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Proteomic and lipidomic analysis of primary mouse hepatocytes exposed to metal and metal oxide nanoparticles

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Abstract

The global analysis of the cellular lipid and protein content upon exposure to metal and metal oxide nanoparticles (NPs) can provide an overview of the possible impact of exposure. Proteomic analysis has been applied to understand the nanoimpact however the relevance of the alteration on the lipidic profile

has been underestimated. In our study, primary mouse hepatocytes were treated with ultra-small (US) TiO_2-USNPs as well as ZnO-NPs, CuO-NPs and Ag-NPs. The protein extracts were analysed by 2D-DIGE and quantified by imaging software and the selected differentially expressed proteins were identified by nLC-ESI-MS/MS. In parallel, lipidomic analysis of the samples was performed using thin layer chromatography (TLC) and analyzed by imaging software. Our findings show an overall ranking of the nanoimpact at the cellular and molecular level: TiO_2-USNPs<ZnO-NPs<Ag-NPs<CuO-NPs. CuO-NPs and Ag-NPs were cytotoxic while ZnO-NPs and CuO-NPs had oxidative capacity. TiO_2-USNPs did not have oxidative capacity and were not cytotoxic. The most common cellular impact of the exposure was the down-regulation of proteins. The proteins identified were involved in urea cycle, lipid metabolism, electron transport chain, metabolism signaling, cellular structure and we could also identify nuclear proteins. CuO-NPs exposure decreased phosphatidylethanolamine and phosphatidylinositol and caused down-regulation of electron transferring protein subunit beta. Ag-NPs exposure caused increased of total lipids and triacylglycerol and decrease of sphingomyelin. TiO_2-USNPs also caused decrease of sphingomyelin as well as up-regulation of ATP synthase and electron transferring protein alfa. ZnO-NPs affected the proteome in a concentration-independent manner with down-regulation of RNA helicase. ZnO-NPs exposure did not affect the cellular lipids. To our knowledge this work represents the first integrated proteomic and lipidomic approach to study the effect of NPs exposure to primary mouse hepatocytes *in vitro.*

Keywords: nanoparticles, hepatocytes, proteomics, lipidomics, mass spectrometry, toxicity

Abbreviations

2D-DIGE: two-dimensional difference gel electrophoresis; NPs: nanoparticles; USNPs: ultra-small nanoparticles; ROS: reactive oxygen species; DLS: dynamic light scattering

1. Introduction

The rapid development of nanotechnology and its applications has led to a growing and widespread use of products containing NPs in a myriad of areas as diverse as electronics, cosmetics, food additives, and medicine [1]. Metal and metal oxide nanoparticles (NPs) such as Silver (Ag) titanium (IV) dioxide (TiO_2), zinc oxide (ZnO), and copper oxide (CuO) are some of the most common industrial NPs additives

for various applications [2, 3]. We have previously shown the cytotoxicity as well as the cellular ultra-structural effects of these NPs on *Saccharomyces cerevisia*e [4]. In this study we focus on the effects of the mentioned NPs on hepatocytes considering that for those NPs that succeed in entering the bloodstream, either after inhalation, via the gastrointestinal tract or dermal absorption, the liver is one of the most important targets. Previous studies have demonstrated high accumulation and retention of NPs in liver after injection and digestion respectively [5-7]. TiO_2–NPs are one of the most studied NPs due to their extensive application in paints, cosmetics, and sunscreens [8, 9]. The interest on ultra-small NPs (USNPs), size range between 1-3 nm, has increased enormously for its applicability to optics and theranostics [10, 11]. The uniqueness of USNPs arises from possessing an extremely large surface area to volume ratio. This property enables them to be regarded as large molecules and accentuating the properties derived from interfacial interactions of the surface atoms with the solvent [12, 13]. A previous study has shown that gold USNPs were able to penetrate deeply into tumor spheroids, showed high levels of accumulation in tumor tissue in mice, and were distributed throughout the cytoplasm and nucleus of cancer cells in vitro and in vivo, whereas at 15 nm, they were found only in the cytoplasm, where they formed aggregates [14]. However, information about the toxicity and effects of TiO_2-USNPs on the cellular response is scarce.

Another NPs of great interest are ZnO- NPs, which due to their remarkable ultra-violet (UV) absorption and optical properties, are included in personal care products such as toothpaste, cosmetics, and textiles [15]. However exposure to ZnO-NPs through inhalation has been shown to cause toxicity through a battery of mechanism including cell stress and inflammation [16]. It has been observed that ZnO-NPs elucidate their toxicity by release of ions which alter Zn homeostasis [17, 18]. This is particularly important in hepatocytes as Zn is an essential trace element required for normal cell growth and function, and Zn deficiency/altered metabolism is observed in many types of liver diseases [19, 20]. CuO-NPs are extensively applied due to their potential applications as gas sensors, catalysts, and superconductors [21]. Cu ions are essential and function as cofactor of many enzymatic reactions and would be cycling between the two redox states. This process can be the source of reactive oxygen species (ROS) [22]. Indeed as hepatocytes are responsible for the Cu ions balance of the body, they are a major target of exposure and line of defense in the case of exposure to CuO-NP. Previous studies have shown that toxicity of CuO-NPs as well as their interference with the Cu ion homeostasis in hepatocytes [23, 24]. Exposure to CuO-NPs has been shown to affect the fatty acid composition *Tetrahymena thermophila* [25]. Toxicity associated with CuO-NPs has been connected with release of Cu ions as well as with oxidative stress. Ag-NPs have been widely used in personal products, food service, medical instruments, and textiles because of their antibacterial effects [26, 27]. Internalized Ag-NPs can release ions which may lead to cellular metabolism

and mitochondrial dysfunction, inducing directly and indirectly ROS generation [2, 28]. Previous studies have also shown the toxicity of Ag-NPs in hepatocytes by affecting homeostasis and reducing albumin release [5] or by stimulating glycogenolysis [29]. Numerous studies have demonstrated that the NPs interaction with serum proteins and cell membranes receptors is determined by the NPs design, affecting cellular uptake, gene and protein expression, and toxicity [30]. It has been reported the interaction of NPs with proteins, lipoproteins and plasma membrane might compromise its fluidity and integrity and/or facilitate the entry of the NPs [31]. However most of the studies showing NPs uptake have been mainly conducted on immortalized cell lines, whereas little is known those effects on primary cells [30]. Primary hepatocytes cultures represent a powerful *in vitro* system, as these cells are directly isolated from the animal keeping the parental specific properties of the liver (*in vivo*) from which they are derived unaltered. The aim of this study is to provide a functional understanding of the impact of the studied NPs in primary hepatocytes. The strategy is to apply a combined OMICs approach, lipidomics and proteomics that could integrated the functional role of lipids in the cellular response. Therefore, the differentially expressed proteins identified in combination with the changes in the lipid composition of the membranes may contribute to understanding the possible effects and exposure risks of the selected NPs. The field of nanotoxicology is aiming to fill gaps on the NP impact and system biology strategies could lead to evaluate possible outcome adverse pathways for human, animals and the environment.

2. Material and Methods

NPs characterization

The following NPs were used in this study: titanium (IV) oxide, 14027, dry nanopowder, rutile, average particle size: 1-3 nm (Plasmachem GmbH, Münster, Germany), ZnO nano powder, 544906, average size <100 nm, Copper (II) oxide nano powder, 544868, average size <50 nm, Ag-NPs aqueous colloidal solution, 0.1 mg/mL, and average particle size: 10 nm were purchased by Sigma (St. Louis, MO, USA). All NPs stock suspensions were prepared by suspending NPs in hepatocytes culture medium. The suspensions were prepared freshly, sonicated in a water bath sonicator for 30 min and vortexed vigorously before each assessment. The average hydrodynamic size by DLS measurement and the zeta potential were determined using a Malvern Zetasizer Nano series V5.03 (PSS0012-16 Malvern Instruments, Worcestershire. UK) and the analysis program DTS (dispersion technology software, Malvern Instruments). Two concentrations of NPs were used in order to assess their size and zeta potential: 5 and 500 mg/L that correspond to the exposure and the stock suspension concentration, respectively. The measurements were conducted in clear disposable capillary cells (DTS1060).

Cell-free dichlorofluorescein (DCFH) assay

The study of the oxidative potential of NPs was measured by a cell free method described by Foucaud *et al.* [32] and modified for this study. Briefly, 2´,7´ dichlorofluoroscein diacetate (DCFH-DA, Molecular Probes D-399) at 2.2 mM was hydrolyzed to DCFH at pH 7.0 with 0.01 N NaOH. The solution was put in the dark for 30 min at room temperature and the chemical reactions was stopped by adding ice cold 0.1 M PBS. Then, horse radish peroxidase (HRP, Sigma P8125) at 20U/ml was added to each sample. To facilitate the comparison between a cellular and cell free system, the solutions were incubated at 37^{o}C in the dark. The fluorescence generated by the DCFH oxidation was measured using a microplate reader at 485 nm excitation and 530 nm emission after 120 min. Freshly diluted hydrogen peroxide (10µM) was used as a positive control. The data were recorded as arbitrary fluorescence units. Two technical and three biological replicates were performed.

Isolation and exposure of primary mice hepatocytes to NPs

Hepatocytes were isolated from C57/6J mice by a collagenase (Roche Diagnostics, Barcelona, Spain) perfusion technique, as described previously [33]. Cells were seeded on fibronectin-coated dishes (3.5 µg/cm2) (2.5 x 10^6 viable cells per plate) and cultured at 37 ºC and 5% CO_2 as described by Palacios *et al.* [34]. The culture medium was Ham's F-12/Leibovitz L-15 (1/1, v/v) supplemented with 2% newborn calf serum, 2 mM L-glutamine, 5 mM glucose, 5 U/mL penicillin, 5 mg/mL streptomycin, 50 mg/L gentamycin, 0.2% fatty acid-free bovine serum albumin (BSA), and 10 nM insulin. After 1 h of adhesion, the medium was changed and the hepatocytes were exposed to different types of NPs for 48 h, frozen in liquid nitrogen and stored at -80 °C. In this study, primary cultures of mouse hepatocytes were treated with the previously described metal and metal oxide NPs (TiO_2, ZnO, CuO, and Ag-NPs) at 1 and 5 mg/L concentrations for 48 h. The choice of the concentrations was based on a previous *in vitro* study of catfish primary hepatocytes and human cells exposed to metal oxide NPs with some modifications [35]. All the experiments were conducted in compliance with institutional guidelines, and the analyses were performed on at least four biological replicates for each treatment (control included) unless specified otherwise. Animal procedures were approved by the University of the Basque Country and Animal Care and Use Committees.

Cell viability assay

The cytotoxicity of NPs was determined using standard MTT assay described previously with slightly modifications [36]. Briefly, primary mouse hepatocyte cells were plated in two 96-well culture plates in 200 µl of culture medium at a density of 1 x 10^5 cells/ml. After incubation for 24 h, NPs at concentrations

of 1 and 5 mg/L were added to respective cells. The cells were then cultivated for an additional 48 h with NPs containing medium changed every day. On the third day, 20 µl of tetrazolium dye MTT solution (5 mg/mL) was added to each well and was further incubated for 4 h. The supernatants were then removed and 200 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal at 37 °C. The absorbance was measured with a VICTOR3™ multi-labeled microplate reader (Perkinelmer Inc., Waltham, MA USA) at 560 nm. The assay was performed twice with three replicates for each sample in each assay.

Preparation of protein extracts

Hepatocytes media was carefully discarded and cells pellets (~ 1.5 x 10^6 cells per sample) were re-suspended in cell washing buffer solution (10mM Tris-base pH 8, 5mM of magnesium acetate) centrifuged at 12,000 g at 4°C for 4 min for three times according to the manufacturer's instructions (GE Healthcare). Later, hepatocytes were re-suspended in lysis buffer (2% ASB14, 8M urea, 5mM magnesium acetate, 20mM Tris-base pH 8.5)[37], left on ice for 10 min, and sonicated intermittently on ice until cells were lysed. Cell debris was removed by centrifugation at 12,000 g at 4^{o}C for 10 min while the supernatant was transferred in new tubes followed by 20% of trichloroacetic acid (TCA) in cold acetone at -20°C overnight.

The protein precipitates were collected by centrifugation at 12,000 g for 5 min, and then the proteins were solubilized again in lysis buffer. Cycles of intermittent sonication followed by centrifugation at 10,000 g for 10 min were performed until all proteins were solubilized in the buffer and no evidence of precipitate was observed. All these steps were carried at 4 °C. Before DIGE labeling, protein concentrations were measured according to Bradford method [38].Bovine serum albumin was used as standard.

Cy-Dye labeling and separation of proteins by 2DE

Protein CyDye labeling and DIGE analysis were performed according to the manufacturer's instructions (GE Healthcare). Samples containing 25µg of solubilized proteins were labeled by 200 pmol of reconstitute CyDye. The quenched Cy3- and Cy5-labeled samples for each experimental sample were then combined with the quenched Cy2-labeled pool internal standard. These samples were then quenched by the addition of 1 µL 10 mM lysine followed by incubation on ice for 10 min. The total proteins (75µg) were mixed and denatured in sample buffer (7M urea, 2M thiourea, 2% ASB 14, 2% DTT, 2% IPG buffer (pH 3-10)), and then rehydrated with rehydration buffer (7M urea, 2M thiourea, 2% ASB 14, 0.2% DTT, 1% IPG buffer (pH 3-10)) and trace amounts of bromophenol blue. A final volume of 200 µl of sample was then distributed evenly along IPG strip pH 3−10NL, 11 cm, covered by mineral oil and passively rehydrated for at least 12 h in dark conditions. Isoelectric focusing was performed on a Protean IEF Cell

(Bio-Rad) at 20^{o}C using wet wicks inserted between the IPG strips and the electrodes. The first dimension was carried using the following program as recommended by the manufacturer's instructions (Bio-Rad): rapid voltage slope at all the steps; step 1, 250 V for 15 min; step 2, 8000 V for 2.5 h, and step 3 at 8000 V until 35000 Vh was reached. After focusing the strips were equilibrated for 15 min in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT and then for 15 min in equilibration buffer containing 2.5% iodoacetamide. The second dimension was carried out on homogeneous 12.5% T Criterion precast gels (Bio-Rad, Hercules, CA) at 120 V for 2h using a Criterion Cell (Bio-Rad). DIGE gels were fixed in 10% methanol and 7.5% acetic acid for 1h in the dark and washed with bi-distilled water for 15 min before image acquisition. After image acquisition the gels were stained by colloidal Coomassie blue staining for subsequent spot picking and protein identification.

Image acquisition and analysis

DIGE gels were scanned using FLA-5100 Fluorescence Image Analyzer (Fuji Medical, Stamford, CT) according to manufacturer's recommendation. DIGE images (16 bit TIFF, 600 PMT) were analyzed by REDFIN software (Ludesi, Malmö, Sweden, http://www.ludesi.com) for spot detection, spot quantification and normalization, spot matching and statistical analysis. The comparison of test spot volumes (Cy3 or Cy5 labelled) with the corresponding internal standard spot volume (Cy2 labeled) gave normalization for each matched spot. This allows a satisfactory quantification and comparison of different gels. Differential expression of proteins was defined on the basis of ≥1.5-fold change between group averages and one-way ANOVA $p \leq 0.05$.

Protein identification by mass spectrometry

Mass spectrometry analysis for protein identification was performed on nano-LC-MS/MS (Bruker Daltonics, Bremen, Germany) after protein spot excision and trypsin in-gel digestion. Briefly, differentially expressed spots excised proteins were treated with 25mM of NH_4HCO_3 in 50% of acetonitrile (ACN) until complete de-staining, dried with 99.5% ACN, and digested with sequencing grade modified trypsin in 25mM NH_4HCO_3 for 16 hours at 37°C. The peptides were extracted twice with 5% formic acid (FA) in 50% ACN and dried in Speed Vac concentrator (THERMO SAVANT, Holbrook, NY, USA). The fractions were desalted using C18 ZipTip (Millipore) following the manufacturer's instructions and the nano-electrospray capillaries were loaded with 6 µl of peptide solutions in 50% ACN in water with 0.1% FA. A 20 mm×100 µm pre column followed by a 100 mm×75 µm analytical column both packed with reverse-phase C18 were used for separation at a flow rate of 300 nl/min. The gradient buffers used were 0.1% formic acid in water (A) and 0.1% formic acid in 100% acetonitrile (B). Separation was performed with a linear gradient for 60 min (100-0% sol. A in 60 min, 0-100% sol. B in 60

min). Automated online tandem MS analyses were performed when peptide ions were sequenced using two alternating fragmentation techniques: collision induced dissociation (CID) and electron transfer dissociation (ETD). The data obtained were analyzed by Bruker Daltonics DataAnalysis 3.4 and the resulting MGF files where used to search for protein in Swissprot (*Mus musculus*) using Mascot Server (2.3) (www.matrixscience.com). The search parameters allowed mass error up to 0.8 Da for MS data and up to two missed trypsin cleavage. Peptide modifications searched for included carbamidomethyl (Cys) as the only fixed modification, and up to two variable modifications from among the following: oxidation (Met), acetyl (N-term), pyroglutamate (Gln) and Met-loss (N-term). Significance threshold in the MASCOT searches was set as $p<0.01$. Peptides were considered reliable if the MS/MS spectra had a MASCOT score above 35 and an expect value below 0.01.

Molecular weight and pI of the identified proteins were calculated with the Expasy compute pI/Mw tool (http:// www.expasy.ch/tools/pi_tool.html).

Extraction, