequence of exposure to either ionic Cd or CdS QDs and reveals several miRNAs that have the potential to be early biomarkers of exposure to these toxicants. | Change made.<br>Line **76 – 80** (page 4): [*…The transcriptomic approach has allowed for the identification of molecular mechanisms of CdS QDs exposure, highlighting potential candidates for exposure biomarkers. This paper describes the miRNA profiles as a consequence of exposure to either ionic Cd or CdS QDs and reveals several miRNAs that have the potential to be early biomarkers of exposure to these toxicants…*]. |
| **8.** Line 98: For example, Titanium dioxide | Change made.<br>Line **100** (page 5): [*…For example, titanium dioxide…*]. |
| **9.** Line 102: cell lines | Change made.<br>Line **104** (page 5): [*…cell lines used were…*]. |

# Differences in toxicity, mitochondrial function and miRNome in human cells exposed *in vitro* to Cd as CdS quantum dots or ionic Cd

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**ABSTRACT**

Cadmium is toxic to humans, although Cd-based quantum dots exerts less toxicity. Human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1) were exposed to ionic Cd, Cd(II), and cadmium sulfide quantum dots (CdS QDs), and cell viability, cell integrity, Cd accumulation, mitochondrial function and miRNome profile were evaluated.

Cell-type and Cd form-specific responses were found: CdS QDs affected cell viability more in HepG2 than in THP-1; respective $IC_{20}$ values were ~ 3 and ~ 50 µg $ml^{-1}$. In both cell types, Cd(II) exerted greater effects on viability.

Mitochondrial membrane function in HepG2 cells was reduced 70% with 40 µg $ml^{-1}$ CdS QDs but was totally inhibited by Cd(II) at corresponding amounts. In THP-1 cells, CdS QDs has less effect on mitochondrial function; 50 µg $ml^{-1}$ CdS QDs or equivalent Cd(II) caused 30% reduction or total inhibition, respectively. The different *in vitro* effects of CdS QDs were unrelated to Cd uptake, which was greater in THP-1 cells.

For both cell types, changes in the expression of miRNAs (miR-222, miR-181a, miR-142-3p, miR-15) were found with CdS QDs, which may be used as biomarkers of hazard nanomaterial exposure. The cell-specific miRNome profiles were indicative of a more conservative autophagic response in THP-1 and as apoptosis as in HepG2.

**Keywords.** miRNA; quantum dot; HepG2; THP-1; cadmium.

**Abbreviations.**

Δψm, mitochondrial membrane potential;

Cd(II), $CdSO_4$ 8/3 -hydrate;

CdS QDs, cadmium sulfide quantum dots;

DMEM, Dulbecco's Modified Eagle's Medium;

ENMs, engineered nanomaterials;

FBS, fetal bovine serum;

FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone;

JC1, tetraethylbenzimidazolylcarbocyanine iodide;

PMA, phorbol 12-myristate 13-acetate;

QDs, quantum dots;

SS, side scatter.

## 1. Introduction

Quantum dots (QDs) have medical applications including fluorescence imaging, biosensing and targeted drug delivery to treat inflammation or drug-resistant cancer cells [1–3]; QDs conjugated with antibodies have been used to distinguish normal from cancerous cells [4]. There is an increasing interest in developing nano-theranostic platforms for simultaneous sensing, imaging and therapy [5]. Given the growing demand for and use of QDs, there is a clear need to understand potential toxicity for organisms and the environment [6]. The likely hazards posed by QDs in the biomedical field are not yet fully understood, although some studies have sought to address this issue [7]. The toxicity associated with cadmium (Cd)-containing QDs has been shown to be higher than for other QDs. This has been assumed to be related to the presence of Cd, leading to the production of excessive reactive oxygen species (ROS), indirectly affecting integrity of proteins, nucleic acid and membranes [8–10]. HepG2 cells, a human hepatocellular carcinoma cell line used as a model for human hepatic tissue [11], have been shown to respond to cadmium sulfide quantum

dots (CdS QDs) exposure by altering the abundance of gene transcripts encoding products associated with apoptosis, oxidative stress response and autophagy [12]. The transcriptomic approach has allowed for the identification of molecular mechanisms of CdS QDs exposure, highlighting potential candidates for exposure biomarkers. This paper describes the miRNA profiles as a consequence of exposure to either ionic Cd or CdS QDs and reveals several miRNAs that have the potential to be early biomarkers of exposure to these toxicants [13,14].

MiRNAs are short (19 - 23 nucleotides) non-coding sequences that are ubiquitous in all life forms. Their biological significance lies in their regulatory control over a wide range of cellular processes, achieved either by targeting the degradation of complementary mRNAs or by repressing the process of translation. There is also evidence to suggest that certain miRNAs can interact with sequences in the 5' and 3' untranslated region of their target mRNA, resulting in an enhancement rather than a reduction in translation [15]. Changes in cellular miRNA profiles have been associated with a number of conditions in humans, including cancer, viral infection, immune disorders and cardiovascular diseases [16–18]. In the plant kingdom, miRNA involvement has been described in the response to heavy metal exposure, including Cd and Cu [19,20]. In yeast (*Saccharomyces cerevisiae*), several miRNAs have been associated with the expression of Cd tolerance [21]. A number of epigenetic effects have been shown to be induced by Cd exposure, including DNA methylation, the post-translational modification of histone tails, and the packaging of DNA around the nucleosome; all have been correlated with the abundances of specific miRNAs [22]. Increasing evidence indicates that *in vitro* and *in vivo* exposure of human cells to environmental organic contaminants and metals can alter miRNA expression [23]. It has been demonstrated that the relative abundance of certain miRNAs is responsive

to nanomaterials, although the global effect of this exposure is not understood [24]. For example, titanium dioxide, zinc oxide and gold nanoparticles change miRNAs expression [25,26].

This study examined the changes in the miRNome of two widely studied human cell lines exposed to various levels of Cd, presented as either CdS QDs or Cd(II). The cell lines used were HepG2, hepatocellular carcinoma cells, and THP-1, human macrophage-like cells. While the literature contains numerous descriptions of therapeutic uses of miRNAs [16], their potential as biomarkers for xenobiotic exposure remains unknown; this is in spite of the fact that miRNAs have been reported to be mediators of cellular responses to environmental contaminants [27]. Moreover, the US Food and Drug Administration (USFDA) considers changes in miRNA levels as a possible genome biomarker [13,14]. MiRNAs could be useful not only as potential biomarkers of several diseases but also as key mediators of the mechanisms linking environmental exposure to toxicity and disease development [28]. The present toxicogenomic study on human cell lines was carried out to assess an *in vitro* (non-animal) test for health risk assessment [29] for exposure to ionic- and nanoscale-Cd. In addition, the study was intended to determine whether CdS QDs could represent a less toxic form of Cd in diagnostic medicine [30].

## 2. Materials and methods

### *2.1 Preparation of CdS QDs suspension medium*

CdS QDs were synthesized at IMEM-CNR (Parma, Italy), as described elsewhere [31]. They were characterized in deionized water by transmission electron microscopy (Hitachi HT7700, Hitachi High Technologies America, Pleasanton, CA). Major details are described in Paesano *et al.* [32]. Their structure is crystalline with a

mean static diameter of 5 nm with approximately 78% Cd. Average particle size ($d_h$) of the aggregates and zeta potential in deionized water were estimated 178.7 nm and +15.0 mV, respectively (Zetasizer Nano Series ZS90, Malvern Instruments, Malvern, UK) [33]. The zeta potential of CdS QDs were comparable in water and in the culture medium used: QDs have approximately neutral charge. For hydrodynamic diameters, difference observed in the experimental systems is due to the presence of divalent cations and serum protein that characterizes the culture medium. Characterization details are given in Appendix A. The CdS QDs were suspended in Milli-Q water at a concentration of 100 µg $ml^{-1}$, and pulsed probe sonication was used to minimize aggregation. For cell treatment, the stock particle suspension was vortexed and sonicated for 30 min, and then diluted as appropriate into complete culture medium.

*2.2 Cell Culture, Treatments and Cell Viability Assay*

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 µg $ml^{-1}$ streptomycin, 100 U $ml^{-1}$ penicillin, 4 mM glutamine; for THP-1 cells, the glutamine concentration was reduced to 2 mM. Cells were cultured in 10-cm Petri dishes under a humidified atmosphere in the presence of 5% $CO_2$. Prior to treatment, THP-1 cells were differentiated into macrophages through an incubation with 0.1 µM of phorbol 12-myristate 13-acetate (PMA) for 3 days.

Cells in complete culture medium were seeded into either 96-well plates, at a density of $15 \times 10^3$ cells/well, or 10-cm diameter dishes at $3 \times 10^6$ cells/dish. The medium was replaced after 24 h with fresh medium containing either CdS QDs or Cd(II) (as $CdSO_4$ 8/3 -hydrate). HepG2 cells were treated with a range of Cd concentration, either as CdS QDs or Cd(II), from 0 to 93.6 µg $ml^{-1}$; the THP-1 cells were treated with

a range of Cd doses from 0 to 124.8 µg $ml^{-1}$. Details of all the Cd treatments are given in Table A.1. Each treatment was carried out in triplicate (biological replicates) and each replicate was measured three times (technical replicates). Cell viability was evaluated after 24 h of incubation in the presence of Cd using the resazurin method [34]. Briefly, the culture medium was replaced with a solution of resazurin (44 µM, Sigma-Aldrich, Saint Louis, MO, USA) in serum-free medium. After 30 min, fluorescence was measured at 572 nm with a multimode plate reader (Perkin Elmer Enspire, Waltham, MA, USA). Potential interference in this assay was excluded by measuring fluorescence of the dye mixed with CdS QDs. The treatment time of 24 h was chosen from literature reports about the internalisation time of QDs [35].

*2.3 Mitochondrial Membrane Function Assay*

Mitochondrial membrane potential (Δψm) was estimated using the JC-1 kit (Abcam Ltd, Cambridge, UK) according to the manufacturer's instructions. The assay relies on the accumulation of the cationic dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in energized mitochondria. When the Δψm is low, JC-1 is present mostly in monomeric form, which can be detected through its emission of green fluorescence (530±15 nm). Conversely, when the Δψm is high, the dye polymerizes, resulting in the emission of red to orange fluorescence (590±17.5 nm). Therefore, a decrease in red fluorescence and an increase in green fluorescence are indicative of depolarization in the mitochondrial membrane. Carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), an $H^+$ ionophore uncoupler of oxidative phosphorylation, was used as a Δψm-depolarization positive control. HepG2 or THP-1 cells were seeded into 96-well plates at a density of $7.5 \times 10^4$ cells per well and were incubated for 24 h to allow adhesion. Cells were then exposed to a range of Cd

treatments (Table A.1) for 24 h in the form of either CdS QDs or Cd(II). After extensive washing in phosphate buffered saline (PBS) to remove adherent particles or QDs aggregates, cells were incubated in the JC-1 solution for 30 min at 37°C in the dark. Following a further PBS rinse, fluorescence emitted by the cells was determined by a multimode plate reader (Perkin Elmer Enspire). Individual experiments were run in triplicate; data were expressed as the relative fluorescence unit (RFU) with respect to the control.

*2.4 Confocal Microscopy*

HepG2 and THP-1 cells were seeded into four-well chamber slides at a density of 5 × $10^4$ cells $ml^{-1}$. After treatment with either CdS QDs or Cd(II) (see Table A.1), cells were transferred to a medium containing 5 µM JC-1 for 30 minutes. Following the staining procedure, the cells were rinsed in complete culture medium, incubated at 37°C and 5% $CO_2$ in a Kit Cell Observer (Carl Zeiss, Jena, Germany) and imaged using an inverted LSM 510 Meta laser scanning microscope (Carl Zeiss). Excitation at 633 nm and reflectance were used to visualize CdS QDs. The status of the JC-1 dye was recorded by excitation at 480 nm and the emission was passed through a 535-595 nm filter. In selected experiments, nuclei were counterstained with DRAQ5™ (Alexis Biochemicals, San Diego, California, USA). In these instances, 5 µM DRAQ5™ was added together with JC-1 and cells were visualized with excitation at 633 nm with emission through a 670 nm long pass filter.

The cytoplasm of THP-1 cells exposed to 50 µg $ml^{-1}$ CdS QDs for 24 h was visualized by incubation with 1 µM calcein-AM (Millipore Merck, Burlington, MA, USA) for 2 h; calcein-loaded cells were excited at 488 nm and fluorescence was measured through a 515-540 nm band pass filter.

*2.5 Cellular Uptake of Cadmium*

The entry of CdS QDs into THP-1 cells exposed to 50 µg $ml^{-1}$ of the nanomaterial for either 4 and 24 h was estimated with a cytofluorimetric assay [12]. After exposure, cells were first harvested by trypsin treatment and centrifugation (800 x *g*, 5 min), after which they were suspended in PBS containing 1% (v/v) FBS. The presence of CdS QDs was revealed by flow cytometry (NovoCyte, ACEA Biosciences, San Diego, CA, USA); specifically, CdS QDs uptake was associated with a higher side scatter (SS) intensity. The experiment involved three biological replicates, each represented by three technical replicates. A similar analysis of Cd entry into HepG2 cells has been reported previously [12]. The cells were thoroughly washed to remove any surface-attached agglomerates of CdS QDs and quantification of Cd accumulated by the cells was then obtained using inductively coupled plasma mass spectrometry (ICP-MS) as described by Peng *et al.* [36]. Confocal microscopy showed that agglomerates of CdS QDs were absent from these preparations. HepG2 or THP-1 cells, exposed to various doses of CdS QDs or Cd(II) (Table A.1) for 24 h, were rinsed three times in PBS, harvested by trypsinization prior to counting, and then digested with 67% $HNO_3$ at 165°C for 3 h. The solution obtained was diluted by adding 2 volumes of water prior to ICP-MS analysis.

*2.6 RNA Isolation and miRNAs Quantification*

To avoid compromising RNA integrity, extractions from HepG2 and THP-1 cells exposed to Cd in the form of either CdS QDs or Cd(II) were performed using a mirVANA™ column-based kit (Life Technologies, Carlsbad, CA, USA). RNA concentration and integrity were monitored by spectrophotometry and gel

electrophoresis, respectively. The abundance of each miRNA was obtained using a TaqMan® Array Human MicroRNA A+B Card Set v3.0 (Applied Biosystems, Foster City, CA, USA), which quantifies 754 miRNAs. A 1-µg aliquot of RNA was reverse-transcribed using Megaplex™ RT Primers (Applied Biosystems), and the subsequent PCR array was run using a 7900HT Fast Real Time PCR system (Applied Biosystems) following the MegaPlex™ Pool Protocol (PN 4399721 RevC). Each sample was analyzed in duplicate. The raw data were analyzed using RQ Manager 1.2 software (Applied Biosystems) and relative abundances were calculated using the $2^{-\Delta\Delta Ct}$ method [37]. The selected reference sequence was non-coding U6 small nuclear RNA. The fold-change threshold applied to define significant changes in abundance was 2 (for increased miRNAs) and 0.5 (for decreased miRNAs).

*2.7 In vitro analysis of autophagy: Western blot assay*

Total cell lysates were obtained as described elsewhere [38]. The monolayers were rinsed with ice-cold PBS and then covered with 60 µl of Lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM NaF, 2 mM imidazole) supplemented with a protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche, Monza, Italy). Equal amounts of proteins from each sample were separated by 4-20% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore, Millipore Merck Corporation, MA, USA); membranes were then incubated in TBS with 10% blocking solution (Western Blocking Reagent, Roche) for 1h and exposed overnight at 4°C to primary antibodies against LC3II (microtubule–associated protein light chain 3, Cell Signaling Technology, Danvers, MA, USA), p62 (ubiquitin-binding protein p62, Abcam Ltd) or tubulin (Sigma-Aldrich)

diluted in TBS-T with 5% BSA. After three washes of 10 min each in TBS-T (50mM Tris Base, 150mM NaCl, pH 7.5), membranes were exposed to the HRP-conjugated secondary anti-rabbit or anti-mouse IgG antibodies for 1h at room temperature (HRP, Cell Signaling Technology). Visualization of protein bands was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Merck). The expression of tubulin was used for loading control. Individual experiment were run in triplicate.

*2.8 Statistic and Bioinformatics Analysis*

The software package SPSS Statistics® v.21 (IBM, Armonk, NY, USA) was used to compare control and treatment effects. Levene, Shapiro-Wilk and Kolmogorov-Smirnov tests were applied to ascertain data normality and variance homogeneity. One-way analysis of variance, followed by the Tukey test was used to identify and order means differing significantly from one another. The significance threshold probability was set at 0.05. To visualize transcriptomic data, hierarchical clustering was performed using the heatmap.2 routine implemented in the R software (www.R-project.org/). Genes targeted by differentially abundant miRNAs were identified using the DIANA-Tarbase v.7 database (diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index)[39]. The KEGG pathway enrichment of these target genes was derived from an analysis based on DIANA-mirPath software [40]. The p-value threshold was set 0.05 and FDR correction was applied. miRTargetLink [41] was used to identify interaction networks among the target genes using information documented in the miRTarBase. Only strong interactions (backed up by strong experimental methods such as the 'reporter gene assay') were taken into consideration. PANTHER (pantherdb.org/) software was used

to search for gene enrichment, and the Gene Ontology database provided functional annotation for the genes targeted by differentially abundant miRNAs.

## 3. Results and Discussion

Experiments were designed to compare the responses of HepG2 and THP-1 cells to Cd exposure in the form of either CdS QDs or Cd(II). Some of the distinguishing features of the two cell types are listed in Table A.2. THP-1 were compared with HepG2 cells because of their different role relative to *in vivo* exposure to Cd. In the body, engineered nanoparticles may be recognized and processed by immune cells, among which macrophages play a crucial role. Macrophages act as the first line of defense against invading agents, including QDs [42]. Hepatocytes are instead involved in the attempt to dispose the eventual toxicant in the liver, which is the major human organ which accumulates both $Cd^{2+}$ and Cd-containing QDs [43].

### *3.1 Cell viability*

When exposed to Cd(II), the viability of both cell types was dose-dependent, as reported elsewhere [44,45]. Specifically, the estimated $IC_{50}$ for HepG2 cells was ~ 4 µg $ml^{-1}$ Cd as Cd(II) and ~ 15 µg $ml^{-1}$ Cd as CdS QDs (corresponding to ~ 20 µg $ml^{-1}$ CdS QDs) (Fig. A.1a). The $IC_{20}$ for CdS QDs was calculated at 3 µg $ml^{-1}$ (~ 2.3 µg $ml^{-1}$ Cd). Measurements taken after a 14-day immersion of CdS QDs in the growth medium showed that the release of $Cd^{2+}$ into solution reached a maximum of approximately 1 – 2%, consistent with previous reports [46,47]. This value occurs for all the growth and treatment conditions reported throughout the paper.

For THP-1 cells, the susceptibility to Cd(II) was comparable, whereas the $IC_{20}$ for CdS QDs was nearly 50 µg $ml^{-1}$, and at ~ 120 µg $ml^{-1}$ viability was still more than

60% (Fig. A.1b). Thus, the sub-toxic dose ($IC_{20}$) of CdS QDs for THP-1 cells was established at 50 µg $ml^{-1}$ (39 µg $ml^{-1}$ Cd). From the literature and from our study, an equivalent dose of $Cd^{2+}$ drastically reduces cell viability [48].

*3.2 Mitochondrial Function and Cell Morphology*

Mitochondrial function is one of the main targets of QDs [49,50]. In HepG2 cells, 2.3 µg $ml^{-1}$ of Cd as CdS QDs at $IC_{20}$ (3 µg $ml^{-1}$ CdS QDs) had a minimal effect on mitochondrial membrane potential; an inhibition of ~ 50% was observed at 31.2 µg $ml^{-1}$ of Cd (40 µg $ml^{-1}$ CdS QDs) (Fig. 1a). In contrast, mitochondrial function was significantly inhibited in the presence of 2.3 µg $ml^{-1}$ Cd as Cd(II) (Fig. 1b). THP-1 cells responded in similar fashion but were largely unaffected by CdS QDs exposure even at 50 µg $ml^{-1}$ (39 µg $ml^{-1}$ Cd) (Fig. 1c), although they were quite susceptible to Cd(II), the dose totally inhibiting mitochondrial membrane potential being 7.8 µg $ml^{-1}$ Cd as Cd(II) (Fig. 1d). Therefore, Cd strongly inhibited mitochondrial function in both cell lines when present as Cd(II) but not as CdS QDs, which caused only a partial inhibition.

Confocal images of JC-1-labeled HepG2 cells exposed to 3 µg $ml^{-1}$ of CdS QDs are shown in Fig. A.2. This condition ($IC_{20}$) failed to induce any significant reduction in JC-1 aggregation; the amount of JC-1 monomer was not altered (Fig. A.2), indicating that mitochondrial function was unaffected by the treatment. In this condition, the cell shapes were also normal. Treatment with 2.3 µg $ml^{-1}$ Cd as Cd(II) led to a significant decrease in JC-1 aggregates (data not shown). In contrast, micrographs of THP-1 cells exposed to 5 µg $ml^{-1}$ Cd in the form of either Cd(II) or CdS QDs (Fig. 2), show a significant alteration in mitochondrial function after exposure to Cd(II). When THP-1 cells were exposed to 50 µg $ml^{-1}$ of CdS QDs, a more significant reduction in JC-1

aggregates was observed (Fig. 2), but cell morphology appeared to be substantially unaffected.

*3.3 Cd Uptake*

Internalization of QDs in human cells occurs *in vitro* within 24 h from exposure [51]. A cytofluorimetric assay was used to demonstrate the capacity of HepG2 and THP-1 cells to accumulate CdS QDs. CdS QDs uptake by HepG2 cells was reported in a previous paper [12]. The same method was applied here for the THP-1 cell line. A significant increase in side scatter (SS) was observed when cells were exposed to 50 µg $ml^{-1}$ of CdS QDs for 4 h and 24 h (Fig. 3), consistent with QDs entry. Separate ICP-MS measurements of cells exposed to CdS QDs for 24 h, with subsequent thorough washing to remove any CdS QDs remaining on the surface, demonstrated a dose-dependent increase in cellular Cd levels (Table A.3). Interestingly, HepG2 cells accumulated greater amounts of Cd upon exposure to CdS QDs than to equivalent amounts of Cd as Cd(II). THP-1 cells accumulated more Cd than HepG2 cells, possibly a result of their phagocytic competence. Also in this case the uptake of Cd as CdS QDs was higher than for Cd as Cd(II). Therefore, the larger negative impacts on viability and mitochondrial function reported for Cd(II) are not due to a greater uptake of Cd.

To evaluate the interaction of THP-1 cells with CdS QDs, calcein-loaded macrophages were treated with 50 µg $ml^{-1}$ of CdS QDs: the majority of the CdS QDs formed aggregates that were clearly evident in reflectance mode (see the grey pseudocolor in the confocal images in Fig. A.3a). The orthogonal projections and 3-D reconstruction indicate that the CdS QDs were grouped in aggregates in close

contact with the cell surface, with images indicating the formation of deep, shallow invaginations in the cell membrane, highly suggestive of internalization (Fig. A.3b).

*3.4 miRNAs Expression Profiling: Comparison Between CdS QDs and* Cd(II)

Significant changes have been reported for miRNAs of human cells exposed to engineered nanomaterials (ENMs) [25]. Table A.4 gives a summary of the effect of Cd exposure on HepG2 and THP-1 miRNomes (the number of assayed miRNAs was 754). For HepG2 cells exposed to 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II), the number of miRNAs with significantly increased or decreased abundance are reported in Fig. 4a as Venn diagrams. Heatmaps showed the abundances of three miRNAs (miR-1267, miR-200a-5p, 26b-3p) which were increased by CdS QDs, but reduced by Cd(II); the opposite trend was evident for three other miRNAs (miR-218-5p, miR-548b-3p, miR-589-3p) (Fig. 5a). A more extensive heatmap is presented in Fig. 1 in Paesano *et al.* (Data in Brief). The analysis demonstrates that exposure to CdS QDs or to Cd(II) had markedly different effects on the HepG2 miRNome. The response of THP-1 cells was more complex, with markedly different effects of high dose CdS QDs (39 µg $ml^{-1}$ Cd) or Cd(II) (5 µg $ml^{-1}$ Cd) on miRNAs abundance (Fig. 6a). Heatmap representations of these data are given in Fig. 2a in Paesano *et al.* (Data in Brief). When THP-1 cells were exposed to lower doses of Cd (5 µg $ml^{-1}$), equivalent to 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II), the effects on miRNAs levels were different: only six common miRNAs were found up-modulated while one down-modulated (Fig. 4b). CdS QDs induced a general increase in miRNAs levels, while Cd(II) produced a decrease (heatmap with individual variations is reported in Fig. 2b in Paesano *et al.* (Data in Brief)). Thus, at this lower level of stress, the two forms of

Cd also had very different effects on the miRNome in THP-1 and HepG2 cells; Cd(II) led to more dramatic consequences as compared with CdS QDs.

*3.5 Comparison between the Cell Line Responses to Cd*

Figs 4c, d and 5b, c show a comparison of the miRNomes for HepG2 and THP-1 cells when exposed to CdS QDs and Cd(II).

Exposure of THP-1 cells to 50 µg $ml^{-1}$ CdS QDs had a similar suppressive effect on cell viability as did exposure of 3 µg $ml^{-1}$ CdS QDs on HepG2 cells (Fig. A.1). However, there was little similarity with respect to the effect of the exposure on the miRNome. Specifically, there was no overlap between the sets of miRNAs that increased in abundance, although there were 17 suppressed miRNAs in common between the two cell types (Fig. 6b). Conversely, 13 of the miRNAs responded differentially, either increasing in abundance in THP-1 cells while decreasing in HepG2 cells, or *vice versa*. Analysis of the relevant heatmaps (Fig. 5b and Fig. 3a in Paesano *et al.* (Data in Brief)) suggests that the two cell types deployed different strategies to maintain viability in response to Cd exposure. Molecular responses to a comparable level of CdS QDs-imposed stress (3 µg $ml^{-1}$ for HepG2 and 6.4 µg $ml^{-1}$ for THP-1 cells) were also quite distinct: 10 miRNAs increased in both cell types, and 2 decreased (Fig. 4c). In THP-1 cells, exposure to the lower dose of CdS QDs mostly increased miRNAs levels. When the stress was imposed by Cd(II), the responses of the two cell types were similar in the number of miRNAs down-modulated, with 39 of these in common (Fig. 4d). The heatmaps presented in Figs 5b, c presents an overview of the effect of the lower dose of CdS QDs and Cd(II) on the miRNome. A comparison between the two cell lines each challenged with CdS QDs at lower (3 or 6.4 µg $ml^{-1}$) and THP-1 at higher dose (50 µg $ml^{-1}$) is shown in Fig. 3b in Paesano *et*

*al.* (Data in Brief). For both THP-1 and HepG2 the lower doses result primarily in up-modulation, whereas THP-1 at 50 µg $ml^{-1}$ is largely down-modulated. A global comparison between the responses of the two cell lines to CdS QDs-imposed stress is also given in Fig. 6c. For THP-1 cells, 130 miRNAs were modulated exclusively in response to 50 µg $ml^{-1}$ of CdS QDs treatment but at 6.4 µg $ml^{-1}$, that value was only 45. For HepG2 cells, 26 miRNAs responded exclusively to 3 µg $ml^{-1}$ CdS QDs. In conclusion, the miRNomes of the two cell lines reacted differently to QDs exposure; however, exposure to Cd(II) caused mainly a reduction in miRNA abundances in both cell lines.

*3.6 In silico analysis: Pathways, GO and Networks Analysis*

The pathways potentially impacted by miRNA modulation under Cd-induced stress were identified using the DIANA-mirPath algorithm [40]. In the case of the HepG2 cell line, Tables A.5 and A.6 show the cellular pathways more likely affected by 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). An equivalent analysis was conducted for THP-1 cells exposed to either 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II) (Tables A.7 and A.8). Although a rather similar set of pathways was impacted in the two cell types, it is noteworthy that the miRNAs involved were markedly different for the two forms of Cd. An *in silico* analysis on the biological significance of the differentially abundant miRNAs was also performed using miRTargetLink and PANTHER software. Gene ontology (GO) enrichment analysis from PANTHER gave results shown summarized below and reported in details in Fig. 4 in Paesano *et al.* (Data in Brief) for HepG2 cells, treated with either CdS QDs or Cd(II). Fig. 5 in Paesano *et al.* (Data in Brief) shows results for THP-1 cells treated with 50 µg $ml^{-1}$ CdS QDs, and Fig. 6 in Paesano *et al.* (Data in Brief) reports THP-1 cells exposed to the lower dose of CdS

QDs or to Cd(II). A comparison for HepG2 showed that in the treatment with CdS QDs the major GO categories involved were: ‘miRNA mediated inhibition of translation’, ‘regulation of RNA polymerase II transcriptional preinitiation complex assembly’ and ‘regulation of gene silencing by miRNA’. In the case of Cd(II) the major target genes were associated with apoptosis, stress response, gene silencing and mitochondrial depolarization.

For THP-1 exposed to the lower dose of CdS QDs (6.4 µg $ml^{-1}$), the main GO categories were ‘positive regulation of cell-cycle phase transition’, ‘regulation of cell-cycle G1/S phase transition’ and ‘positive regulation of production of miRNAs involved in gene silencing by miRNA’. In the case of Cd(II) the gene targets belonged to: ‘regulation of B cell apoptotic process’, ‘release of cytochrome c from mitochondria’, ‘positive regulation of protein insertion into mitochondrial membrane involved in programmed cell death’ and ‘leukocyte apoptotic process’. For THP-1, GO categories related to mitochondrial function were more evident when treated with Cd(II) or with CdS QDs at the higher dose. Indeed, when THP-1 were treated with the higher dose of CdS QDs (50 µg $ml^{-1}$) most of the regulated miRNA belonged to GO categories: ‘regulation of production of miRNAs involved in gene silencing by miRNA’, ‘extrinsic apoptotic signaling pathway in absence of ligand’, ‘regulation of mitochondrial membrane potential’ and ‘cellular response to mechanical stimulus’. A comparison of the GO categories of the target genes in the two cell types revealed for treatment with CdS QDs some commonalities, notably 'epidermal growth factor receptor signaling', 'positive regulation of mitotic cell cycle phase transition' and 'negative regulation of extrinsic apoptosis' (see Fig. 7 in Paesano *et al.* (Data in Brief)). Some common categories were also evident from comparison between the response of cells exposed to CdS QDs and those exposed to Cd(II) (see Fig. 7 in

Paesano *et al.* (Data in Brief)). Although the two cell lines responded differently to CdS QDs, this analysis has highlighted that some targets of regulated miRNAs belong to the same classes of GO, suggesting that they are involved in the same cellular processes. All similarities and differences in response to CdS QDs and to Cd(II) was markedly different both in HepG2 and in THP-1 are shown in Fig. 7 in Paesano *et al.* (Data in Brief).

miRTargetLink software was used to generate regulatory networks using miRNAs modulated in response to CdS QDs in HepG2 and THP-1 cells. From these data, a network was created considering mainly autophagic and apoptotic pathways. The network summarized the response of the two cell types to CdS QDs. Overall, the autophagic pathway seemed activated in THP-1 cells exposed to the higher, but not to the lower dose of CdS QDs. In contrast, in HepG2 cells, exposure to QDs led to activation of the apoptotic process. These networks are illustrated in Figs 8a, b in Paesano *et al.* (Data in Brief).

*3.7 Activation of miRNA Response*

One notable feature of the response of THP-1 cells to 50 µg $ml^{-1}$ CdS QDs was the high number of miRNAs with a decreased abundance. The major pathways likely affected by this response were apoptosis, DNA repair, cell cycling, xenobiotic metabolism and autophagy. In particular, Fig. 7 illustrates a reconstruction *in silico* of miRNAs involved in the regulation of autophagy in the response of THP-1 to the higher dose of CdS QDs (50 µg $ml^{-1}$); however, the same pathway appears to be largely unaffected in THP-1 cells exposed to the lower dose of CdS QDs (6.4 µg $ml^{-1}$, Fig. 9 in Paesano *et al.* (Data in Brief)). *MTOR* transcript was likely repressed, given that the abundance of miR-101, miR-199a, miR-30a and miR-7 was enhanced. At the

same time, the vesicle elongation phase could be repressed by up-regulated miRNAs including miR-101, miR-30a, miR-885-3p and miR-181a. Moreover, miR-30a, which is involved in the repression of Beclin-1, was up-regulated, thus pointing to autophagy suppression. Several other miRNAs that responded positively to exposure also have gene targets that encode proteins involved in autophagy (Fig. 9 in Paesano *et al.* (Data in Brief)). This hypothesis is confirmed by *in vitro* analysis with autophagy markers (LC3II and p62). LC3II is recruited from the cytosol and associates with the phagophore early in autophagy. This localization serves as a general marker for autophagic membranes and for monitoring the process as it develops [53]. p62 is a receptor for cargo destined to be degraded by autophagy, including ubiquitinated protein aggregates destined for clearance. The p62 protein is able to bind ubiquitin and also to LC3II, thereby targeting the autophagosome and facilitating clearance of ubiquitinated proteins [54]. As shown in Fig. 8, the induction of autophagy in THP-1 cells treated with Cd as CdS QDs was confirmed by an increase in LC3II and a constant p62 levels, while the increase in p62 and LC3II levels after exposure to 5 µg $ml^{-1}$ of Cd as Cd(II) (11.4 µg $ml^{-1}$) suggests a blockage of the autophagic flow. Conversely, the miRNAs responding in the CdS QDs-exposed HepG2 cells had little or no association with the regulation of autophagy but were, instead, associated with apoptosis (Fig. 9). In this case, the exposure to QDs does not cause an increase in LC3II, suggesting a normal condition of the autophagic flow (Fig. 8). Thus, autophagy seemed to be preferentially activated over apoptosis in THP-1 cells exposed to the highest dose of Cd (Fig. 10 in Paesano *et al.* (Data in Brief)). Instead, THP-1 cells exposed to the lower dose of CdS QDs did not activate the apoptotic process (Fig. 11 in Paesano *et al.* (Data in Brief)), which was, however,

triggered by the exposure to the equivalent dose of Cd as Cd(II) (Fig. 12 in Paesano *et al.* (Data in Brief)).

A previous analysis of the HepG2 response to CdS QDs exposure had suggested that a number of genes associated with apoptosis were among those up-regulated by the stress [12,55]. The current work demonstrates that exposure to CdS QDs reduced the abundance of both miR-32 and miR-149, which would have favored the release of cytochrome c, mitochondria-related apoptosis inducing factor and endonuclease G and, hence, promoted apoptosis [56,57]. The response to Cd(II) suggests that both the intrinsic and the extrinsic apoptotic pathways were activated, pointing to a larger alteration and damage of cell viability (Fig. 13 in Paesano *et al.* (Data in Brief)). The response of THP-1 cells to CdS QDs exposure was quite different in term of cell viability, mitochondrial function and in the number of miRNAs up- or down-modulated. This may explain why these cells appeared to be less susceptible to the stress than HepG2 cells: autophagy is obviously less clearly indicative of a death process than the triggering of apoptosis. Moreover, at the lower dose of CdS QDs, THP-1 cells do not activate either autophagy or apoptosis, relying on subtler rescue mechanisms (see Figs 9 and 10 in Paesano *et al.* (Data in Brief)).

An overview of the differences and commonalities between the miRNomes of the two cell types in response to the lower or to the higher level of CdS QDs is shown in Table 1 and in Figs 14a, b in Paesano *et al.* (Data in Brief). Of note, two cancer-associated miRNAs, miR-191-3p and miR-133a-3p, are increased in abundance. Table 1 catalogs the miRNAs that were most responsive to the various treatments, including Cd(II), along with functional information regarding their likely target genes [58,59]. miRNAs belonging to the let-7 family were particularly responsive to Cd exposure; these miRNAs have been described as tumor suppressors, given that their

abundance is often much lower in cancerous than in healthy tissues [29,60]. In the THP-1 cells, seven let-7 miRNAs were reduced in abundance after exposure to 50 µg $ml^{-1}$ CdS QDs, whereas there was no effect in cells exposed to the lower dose. Meanwhile, exposure to 11.4 µg $ml^{-1}$ Cd(II) reduced the abundance of eight let-7 miRNAs. Note that in HepG2 cells exposed to 5.2 µg $ml^{-1}$ Cd(II), only three let-7 miRNAs were reduced. In THP-1 cells, miR-15b, which has also been implicated as a tumor suppressor because it affects apoptosis through its targeting of gene *BCL-2* [61], was also reduced by 50 µg $ml^{-1}$ CdS QDs. A low dose of CdS QDs in HepG2 cells reduced expression of miR-15b in HepG2 cells but a comparable dose had no effect on THP-1 cells.

## 4. Conclusion

*In vitro* studies on cellular models have clearly shown the molecular effects of ENMs such as QDs and suggested possible modes of action in relation to their intrinsic physico-chemical properties [62]. This information may be important for defining their hazardous properties, a critical step in the identification of suitable biomarkers of exposure. For similar QDs the metal (e.g. Cd) is largely responsible for the toxicity [63]. *In vivo* evidence shows QDs cause pulmonary inflammation and hepatic toxicity [64,65]. MiRNAs have been suggested as potential biomarkers of exposure to toxins with some having important roles in multiple signaling pathways and apoptosis [28]. One function of miRNAs seems to cover a critical aspect of the general stress response [66] with involvement in the formation of stress-induced response complex (SIRC) which shuttles miRNAs into the nucleus [67]. Some proteins responsive to metal-containing QDs, including metallothionein 1A, cytochrome P450 1A and heme oxygenase, can be used as sensitivity biomarkers [68], but other events and

molecules would be useful to track exposure to QDs. After the oxidative stress which follows ROS production and mitochondrial stress, additional glutathione is synthesized and redistributed via MPAK-Nrf2. In addition TFEB is activated which may promote lysosome formation and stabilization, helping to clear damaged organelles [69]. If the stress continues there can be different types of cell damage [10] including autophagy [70], apoptosis [71] and necrosis [72].

Different studies propose miRNAs as biomarkers of adverse exposure to metal-based nanomaterials [25]. Moreover, the USFDA has recently accepted the use of miRNAs as ‘genome biomarkers’.

Although miRNA profiling has been used to detect the response of different types of cells and organisms to metals and to nanomaterials such as CdTe QDs [73], no available study reports a direct comparison between exposure to the same metal/element as a salt and as a QD constituent. A number of studies have correlated the level of toxicant exposure to the induction of miRNAs in blood [13,14] but there are several potential drawbacks of using miRNA changes to detect any possible ‘genome biomarkers’ of exposure, including molecular instability [74]. The assay of miRNAs expression we used here was based on 'array' quantitative PCR with specific primers and TaqMan probes, which constitutes a gold-standard method for quantitative transcriptional analysis [75]. Exposure to cadmium-based QDs and changes in miRNAs have been correlated and used to explain cytotoxicity in mammalian NIH/3T3 cells [73], in zebrafish liver cells [76], and in the brain of Alzheimer's disease patients [77]. Altering the level of a single miRNA can trigger a cascade of signaling events, potentially culminating in a major effect, either stimulatory or inhibitory, on cell proliferation, apoptosis or other processes. In principle, this raises the possibility of clinical interventions based on the modulation of

specific miRNAs by exposure to inhibitors or enhancers. The data presented here showed that nanosized Cd, rather than ionic Cd, has a ‘soft’ regulatory effect on miRNomes in human cells that is quite different from the ‘toxic’ inhibitory impact of ionic Cd. There are three possible levels of response of human cells to nanomaterials such as CdS QDs. The first of these is cell-type specific, as evidenced in a meta-analysis of Cd-containing QDs [35]. Macrophages appear to be less susceptible to toxicity than hepatocytes, even though they accumulate QDs more readily. The second is physiological, as exemplified by differences in the capacity to maintain mitochondrial structure and function when exposed to the stress agent. The final level relates to the response of the miRNome, which has an impact on the expression of various genes associated with defense or response to damage. It is known that CdS QDs enter HepG2 cells. Previous studies had shown this was followed by entry into lysosomes, triggering lysosomal enzymes with production of ROS and initiation of autophagy [78] or apoptosis [79]. In our work HepG2 cells seem to be programmed for apoptosis when exposed to CdS QDs, whereas for THP-1 cells the outcome is autophagy. Some nanomaterials induce autophagy in cancer cells which could lead to cancer cell death, enabling specific cancer therapies [80]. Autophagy induced by QDs can be seen as an attempt to degrade what is perceived as foreign [81], but, in some instances, as for HepG2 cells, it can lead to apoptosis and cell death [82]. MiRNAs associated with mitochondria [83,84] and cytosolic miRNAs can be transferred into the mitochondria (or generated inside) and initiate this deregulation processes [85]. Mitochondria are known as ROS generators and also targets of ROS [49]. ROS cause mitochondrial swelling, inhibition of respiration and mitochondrial permeability transition [86]. In the cells we studied, mitochondrial function was particularly sensitive to Cd(II) but less sensitive to QDs. In particular, the

relative tolerance of THP-1 cells favors the idea that this cell type is more capable to maintain a stable level of cellular homeostasis employing autophagy. Another potentially significant impact is the activation of miRNAs of the tumor-suppressing let-7 family which were down-regulated by Cd(II) but not by equivalent doses of Cd QDs. The relative low cytotoxicity exhibited by CdS QDs could be of interest in the context of their potential use as carriers of clinically active compounds such as antibiotics [87] or antibodies [88] or in gene delivery, as in gene therapy [89, 90].

## Appendix A. Supplementary data


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## Declaration of Competing Interest

The authors declare no competing financial interest.

## Author Contributions

The manuscript was written with contributions from all authors who have given approval to the final version of the manuscript.

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## Figure captions

**Fig. 1** *The effect of CdS QDs and Cd(II) treatment on mitochondrial membrane potential, as quantified by JC-1 staining.* Cells were exposed for 24 h to Cd in the form of either CdS QDs or Cd(II). The data report the ratio between aggregated and monomeric forms of JC1, and are representative of three independent experiments. The concentrations of CdS QDs and Cd(II) shown are for the Cd in the material. Asterisks ***. ****: $p<0.001$, $<0.0001$ vs. values obtained from non-treated cells.

**Fig. 2** *The effect on THP-1 cell morphology of exposure to Cd in the form of either CdS QDs or* Cd(II)*.* After a 24 h exposure to a high or low dose of either stressor, cell monolayers were labelled with JC-1 to assay mitochondrial function or with DRAQ5 to assay nuclear morphology. CdS QDs, 6.4 µg $ml^{-1}$ equivalent to 5 µg $ml^{-1}$ Cd, induced a modest increase in the amount of JC-1 monomers, suggesting some alteration in mitochondrial function but there was no evidence of marked changes in cell morphology. Cd in the form of Cd(II), 11.4 µg $ml^{-1}$ equivalent to 5 µg $ml^{-1}$ Cd, not only substantially increased the abundance of JC-1 monomers, but also caused loss of the red signal, suggesting a significant alteration in mitochondrial function. In addition, Cd(II) treatment also changed the typical elongated shape into a more rounded form. When THP-1 cells were exposed to a high dose of CdS QDs, 50 µg $ml^{-1}$ equivalent to 39 µg $ml^{-1}$ Cd, most of the CdS QDs aggregated and the presence of JC-1 monomeric forms was only slightly increased. Cell morphology appeared to be substantially unaffected. Bar: 20 µm. The images illustrate representative microscope fields where at least 100 cells were present.

**Fig. 3** *The uptake of CdS QDs into THP-1 cells as measured using a cytofluorimetric assay.* Cells were exposed to 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs for 0 - 24 h. Typical scatter plots are shown, obtained from a representative experiment performed three times with comparable results. FS, forward scatter; SS, side scatter

**Fig. 4** *Venn diagram representation of the effect of exposure to Cd on the miRNome.* **a**, HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). The number of miRNAs increased in abundance were 34 and 29, respectively, while number of miRNAs decreased in abundance were 32 and 102, respectively. Only 11 and 13 miRNAs were increased or reduced in abundance by both treatments, respectively. **b**, THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II). Exposure to CdS QDs increased the abundance of 136 miRNAs, whereas only 15 were reduced. **c**, Comparison between HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs and THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 6.4 µg $ml^{-1}$ CdS QDs. Ten miRNAs responded positively and 2 responded negatively in both cell types. Eight miRNAs responded in opposite directions. **d**, Comparison between HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 5.2 µg $ml^{-1}$ Cd(II) and THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 11.4 µg $ml^{-1}$ Cd(II). Thirty nine miRNAs responded negatively in both cell types, while no miRNA responded positively; 16 miRNAs responded in opposite manner.

**Fig. 5** *A heatmap-based illustration of the HepG2 and THP-1 cell responses to Cd exposure*. The heatmaps show only those miRNAs which were increased or decreased in both cell types or with either treatment. Positively responding miRNAs are shown in red and negatively responding ones in green. **a**, Differentially abundant

miRNAs present in HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). For a large number of miRNAs abundance is reduced when the cells are treated with Cd(II) as compared with cells treated with CdS QDs. **b**, Differentially abundant miRNAs present in HepG2 and THP-1 cells exposed to 2.3 and 5 µg $ml^{-1}$ Cd as 5.2 µg $ml^{-1}$ and 11.4 µg $ml^{-1}$ Cd(II). **c**, Differentially abundant miRNAs present in HepG2 and THP-1 cells exposed to 2.3 and 5 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ and 6.4 µg $ml^{-1}$ CdS QDs.

**Fig. 6** *The effect on the miRNome of exposure to Cd, illustrated by a Venn diagram.* **a**, miRNAs induced in THP-1 cells in response to exposure to either 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs or 5 µg $ml^{-1}$ Cd as 11.4 µg $ml^{-1}$ Cd(II). The abundances of totals of 9 and 18 miRNAs were increased by CdS QDs and Cd(II) treatment, respectively. miRNAs decreased in response to the two treatments were 237 and 129 respectively; of these, 124 responded negatively to both treatments, while 5 miRNAs were decreased by Cd(II) treatment but increased in the presence of CdS QDs. **b**, miRNAs induced in either HepG2 or THP-1 cells in response to exposure to, respectively, 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs and 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs; **c**, miRNAs induced in either HepG2 or THP-1 cells in response to exposure to CdS QDs (all treatments).

**Fig. 7** *The core autophagy pathway and its regulation by miRNAs in THP-1 cells exposed to 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs..* The entire pathway was divided into five steps: induction, vesicle nucleation, elongation, retrieval and fusion. Arrows indicate increase or decrease of miRNA. A green arrow indicated a decrease with

lack of repression of its specific targets. The overall effect seems to bring the cell towards autophagosome formation and autophagy.

**Fig. 8** *The effect of exposure to Cd on autophagy markers in THP-1 and HepG2 cells.* THP-1 and HepG2 cells were incubated for 24h in the presence of different doses of Cd: 2.3 µg $ml^{-1}$ as 3 µg $ml^{-1}$ CdS QDs, 5 µg $ml^{-1}$ as 6.4 µg $ml^{-1}$ CdS QDs or as 11.4 µg $ml^{-1}$ Cd(II) and 39 µg $ml^{-1}$ as 50 µg $ml^{-1}$ CdS QDs. Cells were then extracted and Western Blot analysis of p62 and LC3II was performed as described in Materials and Methods. Tubulin was used for loading control. *Pos* indicates THP-1 cells, treated with rapamycin, 10 nM, 3h, and cloroquine, 100 µM, 2h, exploited as positive controls for autophagy.

**Fig. 9** *The core apoptotic pathway and its regulation by miRNAs in HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs.* The figure depicts events of the intrinsic and extrinsic apoptotic pathways. Arrows indicate increase or decrease of miRNA or gene. A red arrow indicates increased abundance of a specific gene. A green arrow indicates a decrease which permits the expression of its specific target. In this system the activation of the intrinsic pathway leads to apoptosis. At the dose of CdS QDs considered and under the experimental conditions adopted, the proportion of cells which effectively completed apoptosis was limited, as shown by morphological observation (see Fig. A.2).

## Table

**Table 1** Differentially abundant miRNAs in response to Cd exposure and their principal cellular targets, pathways and related diseases

| | | THP-1 | | | HepG2 | | | |
|---|---|---|---|---|---|---|---|---|
| | | 39 µg ml$^{-1}$ Cd | 5 µg ml$^{-1}$ Cd | | 2.3 µg ml$^{-1}$ Cd | | | |
| **Processes**[1] | **miRNA involved**[2] | QDs[3] 50 µg ml$^{-1}$ | QDs[3] 6.4 µg ml$^{-1}$ | Cd(II)[3] 11.4 µg ml$^{-1}$ | QDs[3] 3 µg ml$^{-1}$ | Cd(II)[3] 5.2 µg ml$^{-1}$ | **Target protein**[4] | **Diseases**[5] |
| Apoptosis | miR-34a | / | / | / | / | ↓ | BCL-2 | Cancer |
| | miR-195 | ↓ | / | / | ↓ | ↓ | | |
| | miR-143 | ↓ | / | ↓ | / | ↓ | | |
| | miR-155 | ↓ | ↑ | ↓ | ↓ | / | | |
| | miR-125 | ↓ | ↑ | ↓ | / | / | | |
| | miR-29a | ↓ | / | / | / | ↓ | CDC42, p58α | Cancer/ Huntington's disease |
| | miR-125b | ↓ | / | ↓ | / | / | p53 | Cancer/ Psoriasis |
| | miR-221 | ↓ | / | ↓ | / | / | p27 (KIP1) | |
| | miR-222 | ↑ | ↑ | ↓ | / | ↓ | | |
| | miR-181a | ↑ | ↑ | / | / | / | BIM | Cancer |
| | miR-32 | ↓ | / | ↓ | ↓ | ↓ | | |
| | miR-25 | ↓ | / | ↓ | / | / | | |
| DNA Repair | miR-16 | ↓ | ↑ | ↓ | / | / | UNG2 | Cancer |
| | miR-199 | ↓ | ↑ | ↓ | / | / | | |
| | miR-21 | ↓ | / | ↓ | / | ↓ | hMSH2 | |
| | miR-192 | ↓ | / | ↓ | / | ↓ | ERCC3, ERCC4 | Toxicant exposure biomarker |
| | miR-101 | ↓ | ↑ | ↓ | / | / | DNA-PKcs | |
| | miR-24 | ↓ | ↑ | ↓ | / | / | H2AX | Cancer |
| | miR-96 | ↓ | / | / | / | / | RAD51 | / |
| Cell cycle | miR-16 | ↓ | ↑ | ↓ | / | / | CDK2 | Cancer |
| | miR-449a/b | ↓ | ↑/↓ | ↓ | / | / | CDK6, CDC25A | / |
| | miR-15 | ↓ | / | / | ↑ | / | WEE1, CHK1 | Cancer |
| | miR-125 | ↓ | ↑ | ↓ | / | / | Cyclin A2 | |
| | let-7b | ↓ | / | ↓ | / | ↓ | Cyclin A | |
| Xenobiotic metabolism | miR-27b | ↓ | / | / | / | ↓ | CYP1B1 | Diabetes |
| | miR-126 | ↓ | ↑ | / | ↓ | ↓ | CYP2A3 | Cancer/ Cardiovascular diseases |
| | miR-378 | ↓ | / | / | ↓ | ↓ | CYP2E1 | Cancer |
| | miR-133a | ↓ | ↑ | / | ↑ | ↑ | GSTP1 | |
| Autophagy/ Phagocytosis | let-7a | ↓ | / | ↓ | / | ↓ | several chemokines | Cancer |
| | miR-146a | ↓ | / | / | / | / | | Inflammatory diseases |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | miR-25 | ↓ | / | ↓ | / | / | | Cancer |
| | miR-26a | ↓ | / | ↓ | / | ↑ | | |
| Autophagy/ Phagocytosis | miR-132 | ↓ | ↑ | ↓ | / | ↑ | several chemokines | Alzheimer's disease |
| | miR-140 | ↓ | ↑ | ↓ | / | ↓ | | Cancer |
| | miR-146b | ↓ | / | / | / | / | | Inflammatory diseases |
| | miR-155 | ↓ | ↑ | ↓ | ↓ | / | | Cancer |
| | miR-210 | ↓ | ↑ | ↓ | / | / | | |
| | miR-21 | ↓ | / | ↓ | / | / | | |
| | miR-142-3p | ↓ | / | / | ↓ | / | | Cardiovascular diseases |
| | miR-125b | ↓ | / | ↓ | / | / | | Cancer |
| | miR-17-5p | ↓ | / | ↓ | / | ↓ | | |
| | miR-24 | ↓ | ↑ | ↓ | / | / | | |
| | miR-30b | ↓ | ↑ | ↓ | / | ↓ | | |
| | miR-101 | ↓ | ↑ | ↓ | / | / | | Toxicant exposure biomarker |
| | miR-652-3p | ↓ | / | ↓ | / | ↓ | | / |
| | miR-1275 | ↓ | ↑ | ↓ | / | ↓ | | / |
| | miR-7 | / | ↑ | / | ↓ | / | mTOR | / |
| | miR-199a | ↓ | ↑ | ↓ | / | / | | Cancer |
| | miR-30a | ↓ | ↑ | / | ↓ | ↓ | Beclin | Cancer |

**Note.** [1] The more relevant processes emerging from analysis by DIANA-mirPath software.
[2] The miRNAs evaluated here represent the more significant variations, which have commonalities between different cell types and different treatments. The same were also suggested as exposure biomarkers for different environmental or health related clues [58,59].
[3] The red and green arrows indicate the miRNA is increased or decreased in abundance.
[4,5] Main target proteins and diseases were taken from literature [58,59].

# Differences in toxicity, mitochondrial function and miRNome in human cells exposed *in vitro* to Cd as CdS quantum dots or ionic Cd

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**ABSTRACT**

Cadmium is toxic to humans, although Cd-based quantum dots exerts less toxicity. Human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1) were exposed to ionic Cd, Cd(II), and cadmium sulfide quantum dots (CdS QDs), and cell viability, cell integrity, Cd accumulation, mitochondrial function and miRNome profile were evaluated.

Cell-type and Cd form-specific responses were found: CdS QDs affected cell viability more in HepG2 than in THP-1; respective $IC_{20}$ values were ~ 3 and ~ 50 µg $ml^{-1}$. In both cell types, Cd(II) exerted greater effects on viability.

Mitochondrial membrane function in HepG2 cells was reduced 70% with 40 µg $ml^{-1}$ CdS QDs but was totally inhibited by Cd(II) at corresponding amounts. In THP-1 cells, CdS QDs has less effect on mitochondrial function; 50 µg $ml^{-1}$ CdS QDs or equivalent Cd(II) caused 30% reduction or total inhibition, respectively. The different *in vitro* effects of CdS QDs were unrelated to Cd uptake, which was greater in THP-1 cells.

For both cell types, changes in the expression of miRNAs (miR-222, miR-181a, miR-142-3p, miR-15) were found with CdS QDs, which may be used as biomarkers of hazard nanomaterial exposure. The cell-specific miRNome profiles were indicative of a more conservative autophagic response in THP-1 and as apoptosis as in HepG2.

**Keywords.** miRNA; quantum dot; HepG2; THP-1; cadmium.

**Abbreviations.**

Δψm, mitochondrial membrane potential;

Cd(II), $CdSO_4$ 8/3 -hydrate;

CdS QDs, cadmium sulfide quantum dots;

DMEM, Dulbecco's Modified Eagle's Medium;

ENMs, engineered nanomaterials;

FBS, fetal bovine serum;

FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone;

JC1, tetraethylbenzimidazolylcarbocyanine iodide;

PMA, phorbol 12-myristate 13-acetate;

QDs, quantum dots;

SS, side scatter.

## 1. Introduction

Quantum dots (QDs) have medical applications including fluorescence imaging, biosensing and targeted drug delivery to treat inflammation or drug-resistant cancer cells [1–3]; QDs conjugated with antibodies have been used to distinguish normal from cancerous cells [4]. There is an increasing interest in developing nano-theranostic platforms for simultaneous sensing, imaging and therapy [5]. Given the growing demand for and use of QDs, there is a clear need to understand potential toxicity for organisms and the environment [6]. The likely hazards posed by QDs in the biomedical field are not yet fully understood, although some studies have sought to address this issue [7]. The toxicity associated with cadmium (Cd)-containing QDs has been shown to be higher than for other QDs. This has been assumed to be related to the presence of Cd, leading to the production of excessive reactive oxygen species (ROS), indirectly affecting integrity of proteins, nucleic acid and membranes [8–10]. HepG2 cells, a human hepatocellular carcinoma cell line used as a model for human hepatic tissue [11], have been shown to respond to cadmium sulfide quantum

dots (CdS QDs) exposure by altering the abundance of gene transcripts encoding products associated with apoptosis, oxidative stress response and autophagy [12]. The transcriptomic approach has allowed for the identification of molecular mechanisms of CdS QDs exposure, highlighting potential candidates for exposure biomarkers. This paper describes the miRNA profiles as a consequence of exposure to either ionic Cd or CdS QDs and reveals several miRNAs that have the potential to be early biomarkers of exposure to these toxicants [13,14].

MiRNAs are short (19 - 23 nucleotides) non-coding sequences that are ubiquitous in all life forms. Their biological significance lies in their regulatory control over a wide range of cellular processes, achieved either by targeting the degradation of complementary mRNAs or by repressing the process of translation. There is also evidence to suggest that certain miRNAs can interact with sequences in the 5' and 3' untranslated region of their target mRNA, resulting in an enhancement rather than a reduction in translation [15]. Changes in cellular miRNA profiles have been associated with a number of conditions in humans, including cancer, viral infection, immune disorders and cardiovascular diseases [16–18]. In the plant kingdom, miRNA involvement has been described in the response to heavy metal exposure, including Cd and Cu [19,20]. In yeast (*Saccharomyces cerevisiae*), several miRNAs have been associated with the expression of Cd tolerance [21]. A number of epigenetic effects have been shown to be induced by Cd exposure, including DNA methylation, the post-translational modification of histone tails, and the packaging of DNA around the nucleosome; all have been correlated with the abundances of specific miRNAs [22]. Increasing evidence indicates that *in vitro* and *in vivo* exposure of human cells to environmental organic contaminants and metals can alter miRNA expression [23]. It has been demonstrated that the relative abundance of certain miRNAs is responsive

to nanomaterials, although the global effect of this exposure is not understood [24]. For example, titanium dioxide, zinc oxide and gold nanoparticles change miRNAs expression [25,26].

This study examined the changes in the miRNome of two widely studied human cell lines exposed to various levels of Cd, presented as either CdS QDs or Cd(II). The cell lines used were HepG2, hepatocellular carcinoma cells, and THP-1, human macrophage-like cells. While the literature contains numerous descriptions of therapeutic uses of miRNAs [16], their potential as biomarkers for xenobiotic exposure remains unknown; this is in spite of the fact that miRNAs have been reported to be mediators of cellular responses to environmental contaminants [27]. Moreover, the US Food and Drug Administration (USFDA) considers changes in miRNA levels as a possible genome biomarker [13,14]. MiRNAs could be useful not only as potential biomarkers of several diseases but also as key mediators of the mechanisms linking environmental exposure to toxicity and disease development [28]. The present toxicogenomic study on human cell lines was carried out to assess an *in vitro* (non-animal) test for health risk assessment [29] for exposure to ionic- and nanoscale-Cd. In addition, the study was intended to determine whether CdS QDs could represent a less toxic form of Cd in diagnostic medicine [30].

## 2. Materials and methods

### *2.1 Preparation of CdS QDs suspension medium*

CdS QDs were synthesized at IMEM-CNR (Parma, Italy), as described elsewhere [31]. They were characterized in deionized water by transmission electron microscopy (Hitachi HT7700, Hitachi High Technologies America, Pleasanton, CA). Major details are described in Paesano *et al.* [32]. Their structure is crystalline with a

mean static diameter of 5 nm with approximately 78% Cd. Average particle size ($d_h$) of the aggregates and zeta potential in deionized water were estimated 178.7 nm and +15.0 mV, respectively (Zetasizer Nano Series ZS90, Malvern Instruments, Malvern, UK) [33]. The zeta potential of CdS QDs were comparable in water and in the culture medium used: QDs have approximately neutral charge. For hydrodynamic diameters, difference observed in the experimental systems is due to the presence of divalent cations and serum protein that characterizes the culture medium. Characterization details are given in Appendix A. The CdS QDs were suspended in Milli-Q water at a concentration of 100 µg $ml^{-1}$, and pulsed probe sonication was used to minimize aggregation. For cell treatment, the stock particle suspension was vortexed and sonicated for 30 min, and then diluted as appropriate into complete culture medium.

*2.2 Cell Culture, Treatments and Cell Viability Assay*

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 µg $ml^{-1}$ streptomycin, 100 U $ml^{-1}$ penicillin, 4 mM glutamine; for THP-1 cells, the glutamine concentration was reduced to 2 mM. Cells were cultured in 10-cm Petri dishes under a humidified atmosphere in the presence of 5% $CO_2$. Prior to treatment, THP-1 cells were differentiated into macrophages through an incubation with 0.1 µM of phorbol 12-myristate 13-acetate (PMA) for 3 days.

Cells in complete culture medium were seeded into either 96-well plates, at a density of $15 \times 10^3$ cells/well, or 10-cm diameter dishes at $3 \times 10^6$ cells/dish. The medium was replaced after 24 h with fresh medium containing either CdS QDs or Cd(II) (as $CdSO_4$ 8/3 -hydrate). HepG2 cells were treated with a range of Cd concentration, either as CdS QDs or Cd(II), from 0 to 93.6 µg $ml^{-1}$; the THP-1 cells were treated with

a range of Cd doses from 0 to 124.8 µg $ml^{-1}$. Details of all the Cd treatments are given in Table A.1. Each treatment was carried out in triplicate (biological replicates) and each replicate was measured three times (technical replicates). Cell viability was evaluated after 24 h of incubation in the presence of Cd using the resazurin method [34]. Briefly, the culture medium was replaced with a solution of resazurin (44 µM, Sigma-Aldrich, Saint Louis, MO, USA) in serum-free medium. After 30 min, fluorescence was measured at 572 nm with a multimode plate reader (Perkin Elmer Enspire, Waltham, MA, USA). Potential interference in this assay was excluded by measuring fluorescence of the dye mixed with CdS QDs. The treatment time of 24 h was chosen from literature reports about the internalisation time of QDs [35].

*2.3 Mitochondrial Membrane Function Assay*

Mitochondrial membrane potential (Δψm) was estimated using the JC-1 kit (Abcam Ltd, Cambridge, UK) according to the manufacturer's instructions. The assay relies on the accumulation of the cationic dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in energized mitochondria. When the Δψm is low, JC-1 is present mostly in monomeric form, which can be detected through its emission of green fluorescence (530±15 nm). Conversely, when the Δψm is high, the dye polymerizes, resulting in the emission of red to orange fluorescence (590±17.5 nm). Therefore, a decrease in red fluorescence and an increase in green fluorescence are indicative of depolarization in the mitochondrial membrane. Carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), an $H^+$ ionophore uncoupler of oxidative phosphorylation, was used as a Δψm-depolarization positive control. HepG2 or THP-1 cells were seeded into 96-well plates at a density of $7.5 \times 10^4$ cells per well and were incubated for 24 h to allow adhesion. Cells were then exposed to a range of Cd

treatments (Table A.1) for 24 h in the form of either CdS QDs or Cd(II). After extensive washing in phosphate buffered saline (PBS) to remove adherent particles or QDs aggregates, cells were incubated in the JC-1 solution for 30 min at 37°C in the dark. Following a further PBS rinse, fluorescence emitted by the cells was determined by a multimode plate reader (Perkin Elmer Enspire). Individual experiments were run in triplicate; data were expressed as the relative fluorescence unit (RFU) with respect to the control.

*2.4 Confocal Microscopy*

HepG2 and THP-1 cells were seeded into four-well chamber slides at a density of 5 × $10^4$ cells $ml^{-1}$. After treatment with either CdS QDs or Cd(II) (see Table A.1), cells were transferred to a medium containing 5 µM JC-1 for 30 minutes. Following the staining procedure, the cells were rinsed in complete culture medium, incubated at 37°C and 5% $CO_2$ in a Kit Cell Observer (Carl Zeiss, Jena, Germany) and imaged using an inverted LSM 510 Meta laser scanning microscope (Carl Zeiss). Excitation at 633 nm and reflectance were used to visualize CdS QDs. The status of the JC-1 dye was recorded by excitation at 480 nm and the emission was passed through a 535-595 nm filter. In selected experiments, nuclei were counterstained with DRAQ5™ (Alexis Biochemicals, San Diego, California, USA). In these instances, 5 µM DRAQ5™ was added together with JC-1 and cells were visualized with excitation at 633 nm with emission through a 670 nm long pass filter.

The cytoplasm of THP-1 cells exposed to 50 µg $ml^{-1}$ CdS QDs for 24 h was visualized by incubation with 1 µM calcein-AM (Millipore Merck, Burlington, MA, USA) for 2 h; calcein-loaded cells were excited at 488 nm and fluorescence was measured through a 515-540 nm band pass filter.

*2.5 Cellular Uptake of Cadmium*

The entry of CdS QDs into THP-1 cells exposed to 50 µg $ml^{-1}$ of the nanomaterial for either 4 and 24 h was estimated with a cytofluorimetric assay [12]. After exposure, cells were first harvested by trypsin treatment and centrifugation (800 x *g*, 5 min), after which they were suspended in PBS containing 1% (v/v) FBS. The presence of CdS QDs was revealed by flow cytometry (NovoCyte, ACEA Biosciences, San Diego, CA, USA); specifically, CdS QDs uptake was associated with a higher side scatter (SS) intensity. The experiment involved three biological replicates, each represented by three technical replicates. A similar analysis of Cd entry into HepG2 cells has been reported previously [12]. The cells were thoroughly washed to remove any surface-attached agglomerates of CdS QDs and quantification of Cd accumulated by the cells was then obtained using inductively coupled plasma mass spectrometry (ICP-MS) as described by Peng *et al.* [36]. Confocal microscopy showed that agglomerates of CdS QDs were absent from these preparations. HepG2 or THP-1 cells, exposed to various doses of CdS QDs or Cd(II) (Table A.1) for 24 h, were rinsed three times in PBS, harvested by trypsinization prior to counting, and then digested with 67% $HNO_3$ at 165°C for 3 h. The solution obtained was diluted by adding 2 volumes of water prior to ICP-MS analysis.

*2.6 RNA Isolation and miRNAs Quantification*

To avoid compromising RNA integrity, extractions from HepG2 and THP-1 cells exposed to Cd in the form of either CdS QDs or Cd(II) were performed using a mirVANA™ column-based kit (Life Technologies, Carlsbad, CA, USA). RNA concentration and integrity were monitored by spectrophotometry and gel

electrophoresis, respectively. The abundance of each miRNA was obtained using a TaqMan® Array Human MicroRNA A+B Card Set v3.0 (Applied Biosystems, Foster City, CA, USA), which quantifies 754 miRNAs. A 1-µg aliquot of RNA was reverse-transcribed using Megaplex™ RT Primers (Applied Biosystems), and the subsequent PCR array was run using a 7900HT Fast Real Time PCR system (Applied Biosystems) following the MegaPlex™ Pool Protocol (PN 4399721 RevC). Each sample was analyzed in duplicate. The raw data were analyzed using RQ Manager 1.2 software (Applied Biosystems) and relative abundances were calculated using the $2^{-\Delta\Delta Ct}$ method [37]. The selected reference sequence was non-coding U6 small nuclear RNA. The fold-change threshold applied to define significant changes in abundance was 2 (for increased miRNAs) and 0.5 (for decreased miRNAs).

*2.7 In vitro analysis of autophagy: Western blot assay*

Total cell lysates were obtained as described elsewhere [38]. The monolayers were rinsed with ice-cold PBS and then covered with 60 µl of Lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM NaF, 2 mM imidazole) supplemented with a protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche, Monza, Italy). Equal amounts of proteins from each sample were separated by 4-20% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore, Millipore Merck Corporation, MA, USA); membranes were then incubated in TBS with 10% blocking solution (Western Blocking Reagent, Roche) for 1h and exposed overnight at 4°C to primary antibodies against LC3II (microtubule–associated protein light chain 3, Cell Signaling Technology, Danvers, MA, USA), p62 (ubiquitin-binding protein p62, Abcam Ltd) or tubulin (Sigma-Aldrich)

diluted in TBS-T with 5% BSA. After three washes of 10 min each in TBS-T (50mM Tris Base, 150mM NaCl, pH 7.5), membranes were exposed to the HRP-conjugated secondary anti-rabbit or anti-mouse IgG antibodies for 1h at room temperature (HRP, Cell Signaling Technology). Visualization of protein bands was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Merck). The expression of tubulin was used for loading control. Individual experiment were run in triplicate.

*2.8 Statistic and Bioinformatics Analysis*

The software package SPSS Statistics® v.21 (IBM, Armonk, NY, USA) was used to compare control and treatment effects. Levene, Shapiro-Wilk and Kolmogorov-Smirnov tests were applied to ascertain data normality and variance homogeneity. One-way analysis of variance, followed by the Tukey test was used to identify and order means differing significantly from one another. The significance threshold probability was set at 0.05. To visualize transcriptomic data, hierarchical clustering was performed using the heatmap.2 routine implemented in the R software (www.R-project.org/). Genes targeted by differentially abundant miRNAs were identified using the DIANA-Tarbase v.7 database (diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index)[39]. The KEGG pathway enrichment of these target genes was derived from an analysis based on DIANA-mirPath software [40]. The p-value threshold was set 0.05 and FDR correction was applied. miRTargetLink [41] was used to identify interaction networks among the target genes using information documented in the miRTarBase. Only strong interactions (backed up by strong experimental methods such as the 'reporter gene assay') were taken into consideration. PANTHER (pantherdb.org/) software was used

to search for gene enrichment, and the Gene Ontology database provided functional annotation for the genes targeted by differentially abundant miRNAs.

## 3. Results and Discussion

Experiments were designed to compare the responses of HepG2 and THP-1 cells to Cd exposure in the form of either CdS QDs or Cd(II). Some of the distinguishing features of the two cell types are listed in Table A.2. THP-1 were compared with HepG2 cells because of their different role relative to *in vivo* exposure to Cd. In the body, engineered nanoparticles may be recognized and processed by immune cells, among which macrophages play a crucial role. Macrophages act as the first line of defense against invading agents, including QDs [42]. Hepatocytes are instead involved in the attempt to dispose the eventual toxicant in the liver, which is the major human organ which accumulates both $Cd^{2+}$ and Cd-containing QDs [43].

### *3.1 Cell viability*

When exposed to Cd(II), the viability of both cell types was dose-dependent, as reported elsewhere [44,45]. Specifically, the estimated $IC_{50}$ for HepG2 cells was ~ 4 µg $ml^{-1}$ Cd as Cd(II) and ~ 15 µg $ml^{-1}$ Cd as CdS QDs (corresponding to ~ 20 µg $ml^{-1}$ CdS QDs) (Fig. A.1a). The $IC_{20}$ for CdS QDs was calculated at 3 µg $ml^{-1}$ (~ 2.3 µg $ml^{-1}$ Cd). Measurements taken after a 14-day immersion of CdS QDs in the growth medium showed that the release of $Cd^{2+}$ into solution reached a maximum of approximately 1 – 2%, consistent with previous reports [46,47]. This value occurs for all the growth and treatment conditions reported throughout the paper.

For THP-1 cells, the susceptibility to Cd(II) was comparable, whereas the $IC_{20}$ for CdS QDs was nearly 50 µg $ml^{-1}$, and at ~ 120 µg $ml^{-1}$ viability was still more than

60% (Fig. A.1b). Thus, the sub-toxic dose ($IC_{20}$) of CdS QDs for THP-1 cells was established at 50 µg $ml^{-1}$ (39 µg $ml^{-1}$ Cd). From the literature and from our study, an equivalent dose of $Cd^{2+}$ drastically reduces cell viability [48].

*3.2 Mitochondrial Function and Cell Morphology*

Mitochondrial function is one of the main targets of QDs [49,50]. In HepG2 cells, 2.3 µg $ml^{-1}$ of Cd as CdS QDs at $IC_{20}$ (3 µg $ml^{-1}$ CdS QDs) had a minimal effect on mitochondrial membrane potential; an inhibition of ~ 50% was observed at 31.2 µg $ml^{-1}$ of Cd (40 µg $ml^{-1}$ CdS QDs) (Fig. 1a). In contrast, mitochondrial function was significantly inhibited in the presence of 2.3 µg $ml^{-1}$ Cd as Cd(II) (Fig. 1b). THP-1 cells responded in similar fashion but were largely unaffected by CdS QDs exposure even at 50 µg $ml^{-1}$ (39 µg $ml^{-1}$ Cd) (Fig. 1c), although they were quite susceptible to Cd(II), the dose totally inhibiting mitochondrial membrane potential being 7.8 µg $ml^{-1}$ Cd as Cd(II) (Fig. 1d). Therefore, Cd strongly inhibited mitochondrial function in both cell lines when present as Cd(II) but not as CdS QDs, which caused only a partial inhibition.

Confocal images of JC-1-labeled HepG2 cells exposed to 3 µg $ml^{-1}$ of CdS QDs are shown in Fig. A.2. This condition ($IC_{20}$) failed to induce any significant reduction in JC-1 aggregation; the amount of JC-1 monomer was not altered (Fig. A.2), indicating that mitochondrial function was unaffected by the treatment. In this condition, the cell shapes were also normal. Treatment with 2.3 µg $ml^{-1}$ Cd as Cd(II) led to a significant decrease in JC-1 aggregates (data not shown). In contrast, micrographs of THP-1 cells exposed to 5 µg $ml^{-1}$ Cd in the form of either Cd(II) or CdS QDs (Fig. 2), show a significant alteration in mitochondrial function after exposure to Cd(II). When THP-1 cells were exposed to 50 µg $ml^{-1}$ of CdS QDs, a more significant reduction in JC-1

aggregates was observed (Fig. 2), but cell morphology appeared to be substantially unaffected.

*3.3 Cd Uptake*

Internalization of QDs in human cells occurs *in vitro* within 24 h from exposure [51]. A cytofluorimetric assay was used to demonstrate the capacity of HepG2 and THP-1 cells to accumulate CdS QDs. CdS QDs uptake by HepG2 cells was reported in a previous paper [12]. The same method was applied here for the THP-1 cell line. A significant increase in side scatter (SS) was observed when cells were exposed to 50 µg $ml^{-1}$ of CdS QDs for 4 h and 24 h (Fig. 3), consistent with QDs entry. Separate ICP-MS measurements of cells exposed to CdS QDs for 24 h, with subsequent thorough washing to remove any CdS QDs remaining on the surface, demonstrated a dose-dependent increase in cellular Cd levels (Table A.3). Interestingly, HepG2 cells accumulated greater amounts of Cd upon exposure to CdS QDs than to equivalent amounts of Cd as Cd(II). THP-1 cells accumulated more Cd than HepG2 cells, possibly a result of their phagocytic competence. Also in this case the uptake of Cd as CdS QDs was higher than for Cd as Cd(II). Therefore, the larger negative impacts on viability and mitochondrial function reported for Cd(II) are not due to a greater uptake of Cd.

To evaluate the interaction of THP-1 cells with CdS QDs, calcein-loaded macrophages were treated with 50 µg $ml^{-1}$ of CdS QDs: the majority of the CdS QDs formed aggregates that were clearly evident in reflectance mode (see the grey pseudocolor in the confocal images in Fig. A.3a). The orthogonal projections and 3-D reconstruction indicate that the CdS QDs were grouped in aggregates in close

contact with the cell surface, with images indicating the formation of deep, shallow invaginations in the cell membrane, highly suggestive of internalization (Fig. A.3b).

*3.4 miRNAs Expression Profiling: Comparison Between CdS QDs and* Cd(II)

Significant changes have been reported for miRNAs of human cells exposed to engineered nanomaterials (ENMs) [25]. Table A.4 gives a summary of the effect of Cd exposure on HepG2 and THP-1 miRNomes (the number of assayed miRNAs was 754). For HepG2 cells exposed to 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II), the number of miRNAs with significantly increased or decreased abundance are reported in Fig. 4a as Venn diagrams. Heatmaps showed the abundances of three miRNAs (miR-1267, miR-200a-5p, 26b-3p) which were increased by CdS QDs, but reduced by Cd(II); the opposite trend was evident for three other miRNAs (miR-218-5p, miR-548b-3p, miR-589-3p) (Fig. 5a). A more extensive heatmap is presented in Fig. 1 in Paesano *et al.* (Data in Brief). The analysis demonstrates that exposure to CdS QDs or to Cd(II) had markedly different effects on the HepG2 miRNome. The response of THP-1 cells was more complex, with markedly different effects of high dose CdS QDs (39 µg $ml^{-1}$ Cd) or Cd(II) (5 µg $ml^{-1}$ Cd) on miRNAs abundance (Fig. 6a). Heatmap representations of these data are given in Fig. 2a in Paesano *et al.* (Data in Brief). When THP-1 cells were exposed to lower doses of Cd (5 µg $ml^{-1}$), equivalent to 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II), the effects on miRNAs levels were different: only six common miRNAs were found up-modulated while one down-modulated (Fig. 4b). CdS QDs induced a general increase in miRNAs levels, while Cd(II) produced a decrease (heatmap with individual variations is reported in Fig. 2b in Paesano *et al.* (Data in Brief)). Thus, at this lower level of stress, the two forms of

Cd also had very different effects on the miRNome in THP-1 and HepG2 cells; Cd(II) led to more dramatic consequences as compared with CdS QDs.

*3.5 Comparison between the Cell Line Responses to Cd*

Figs 4c, d and 5b, c show a comparison of the miRNomes for HepG2 and THP-1 cells when exposed to CdS QDs and Cd(II).

Exposure of THP-1 cells to 50 µg $ml^{-1}$ CdS QDs had a similar suppressive effect on cell viability as did exposure of 3 µg $ml^{-1}$ CdS QDs on HepG2 cells (Fig. A.1). However, there was little similarity with respect to the effect of the exposure on the miRNome. Specifically, there was no overlap between the sets of miRNAs that increased in abundance, although there were 17 suppressed miRNAs in common between the two cell types (Fig. 6b). Conversely, 13 of the miRNAs responded differentially, either increasing in abundance in THP-1 cells while decreasing in HepG2 cells, or *vice versa*. Analysis of the relevant heatmaps (Fig. 5b and Fig. 3a in Paesano *et al.* (Data in Brief)) suggests that the two cell types deployed different strategies to maintain viability in response to Cd exposure. Molecular responses to a comparable level of CdS QDs-imposed stress (3 µg $ml^{-1}$ for HepG2 and 6.4 µg $ml^{-1}$ for THP-1 cells) were also quite distinct: 10 miRNAs increased in both cell types, and 2 decreased (Fig. 4c). In THP-1 cells, exposure to the lower dose of CdS QDs mostly increased miRNAs levels. When the stress was imposed by Cd(II), the responses of the two cell types were similar in the number of miRNAs down-modulated, with 39 of these in common (Fig. 4d). The heatmaps presented in Figs 5b, c presents an overview of the effect of the lower dose of CdS QDs and Cd(II) on the miRNome. A comparison between the two cell lines each challenged with CdS QDs at lower (3 or 6.4 µg $ml^{-1}$) and THP-1 at higher dose (50 µg $ml^{-1}$) is shown in Fig. 3b in Paesano *et*

*al.* (Data in Brief). For both THP-1 and HepG2 the lower doses result primarily in up-modulation, whereas THP-1 at 50 µg $ml^{-1}$ is largely down-modulated. A global comparison between the responses of the two cell lines to CdS QDs-imposed stress is also given in Fig. 6c. For THP-1 cells, 130 miRNAs were modulated exclusively in response to 50 µg $ml^{-1}$ of CdS QDs treatment but at 6.4 µg $ml^{-1}$, that value was only 45. For HepG2 cells, 26 miRNAs responded exclusively to 3 µg $ml^{-1}$ CdS QDs. In conclusion, the miRNomes of the two cell lines reacted differently to QDs exposure; however, exposure to Cd(II) caused mainly a reduction in miRNA abundances in both cell lines.

*3.6 In silico analysis: Pathways, GO and Networks Analysis*

The pathways potentially impacted by miRNA modulation under Cd-induced stress were identified using the DIANA-mirPath algorithm [40]. In the case of the HepG2 cell line, Tables A.5 and A.6 show the cellular pathways more likely affected by 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). An equivalent analysis was conducted for THP-1 cells exposed to either 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II) (Tables A.7 and A.8). Although a rather similar set of pathways was impacted in the two cell types, it is noteworthy that the miRNAs involved were markedly different for the two forms of Cd. An *in silico* analysis on the biological significance of the differentially abundant miRNAs was also performed using miRTargetLink and PANTHER software. Gene ontology (GO) enrichment analysis from PANTHER gave results shown summarized below and reported in details in Fig. 4 in Paesano *et al.* (Data in Brief) for HepG2 cells, treated with either CdS QDs or Cd(II). Fig. 5 in Paesano *et al.* (Data in Brief) shows results for THP-1 cells treated with 50 µg $ml^{-1}$ CdS QDs, and Fig. 6 in Paesano *et al.* (Data in Brief) reports THP-1 cells exposed to the lower dose of CdS

QDs or to Cd(II). A comparison for HepG2 showed that in the treatment with CdS QDs the major GO categories involved were: ‘miRNA mediated inhibition of translation’, ‘regulation of RNA polymerase II transcriptional preinitiation complex assembly’ and ‘regulation of gene silencing by miRNA’. In the case of Cd(II) the major target genes were associated with apoptosis, stress response, gene silencing and mitochondrial depolarization.

For THP-1 exposed to the lower dose of CdS QDs (6.4 µg $ml^{-1}$), the main GO categories were ‘positive regulation of cell-cycle phase transition’, ‘regulation of cell-cycle G1/S phase transition’ and ‘positive regulation of production of miRNAs involved in gene silencing by miRNA’. In the case of Cd(II) the gene targets belonged to: ‘regulation of B cell apoptotic process’, ‘release of cytochrome c from mitochondria’, ‘positive regulation of protein insertion into mitochondrial membrane involved in programmed cell death’ and ‘leukocyte apoptotic process’. For THP-1, GO categories related to mitochondrial function were more evident when treated with Cd(II) or with CdS QDs at the higher dose. Indeed, when THP-1 were treated with the higher dose of CdS QDs (50 µg $ml^{-1}$) most of the regulated miRNA belonged to GO categories: ‘regulation of production of miRNAs involved in gene silencing by miRNA’, ‘extrinsic apoptotic signaling pathway in absence of ligand’, ‘regulation of mitochondrial membrane potential’ and ‘cellular response to mechanical stimulus’. A comparison of the GO categories of the target genes in the two cell types revealed for treatment with CdS QDs some commonalities, notably 'epidermal growth factor receptor signaling', 'positive regulation of mitotic cell cycle phase transition' and 'negative regulation of extrinsic apoptosis' (see Fig. 7 in Paesano *et al.* (Data in Brief)). Some common categories were also evident from comparison between the response of cells exposed to CdS QDs and those exposed to Cd(II) (see Fig. 7 in

Paesano *et al.* (Data in Brief)). Although the two cell lines responded differently to CdS QDs, this analysis has highlighted that some targets of regulated miRNAs belong to the same classes of GO, suggesting that they are involved in the same cellular processes. All similarities and differences in response to CdS QDs and to Cd(II) was markedly different both in HepG2 and in THP-1 are shown in Fig. 7 in Paesano *et al.* (Data in Brief).

miRTargetLink software was used to generate regulatory networks using miRNAs modulated in response to CdS QDs in HepG2 and THP-1 cells. From these data, a network was created considering mainly autophagic and apoptotic pathways. The network summarized the response of the two cell types to CdS QDs. Overall, the autophagic pathway seemed activated in THP-1 cells exposed to the higher, but not to the lower dose of CdS QDs. In contrast, in HepG2 cells, exposure to QDs led to activation of the apoptotic process. These networks are illustrated in Figs 8a, b in Paesano *et al.* (Data in Brief).

*3.7 Activation of miRNA Response*

One notable feature of the response of THP-1 cells to 50 µg $ml^{-1}$ CdS QDs was the high number of miRNAs with a decreased abundance. The major pathways likely affected by this response were apoptosis, DNA repair, cell cycling, xenobiotic metabolism and autophagy. In particular, Fig. 7 illustrates a reconstruction *in silico* of miRNAs involved in the regulation of autophagy in the response of THP-1 to the higher dose of CdS QDs (50 µg $ml^{-1}$); however, the same pathway appears to be largely unaffected in THP-1 cells exposed to the lower dose of CdS QDs (6.4 µg $ml^{-1}$, Fig. 9 in Paesano *et al.* (Data in Brief)). *MTOR* transcript was likely repressed, given that the abundance of miR-101, miR-199a, miR-30a and miR-7 was enhanced. At the

same time, the vesicle elongation phase could be repressed by up-regulated miRNAs including miR-101, miR-30a, miR-885-3p and miR-181a. Moreover, miR-30a, which is involved in the repression of Beclin-1, was up-regulated, thus pointing to autophagy suppression. Several other miRNAs that responded positively to exposure also have gene targets that encode proteins involved in autophagy (Fig. 9 in Paesano *et al.* (Data in Brief)). This hypothesis is confirmed by *in vitro* analysis with autophagy markers (LC3II and p62). LC3II is recruited from the cytosol and associates with the phagophore early in autophagy. This localization serves as a general marker for autophagic membranes and for monitoring the process as it develops [53]. p62 is a receptor for cargo destined to be degraded by autophagy, including ubiquitinated protein aggregates destined for clearance. The p62 protein is able to bind ubiquitin and also to LC3II, thereby targeting the autophagosome and facilitating clearance of ubiquitinated proteins [54]. As shown in Fig. 8, the induction of autophagy in THP-1 cells treated with Cd as CdS QDs was confirmed by an increase in LC3II and a constant p62 levels, while the increase in p62 and LC3II levels after exposure to 5 µg $ml^{-1}$ of Cd as Cd(II) (11.4 µg $ml^{-1}$) suggests a blockage of the autophagic flow. Conversely, the miRNAs responding in the CdS QDs-exposed HepG2 cells had little or no association with the regulation of autophagy but were, instead, associated with apoptosis (Fig. 9). In this case, the exposure to QDs does not cause an increase in LC3II, suggesting a normal condition of the autophagic flow (Fig. 8). Thus, autophagy seemed to be preferentially activated over apoptosis in THP-1 cells exposed to the highest dose of Cd (Fig. 10 in Paesano *et al.* (Data in Brief)). Instead, THP-1 cells exposed to the lower dose of CdS QDs did not activate the apoptotic process (Fig. 11 in Paesano *et al.* (Data in Brief)), which was, however,

triggered by the exposure to the equivalent dose of Cd as Cd(II) (Fig. 12 in Paesano *et al.* (Data in Brief)).

A previous analysis of the HepG2 response to CdS QDs exposure had suggested that a number of genes associated with apoptosis were among those up-regulated by the stress [12,55]. The current work demonstrates that exposure to CdS QDs reduced the abundance of both miR-32 and miR-149, which would have favored the release of cytochrome c, mitochondria-related apoptosis inducing factor and endonuclease G and, hence, promoted apoptosis [56,57]. The response to Cd(II) suggests that both the intrinsic and the extrinsic apoptotic pathways were activated, pointing to a larger alteration and damage of cell viability (Fig. 13 in Paesano *et al.* (Data in Brief)). The response of THP-1 cells to CdS QDs exposure was quite different in term of cell viability, mitochondrial function and in the number of miRNAs up- or down-modulated. This may explain why these cells appeared to be less susceptible to the stress than HepG2 cells: autophagy is obviously less clearly indicative of a death process than the triggering of apoptosis. Moreover, at the lower dose of CdS QDs, THP-1 cells do not activate either autophagy or apoptosis, relying on subtler rescue mechanisms (see Figs 9 and 10 in Paesano *et al.* (Data in Brief)).

An overview of the differences and commonalities between the miRNomes of the two cell types in response to the lower or to the higher level of CdS QDs is shown in Table 1 and in Figs 14a, b in Paesano *et al.* (Data in Brief). Of note, two cancer-associated miRNAs, miR-191-3p and miR-133a-3p, are increased in abundance. Table 1 catalogs the miRNAs that were most responsive to the various treatments, including Cd(II), along with functional information regarding their likely target genes [58,59]. miRNAs belonging to the let-7 family were particularly responsive to Cd exposure; these miRNAs have been described as tumor suppressors, given that their

abundance is often much lower in cancerous than in healthy tissues [29,60]. In the THP-1 cells, seven let-7 miRNAs were reduced in abundance after exposure to 50 µg $ml^{-1}$ CdS QDs, whereas there was no effect in cells exposed to the lower dose. Meanwhile, exposure to 11.4 µg $ml^{-1}$ Cd(II) reduced the abundance of eight let-7 miRNAs. Note that in HepG2 cells exposed to 5.2 µg $ml^{-1}$ Cd(II), only three let-7 miRNAs were reduced. In THP-1 cells, miR-15b, which has also been implicated as a tumor suppressor because it affects apoptosis through its targeting of gene *BCL-2* [61], was also reduced by 50 µg $ml^{-1}$ CdS QDs. A low dose of CdS QDs in HepG2 cells reduced expression of miR-15b in HepG2 cells but a comparable dose had no effect on THP-1 cells.

## 4. Conclusion

*In vitro* studies on cellular models have clearly shown the molecular effects of ENMs such as QDs and suggested possible modes of action in relation to their intrinsic physico-chemical properties [62]. This information may be important for defining their hazardous properties, a critical step in the identification of suitable biomarkers of exposure. For similar QDs the metal (e.g. Cd) is largely responsible for the toxicity [63]. *In vivo* evidence shows QDs cause pulmonary inflammation and hepatic toxicity [64,65]. MiRNAs have been suggested as potential biomarkers of exposure to toxins with some having important roles in multiple signaling pathways and apoptosis [28]. One function of miRNAs seems to cover a critical aspect of the general stress response [66] with involvement in the formation of stress-induced response complex (SIRC) which shuttles miRNAs into the nucleus [67]. Some proteins responsive to metal-containing QDs, including metallothionein 1A, cytochrome P450 1A and heme oxygenase, can be used as sensitivity biomarkers [68], but other events and

molecules would be useful to track exposure to QDs. After the oxidative stress which follows ROS production and mitochondrial stress, additional glutathione is synthesized and redistributed via MPAK-Nrf2. In addition TFEB is activated which may promote lysosome formation and stabilization, helping to clear damaged organelles [69]. If the stress continues there can be different types of cell damage [10] including autophagy [70], apoptosis [71] and necrosis [72].

Different studies propose miRNAs as biomarkers of adverse exposure to metal-based nanomaterials [25]. Moreover, the USFDA has recently accepted the use of miRNAs as ‘genome biomarkers’.

Although miRNA profiling has been used to detect the response of different types of cells and organisms to metals and to nanomaterials such as CdTe QDs [73], no available study reports a direct comparison between exposure to the same metal/element as a salt and as a QD constituent. A number of studies have correlated the level of toxicant exposure to the induction of miRNAs in blood [13,14] but there are several potential drawbacks of using miRNA changes to detect any possible ‘genome biomarkers’ of exposure, including molecular instability [74]. The assay of miRNAs expression we used here was based on 'array' quantitative PCR with specific primers and TaqMan probes, which constitutes a gold-standard method for quantitative transcriptional analysis [75]. Exposure to cadmium-based QDs and changes in miRNAs have been correlated and used to explain cytotoxicity in mammalian NIH/3T3 cells [73], in zebrafish liver cells [76], and in the brain of Alzheimer's disease patients [77]. Altering the level of a single miRNA can trigger a cascade of signaling events, potentially culminating in a major effect, either stimulatory or inhibitory, on cell proliferation, apoptosis or other processes. In principle, this raises the possibility of clinical interventions based on the modulation of

specific miRNAs by exposure to inhibitors or enhancers. The data presented here showed that nanosized Cd, rather than ionic Cd, has a ‘soft’ regulatory effect on miRNomes in human cells that is quite different from the ‘toxic’ inhibitory impact of ionic Cd. There are three possible levels of response of human cells to nanomaterials such as CdS QDs. The first of these is cell-type specific, as evidenced in a meta-analysis of Cd-containing QDs [35]. Macrophages appear to be less susceptible to toxicity than hepatocytes, even though they accumulate QDs more readily. The second is physiological, as exemplified by differences in the capacity to maintain mitochondrial structure and function when exposed to the stress agent. The final level relates to the response of the miRNome, which has an impact on the expression of various genes associated with defense or response to damage. It is known that CdS QDs enter HepG2 cells. Previous studies had shown this was followed by entry into lysosomes, triggering lysosomal enzymes with production of ROS and initiation of autophagy [78] or apoptosis [79]. In our work HepG2 cells seem to be programmed for apoptosis when exposed to CdS QDs, whereas for THP-1 cells the outcome is autophagy. Some nanomaterials induce autophagy in cancer cells which could lead to cancer cell death, enabling specific cancer therapies [80]. Autophagy induced by QDs can be seen as an attempt to degrade what is perceived as foreign [81], but, in some instances, as for HepG2 cells, it can lead to apoptosis and cell death [82]. MiRNAs associated with mitochondria [83,84] and cytosolic miRNAs can be transferred into the mitochondria (or generated inside) and initiate this deregulation processes [85]. Mitochondria are known as ROS generators and also targets of ROS [49]. ROS cause mitochondrial swelling, inhibition of respiration and mitochondrial permeability transition [86]. In the cells we studied, mitochondrial function was particularly sensitive to Cd(II) but less sensitive to QDs. In particular, the

relative tolerance of THP-1 cells favors the idea that this cell type is more capable to maintain a stable level of cellular homeostasis employing autophagy. Another potentially significant impact is the activation of miRNAs of the tumor-suppressing let-7 family which were down-regulated by Cd(II) but not by equivalent doses of Cd QDs. The relative low cytotoxicity exhibited by CdS QDs could be of interest in the context of their potential use as carriers of clinically active compounds such as antibiotics [87] or antibodies [88] or in gene delivery, as in gene therapy [89, 90].

## Appendix A. Supplementary data


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## Declaration of Competing Interest

The authors declare no competing financial interest.

## Author Contributions

The manuscript was written with contributions from all authors who have given approval to the final version of the manuscript.

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## Figure captions

**Fig. 1** *The effect of CdS QDs and Cd(II) treatment on mitochondrial membrane potential, as quantified by JC-1 staining.* Cells were exposed for 24 h to Cd in the form of either CdS QDs or Cd(II). The data report the ratio between aggregated and monomeric forms of JC1, and are representative of three independent experiments. The concentrations of CdS QDs and Cd(II) shown are for the Cd in the material. Asterisks ***. ****: $p<0.001$, $<0.0001$ vs. values obtained from non-treated cells.

**Fig. 2** *The effect on THP-1 cell morphology of exposure to Cd in the form of either CdS QDs or* Cd(II)*.* After a 24 h exposure to a high or low dose of either stressor, cell monolayers were labelled with JC-1 to assay mitochondrial function or with DRAQ5 to assay nuclear morphology. CdS QDs, 6.4 µg $ml^{-1}$ equivalent to 5 µg $ml^{-1}$ Cd, induced a modest increase in the amount of JC-1 monomers, suggesting some alteration in mitochondrial function but there was no evidence of marked changes in cell morphology. Cd in the form of Cd(II), 11.4 µg $ml^{-1}$ equivalent to 5 µg $ml^{-1}$ Cd, not only substantially increased the abundance of JC-1 monomers, but also caused loss of the red signal, suggesting a significant alteration in mitochondrial function. In addition, Cd(II) treatment also changed the typical elongated shape into a more rounded form. When THP-1 cells were exposed to a high dose of CdS QDs, 50 µg $ml^{-1}$ equivalent to 39 µg $ml^{-1}$ Cd, most of the CdS QDs aggregated and the presence of JC-1 monomeric forms was only slightly increased. Cell morphology appeared to be substantially unaffected. Bar: 20 µm. The images illustrate representative microscope fields where at least 100 cells were present.

**Fig. 3** *The uptake of CdS QDs into THP-1 cells as measured using a cytofluorimetric assay.* Cells were exposed to 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs for 0 - 24 h. Typical scatter plots are shown, obtained from a representative experiment performed three times with comparable results. FS, forward scatter; SS, side scatter

**Fig. 4** *Venn diagram representation of the effect of exposure to Cd on the miRNome.* **a**, HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). The number of miRNAs increased in abundance were 34 and 29, respectively, while number of miRNAs decreased in abundance were 32 and 102, respectively. Only 11 and 13 miRNAs were increased or reduced in abundance by both treatments, respectively. **b**, THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 6.4 µg $ml^{-1}$ CdS QDs or 11.4 µg $ml^{-1}$ Cd(II). Exposure to CdS QDs increased the abundance of 136 miRNAs, whereas only 15 were reduced. **c**, Comparison between HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs and THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 6.4 µg $ml^{-1}$ CdS QDs. Ten miRNAs responded positively and 2 responded negatively in both cell types. Eight miRNAs responded in opposite directions. **d**, Comparison between HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 5.2 µg $ml^{-1}$ Cd(II) and THP-1 cells exposed to 5 µg $ml^{-1}$ Cd as 11.4 µg $ml^{-1}$ Cd(II). Thirty nine miRNAs responded negatively in both cell types, while no miRNA responded positively; 16 miRNAs responded in opposite manner.

**Fig. 5** *A heatmap-based illustration of the HepG2 and THP-1 cell responses to Cd exposure*. The heatmaps show only those miRNAs which were increased or decreased in both cell types or with either treatment. Positively responding miRNAs are shown in red and negatively responding ones in green. **a**, Differentially abundant

miRNAs present in HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs or 5.2 µg $ml^{-1}$ Cd(II). For a large number of miRNAs abundance is reduced when the cells are treated with Cd(II) as compared with cells treated with CdS QDs. **b**, Differentially abundant miRNAs present in HepG2 and THP-1 cells exposed to 2.3 and 5 µg $ml^{-1}$ Cd as 5.2 µg $ml^{-1}$ and 11.4 µg $ml^{-1}$ Cd(II). **c**, Differentially abundant miRNAs present in HepG2 and THP-1 cells exposed to 2.3 and 5 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ and 6.4 µg $ml^{-1}$ CdS QDs.

**Fig. 6** *The effect on the miRNome of exposure to Cd, illustrated by a Venn diagram.* **a**, miRNAs induced in THP-1 cells in response to exposure to either 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs or 5 µg $ml^{-1}$ Cd as 11.4 µg $ml^{-1}$ Cd(II). The abundances of totals of 9 and 18 miRNAs were increased by CdS QDs and Cd(II) treatment, respectively. miRNAs decreased in response to the two treatments were 237 and 129 respectively; of these, 124 responded negatively to both treatments, while 5 miRNAs were decreased by Cd(II) treatment but increased in the presence of CdS QDs. **b**, miRNAs induced in either HepG2 or THP-1 cells in response to exposure to, respectively, 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs and 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs; **c**, miRNAs induced in either HepG2 or THP-1 cells in response to exposure to CdS QDs (all treatments).

**Fig. 7** *The core autophagy pathway and its regulation by miRNAs in THP-1 cells exposed to 39 µg $ml^{-1}$ Cd as 50 µg $ml^{-1}$ CdS QDs..* The entire pathway was divided into five steps: induction, vesicle nucleation, elongation, retrieval and fusion. Arrows indicate increase or decrease of miRNA. A green arrow indicated a decrease with

lack of repression of its specific targets. The overall effect seems to bring the cell towards autophagosome formation and autophagy.

**Fig. 8** *The effect of exposure to Cd on autophagy markers in THP-1 and HepG2 cells.* THP-1 and HepG2 cells were incubated for 24h in the presence of different doses of Cd: 2.3 µg $ml^{-1}$ as 3 µg $ml^{-1}$ CdS QDs, 5 µg $ml^{-1}$ as 6.4 µg $ml^{-1}$ CdS QDs or as 11.4 µg $ml^{-1}$ Cd(II) and 39 µg $ml^{-1}$ as 50 µg $ml^{-1}$ CdS QDs. Cells were then extracted and Western Blot analysis of p62 and LC3II was performed as described in Materials and Methods. Tubulin was used for loading control. *Pos* indicates THP-1 cells, treated with rapamycin, 10 nM, 3h, and cloroquine, 100 µM, 2h, exploited as positive controls for autophagy.

**Fig. 9** *The core apoptotic pathway and its regulation by miRNAs in HepG2 cells exposed to 2.3 µg $ml^{-1}$ Cd as 3 µg $ml^{-1}$ CdS QDs.* The figure depicts events of the intrinsic and extrinsic apoptotic pathways. Arrows indicate increase or decrease of miRNA or gene. A red arrow indicates increased abundance of a specific gene. A green arrow indicates a decrease which permits the expression of its specific target. In this system the activation of the intrinsic pathway leads to apoptosis. At the dose of CdS QDs considered and under the experimental conditions adopted, the proportion of cells which effectively completed apoptosis was limited, as shown by morphological observation (see Fig. A.2).

## Table

**Table 1** Differentially abundant miRNAs in response to Cd exposure and their principal cellular targets, pathways and related diseases

| | | THP-1 | | | HepG2 | | | |
|---|---|---|---|---|---|---|---|---|
| | | 39 µg ml$^{-1}$ Cd | 5 µg ml$^{-1}$ Cd | | 2.3 µg ml$^{-1}$ Cd | | | |
| **Processes**[1] | **miRNA involved**[2] | QDs[3] 50 µg ml$^{-1}$ | QDs[3] 6.4 µg ml$^{-1}$ | Cd(II)[3] 11.4 µg ml$^{-1}$ | QDs[3] 3 µg ml$^{-1}$ | Cd(II)[3] 5.2 µg ml$^{-1}$ | **Target protein**[4] | **Diseases**[5] |
| Apoptosis | miR-34a | / | / | / | / | ↓ | BCL-2 | Cancer |
| | miR-195 | ↓ | / | / | ↓ | ↓ | | |
| | miR-143 | ↓ | / | ↓ | / | ↓ | | |
| | miR-155 | ↓ | ↑ | ↓ | ↓ | / | | |
| | miR-125 | ↓ | ↑ | ↓ | / | / | | |
| | miR-29a | ↓ | / | / | / | ↓ | CDC42, p58α | Cancer/ Huntington's disease |
| | miR-125b | ↓ | / | ↓ | / | / | p53 | Cancer/ Psoriasis |
| | miR-221 | ↓ | / | ↓ | / | / | p27 (KIP1) | |
| | miR-222 | ↑ | ↑ | ↓ | / | ↓ | | |
| | miR-181a | ↑ | ↑ | / | / | / | BIM | Cancer |
| | miR-32 | ↓ | / | ↓ | ↓ | ↓ | | |
| | miR-25 | ↓ | / | ↓ | / | / | | |
| DNA Repair | miR-16 | ↓ | ↑ | ↓ | / | / | UNG2 | Cancer |
| | miR-199 | ↓ | ↑ | ↓ | / | / | | |
| | miR-21 | ↓ | / | ↓ | / | ↓ | hMSH2 | |
| | miR-192 | ↓ | / | ↓ | / | ↓ | ERCC3, ERCC4 | Toxicant exposure biomarker |
| | miR-101 | ↓ | ↑ | ↓ | / | / | DNA-PKcs | |
| | miR-24 | ↓ | ↑ | ↓ | / | / | H2AX | Cancer |
| | miR-96 | ↓ | / | / | / | / | RAD51 | / |
| Cell cycle | miR-16 | ↓ | ↑ | ↓ | / | / | CDK2 | Cancer |
| | miR-449a/b | ↓ | ↑/↓ | ↓ | / | / | CDK6, CDC25A | / |
| | miR-15 | ↓ | / | / | ↑ | / | WEE1, CHK1 | Cancer |
| | miR-125 | ↓ | ↑ | ↓ | / | / | Cyclin A2 | |
| | let-7b | ↓ | / | ↓ | / | ↓ | Cyclin A | |
| Xenobiotic metabolism | miR-27b | ↓ | / | / | / | ↓ | CYP1B1 | Diabetes |
| | miR-126 | ↓ | ↑ | / | ↓ | ↓ | CYP2A3 | Cancer/ Cardiovascular diseases |
| | miR-378 | ↓ | / | / | ↓ | ↓ | CYP2E1 | Cancer |
| | miR-133a | ↓ | ↑ | / | ↑ | ↑ | GSTP1 | |
| Autophagy/ Phagocytosis | let-7a | ↓ | / | ↓ | / | ↓ | several chemokines | Cancer |
| | miR-146a | ↓ | / | / | / | / | | Inflammatory diseases |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | miR-25 | ↓ | / | ↓ | / | / | | Cancer |
| | miR-26a | ↓ | / | ↓ | / | ↑ | | |
| Autophagy/ Phagocytosis | miR-132 | ↓ | ↑ | ↓ | / | ↑ | several chemokines | Alzheimer's disease |
| | miR-140 | ↓ | ↑ | ↓ | / | ↓ | | Cancer |
| | miR-146b | ↓ | / | / | / | / | | Inflammatory diseases |
| | miR-155 | ↓ | ↑ | ↓ | ↓ | / | | Cancer |
| | miR-210 | ↓ | ↑ | ↓ | / | / | | |
| | miR-21 | ↓ | / | ↓ | / | / | | |
| | miR-142-3p | ↓ | / | / | ↓ | / | | Cardiovascular diseases |
| | miR-125b | ↓ | / | ↓ | / | / | | Cancer |
| | miR-17-5p | ↓ | / | ↓ | / | ↓ | | |
| | miR-24 | ↓ | ↑ | ↓ | / | / | | |
| | miR-30b | ↓ | ↑ | ↓ | / | ↓ | | |
| | miR-101 | ↓ | ↑ | ↓ | / | / | | Toxicant exposure biomarker |
| | miR-652-3p | ↓ | / | ↓ | / | ↓ | | / |
| | miR-1275 | ↓ | ↑ | ↓ | / | ↓ | | / |
| | miR-7 | / | ↑ | / | ↓ | / | mTOR | / |
| | miR-199a | ↓ | ↑ | ↓ | / | / | | Cancer |
| | miR-30a | ↓ | ↑ | / | ↓ | ↓ | Beclin | Cancer |

**Note.** [1] The more relevant processes emerging from analysis by DIANA-mirPath software.
[2] The miRNAs evaluated here represent the more significant variations, which have commonalities between different cell types and different treatments. The same were also suggested as exposure biomarkers for different environmental or health related clues [58,59].
[3] The red and green arrows indicate the miRNA is increased or decreased in abundance.
[4,5] Main target proteins and diseases were taken from literature [58,59].

**Figure 1**

Click here to download high resolution image

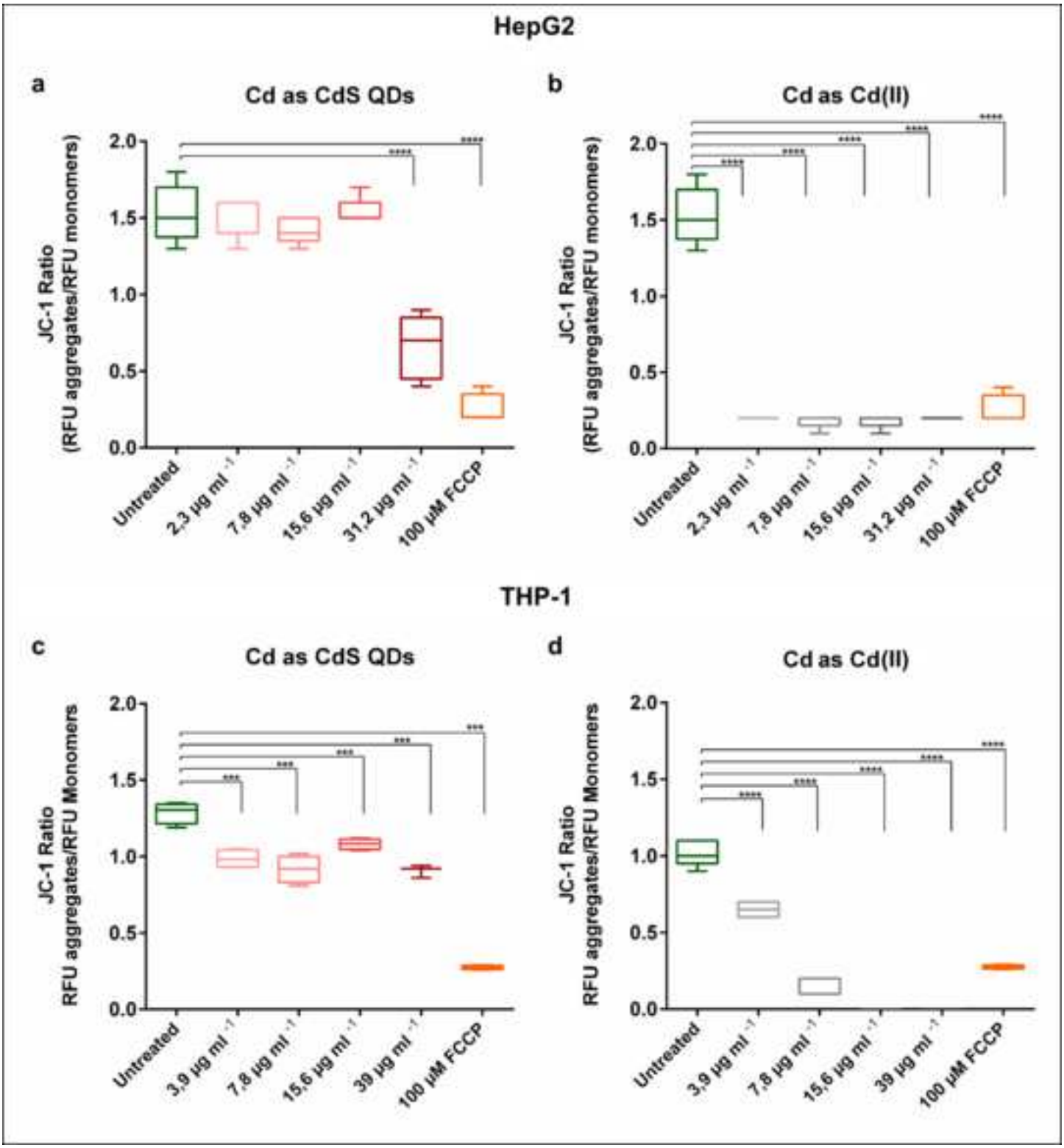

**Figure 2**

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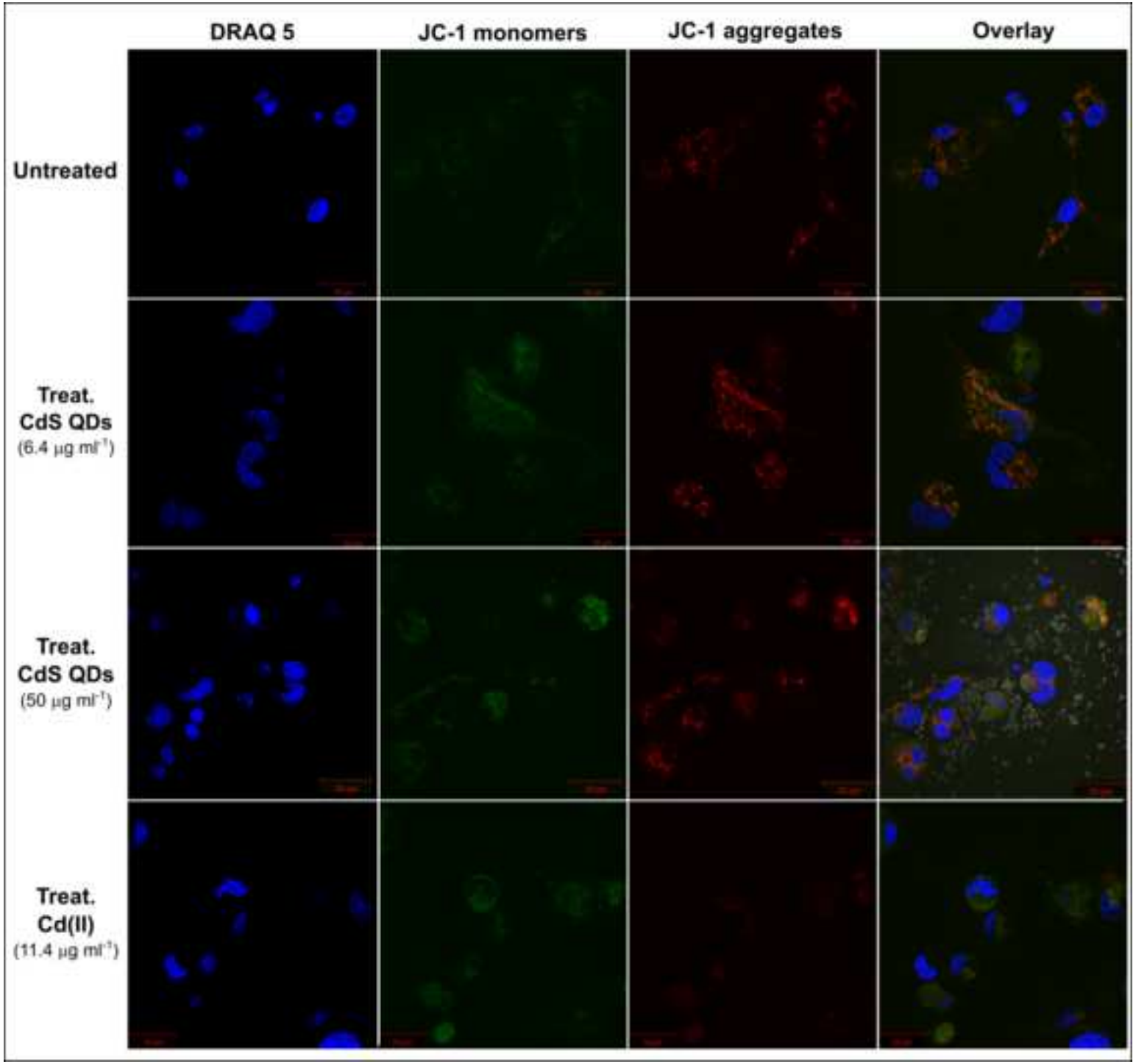

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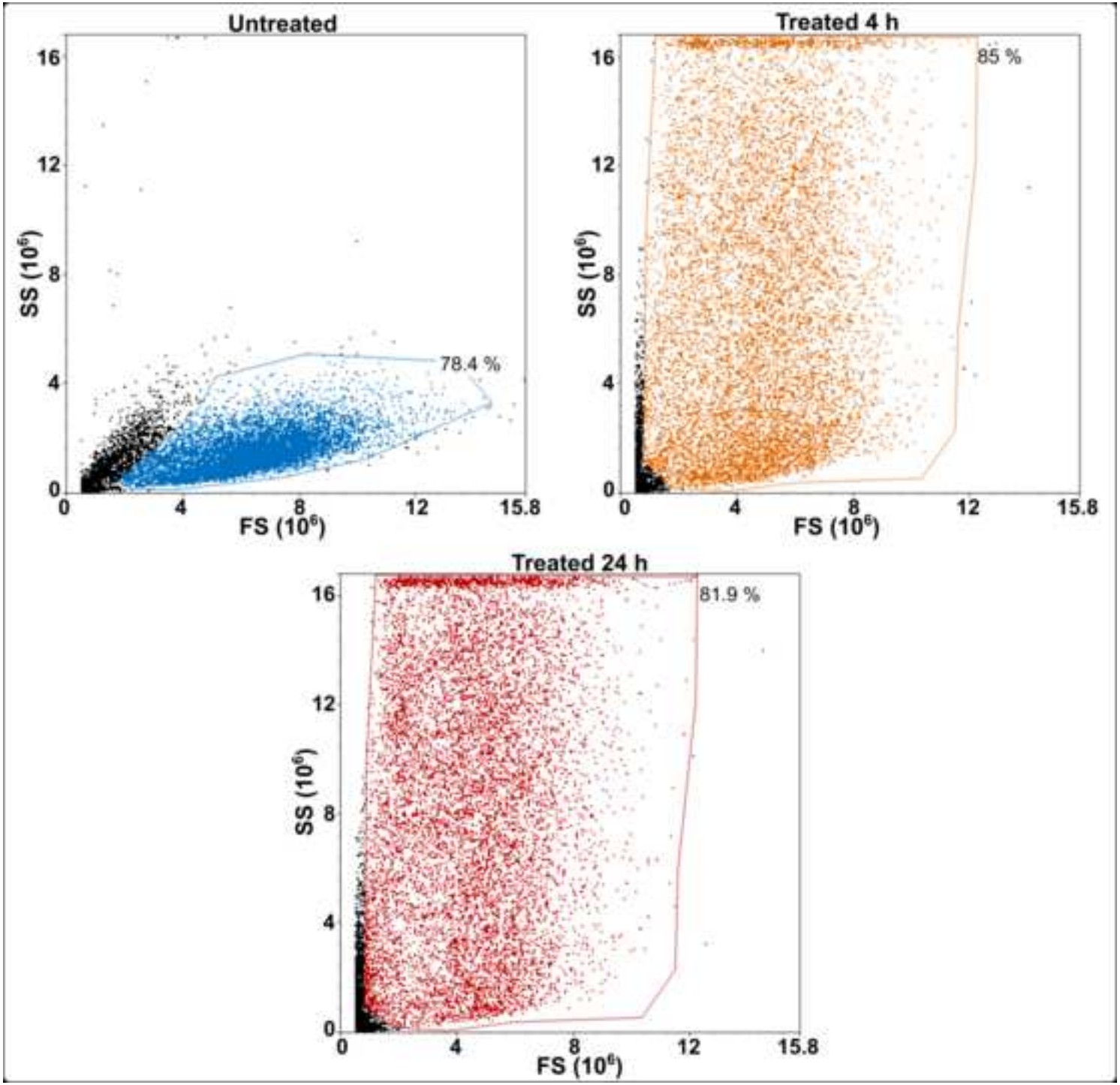

Figure 4
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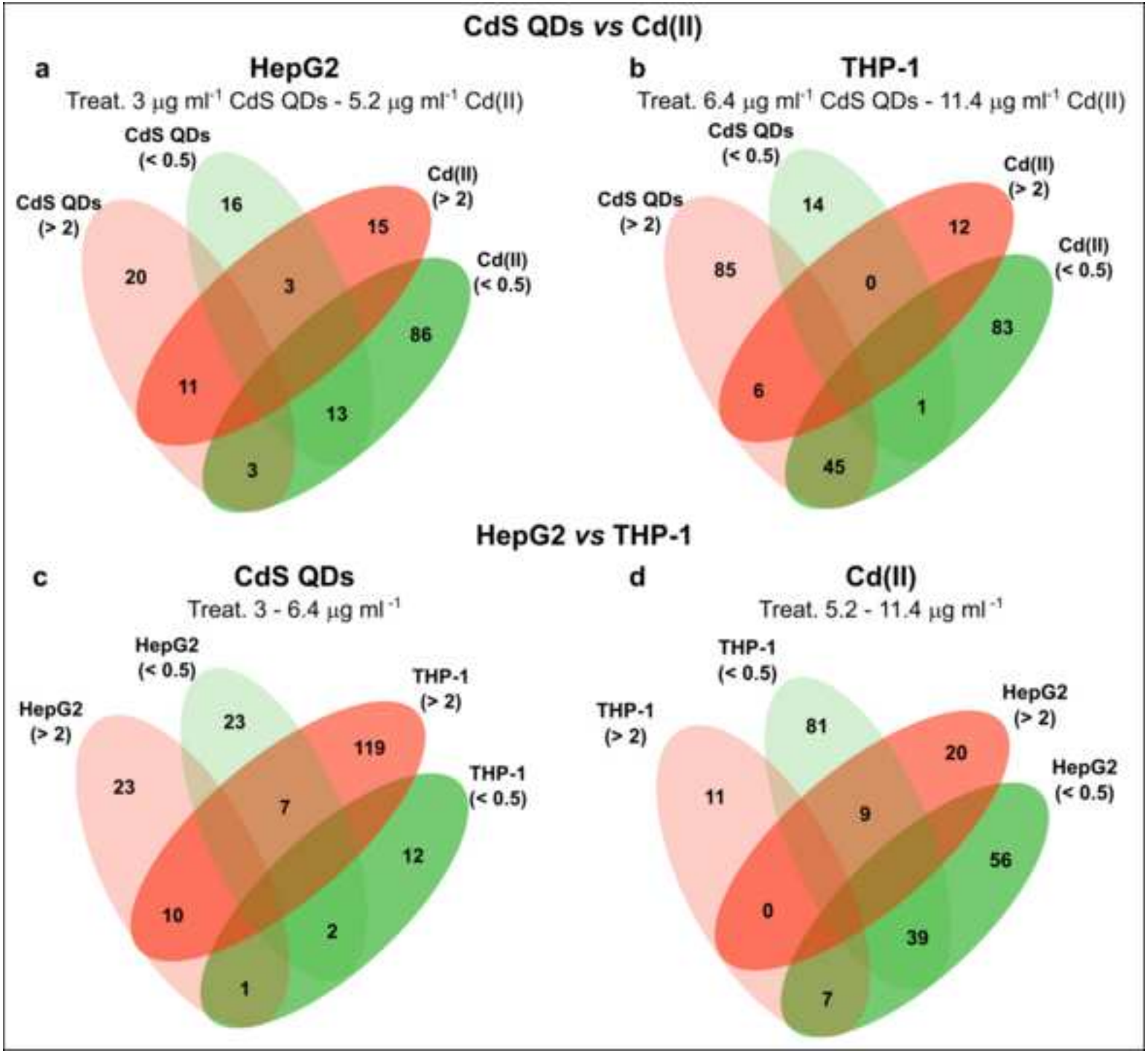

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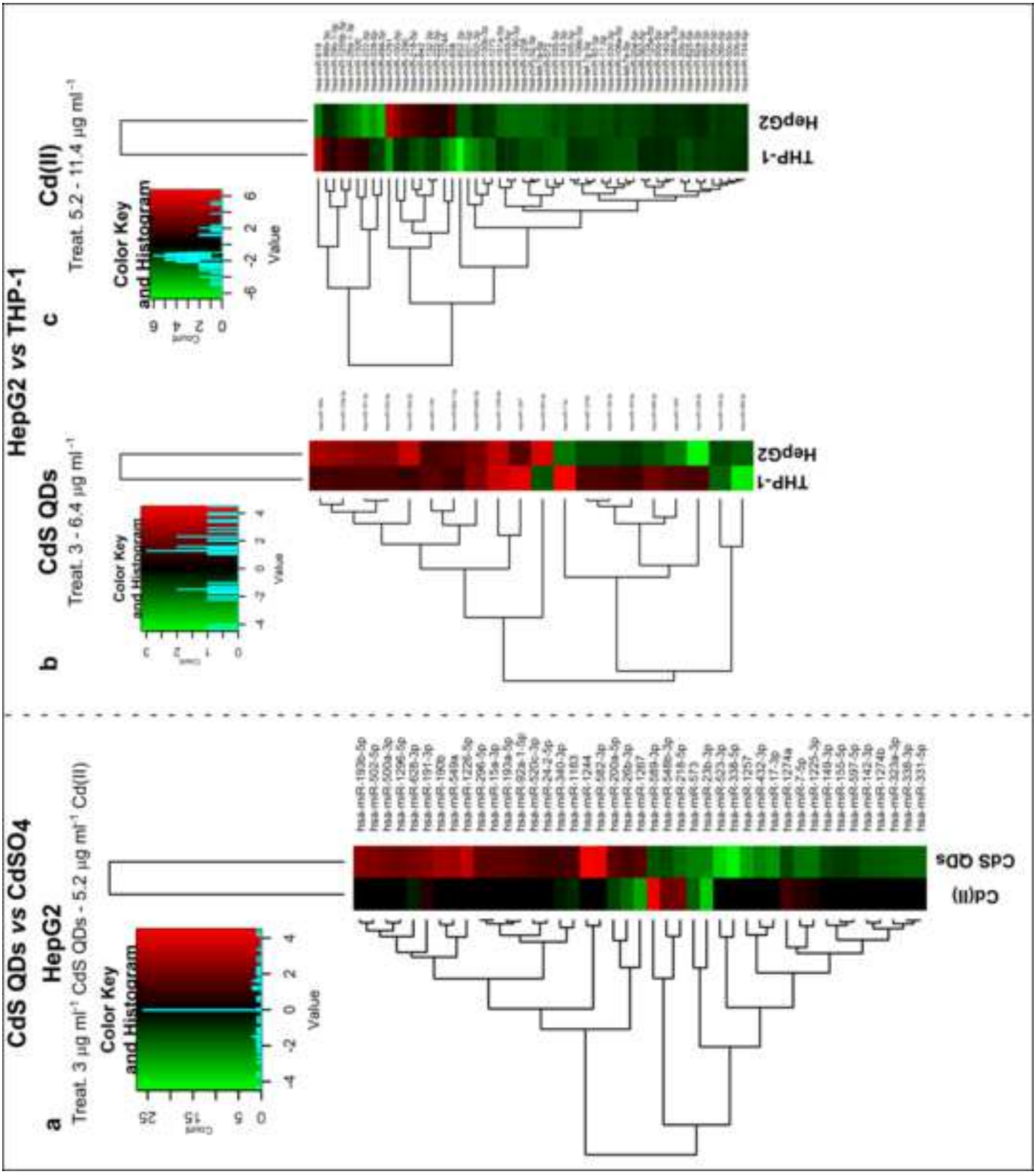

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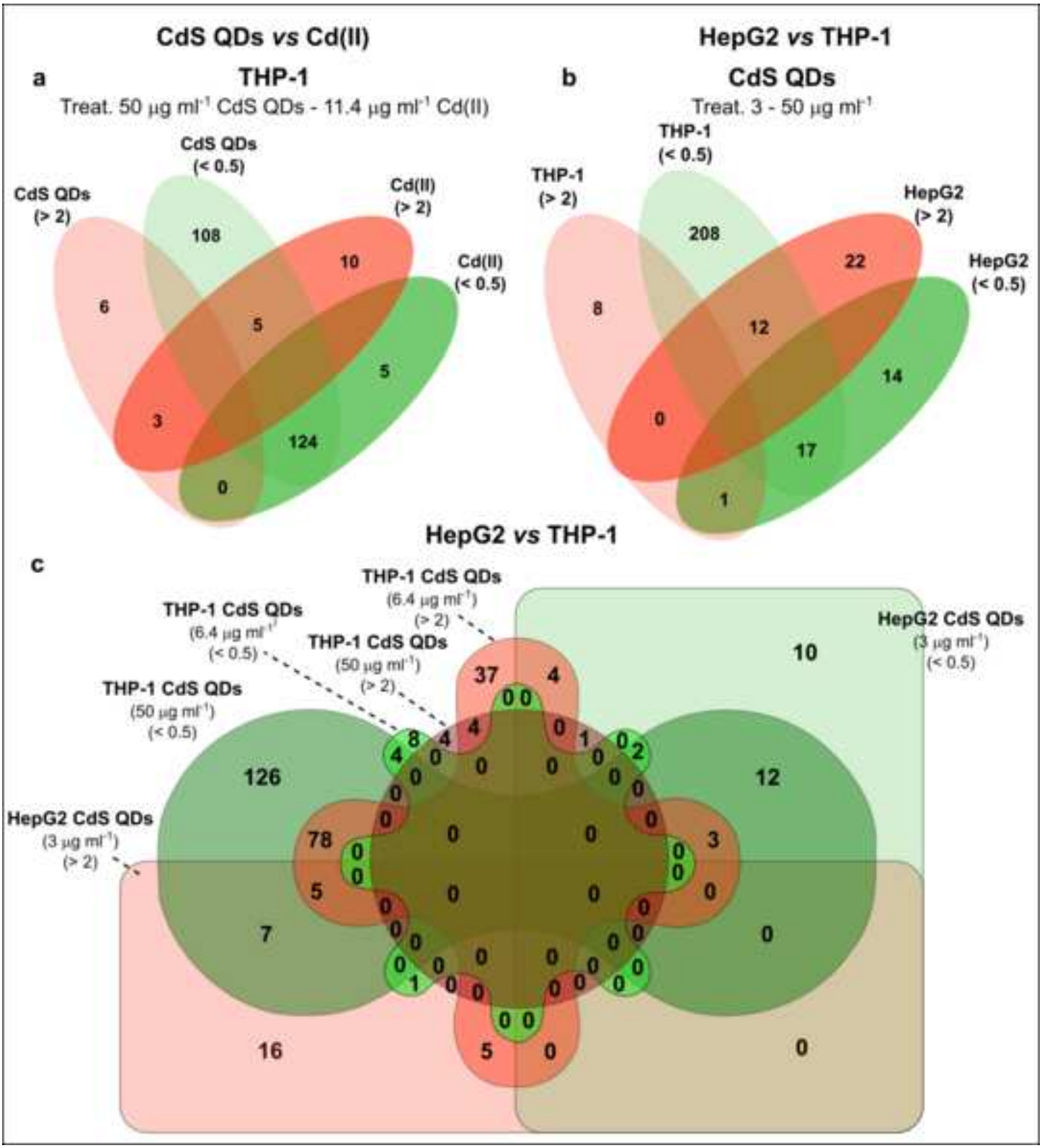

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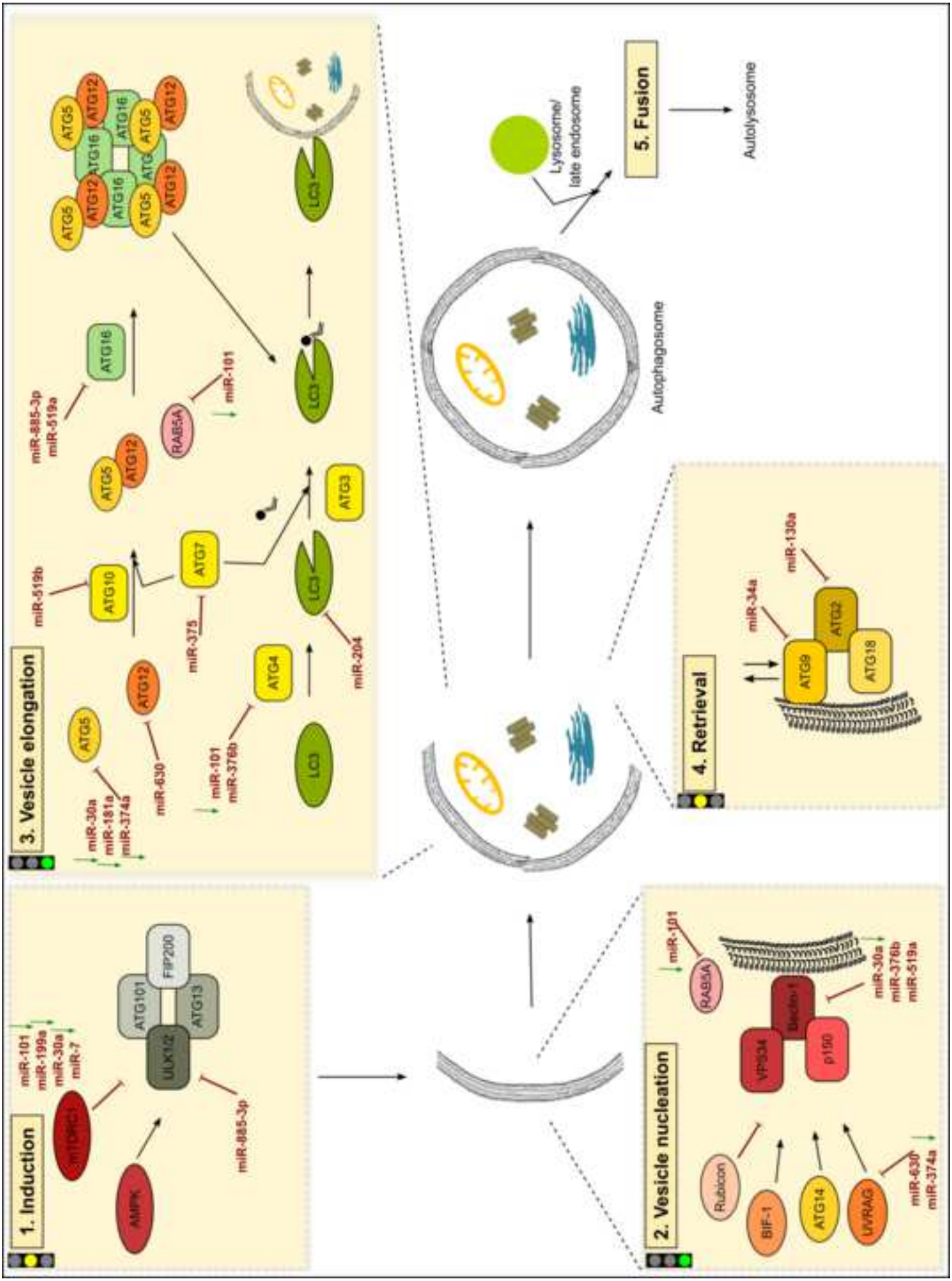

Figure 8
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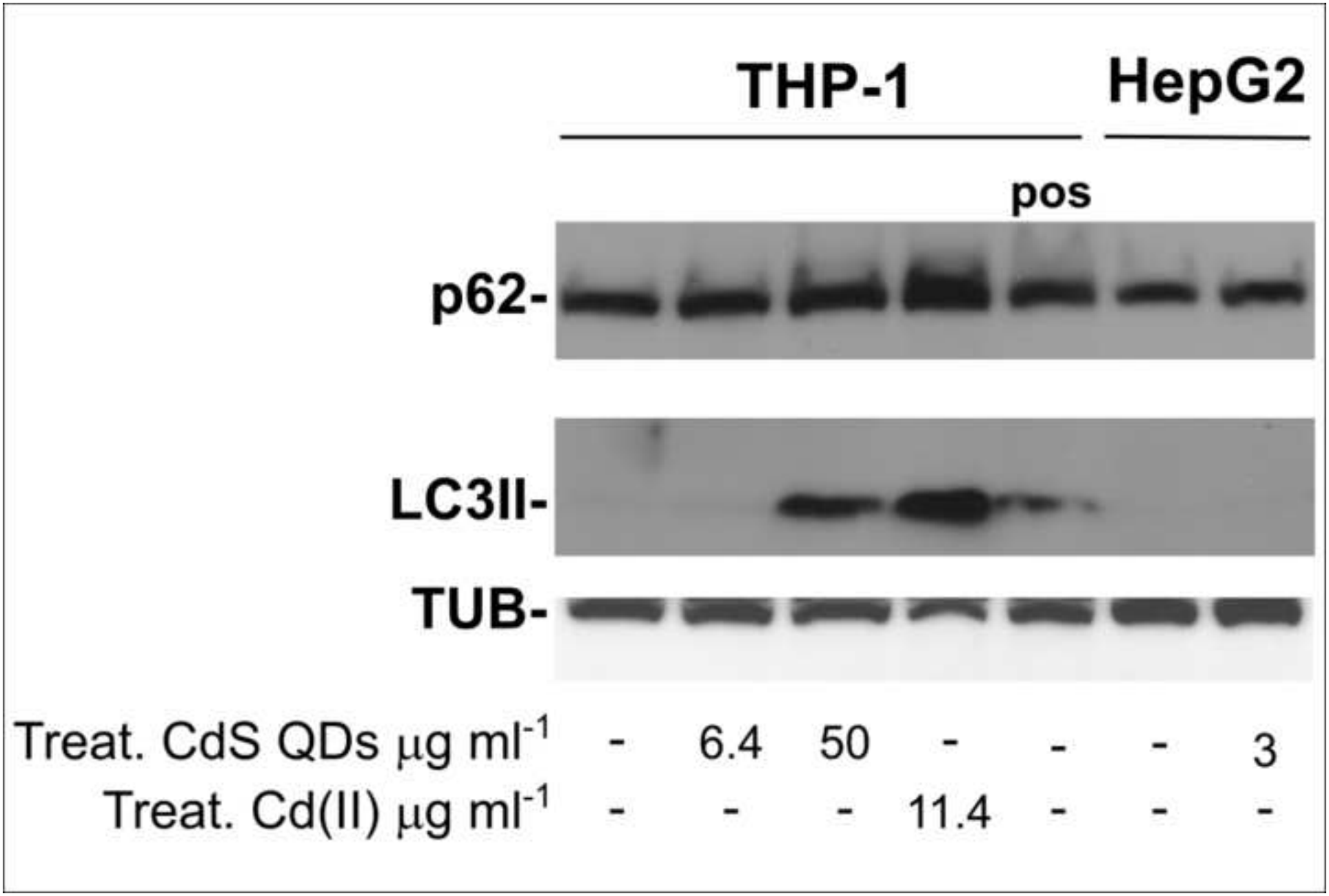

**Figure 9**

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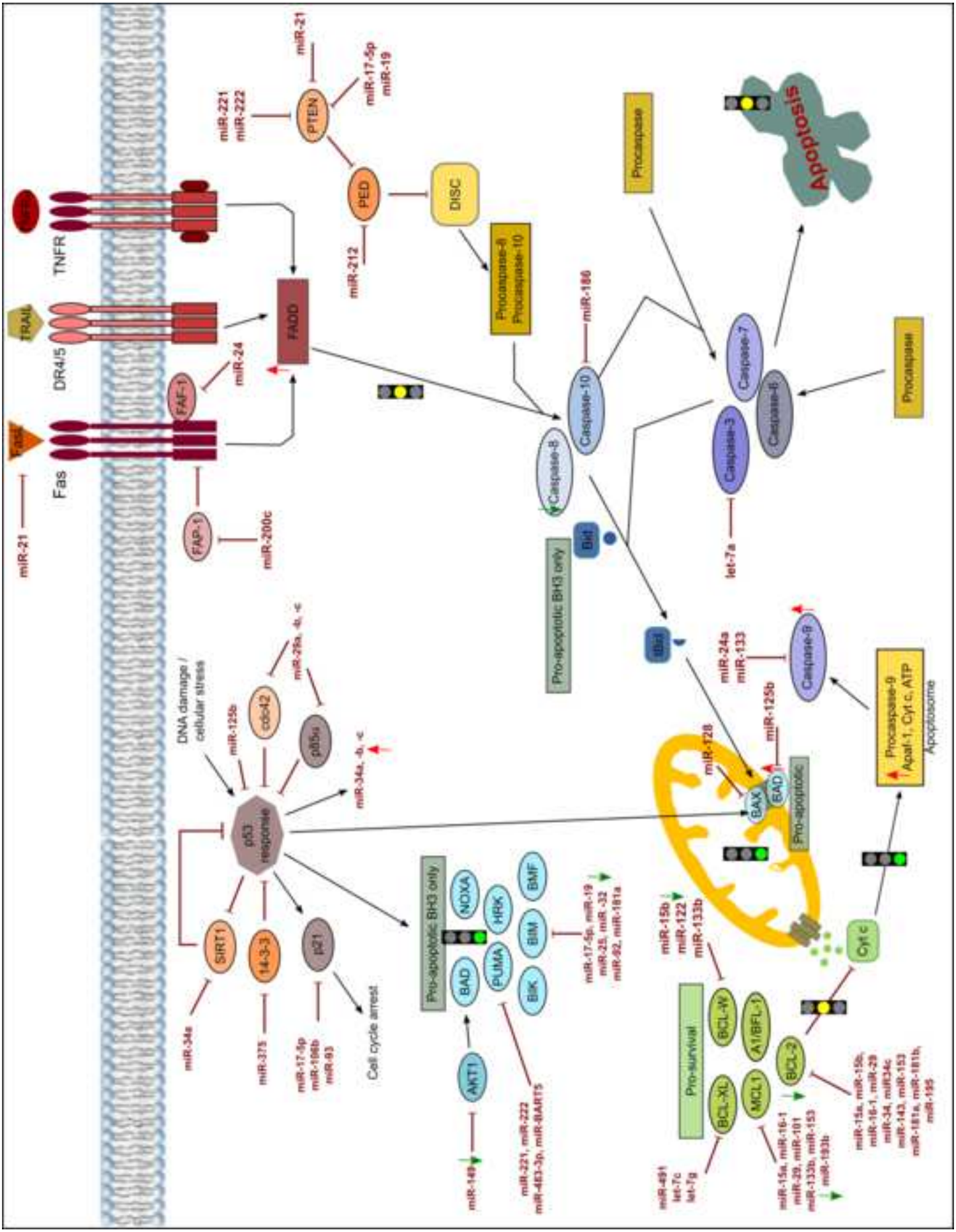


**Appendix A**

**Click here to download Supplementary Material: Revisioned_Appendix A.docx**

*Abstract


## ABSTRACT

Cadmium is toxic to humans, although Cd-based quantum dots exerts less toxicity. Human hepatocellular carcinoma cells (HepG2) and macrophages (THP-1) were exposed to ionic Cd, Cd(II), and cadmium sulfide quantum dots (CdS QDs), and cell viability, cell integrity, Cd accumulation, mitochondrial function and miRNome profile were evaluated.

Cell-type and Cd form-specific responses were found: CdS QDs affected cell viability more in HepG2 than in THP-1; respective $IC_{20}$ values were ~ 3 and ~ 50 µg $ml^{-1}$. In both cell types, Cd(II) exerted greater effects on viability.

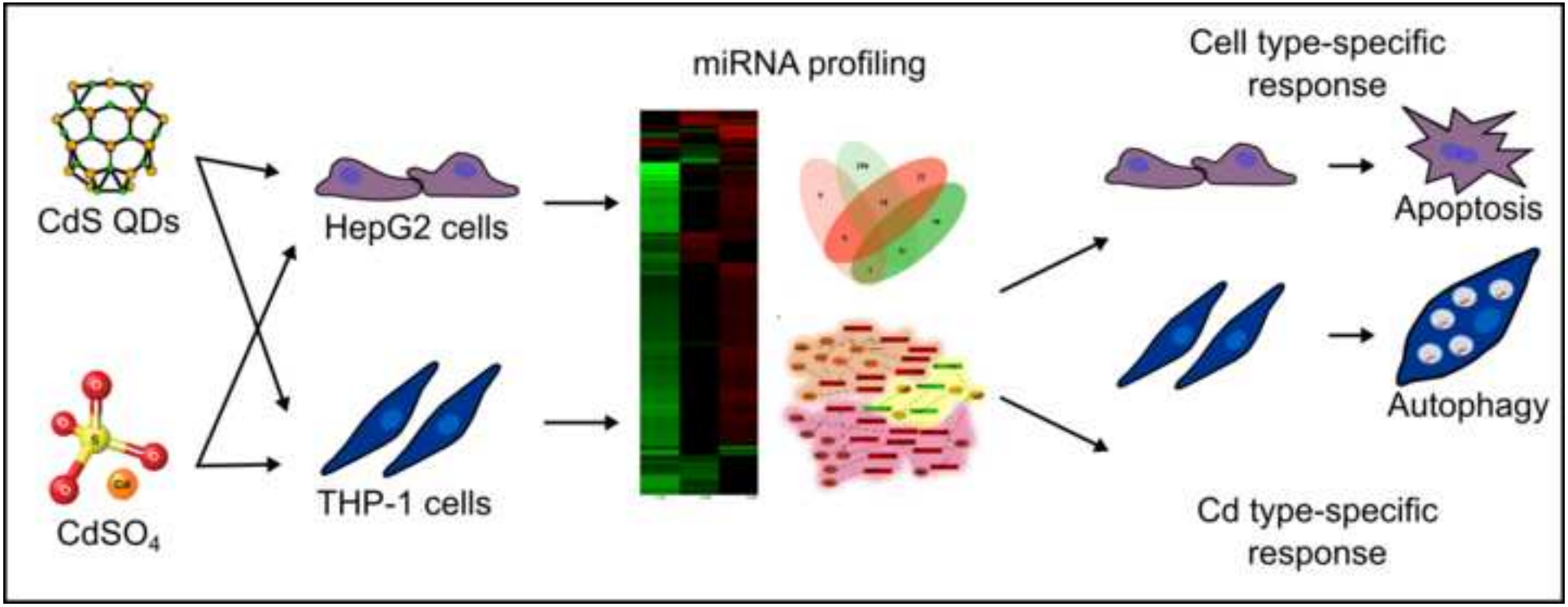
Mitochondrial membrane function in HepG2 cells was reduced 70% with 40 µg $ml^{-1}$ CdS QDs but was totally inhibited by Cd(II) at corresponding amounts. In THP-1 cells, CdS QDs has less effect on mitochondrial function; 50 µg $ml^{-1}$ CdS QDs or equivalent Cd(II) caused 30% reduction or total inhibition, respectively. The different *in vitro* effects of CdS QDs were unrelated to Cd uptake, which was greater in THP-1 cells.

For both cell types, changes in the expression of miRNAs (miR-222, miR-181a, miR-142-3p, miR-15) were found with CdS QDs, which may be used as biomarkers of hazard nanomaterial exposure. The cell-specific miRNome profiles were indicative of a more conservative autophagic response in THP-1 and as apoptosis as in HepG2.

## HIGHLIGHTS

- In two human cell lines, Cd toxicity varied depending on its form: nano or ionic.
- Cells were more sensitive to ionic Cd than to Cd as quantum dots.
- HepG2 cells were more sensitive than THP-1 but this did not correlate to Cd uptake.
- Cell-type and Cd-type responses were correlated with the miRNome.
- *In silico* and *in vitro* pathway analysis suggests apoptosis (HepG2) or autophagy (THP-1).

**Graphical Abstract**



## Novelty Statement

This paper describes a novel application of the miRNome to the risk assessment of engineered nanomaterials. Our results show that cadmium induced different effects on HepG2 and THP-1 cells viability and mitochondrial function in nano and ionic forms. The miRNome was found to be specific to both cell type and Cd form, suggesting great potential as a tool to identify biomarkers for environmental and health risk assessment. *In silico* miRNomes analysis suggested HepG2 cells exposed to a low concentration of quantum dots were subject to apoptosis. At a similar concentration, THP-1 cells were little affected but at higher levels, they tended towards autophagy.

***Credit Author Statement**

CRediT author statement

The manuscript was written with contributions from all authors

*Declaration of Interest Statement

**Declaration of interests**

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: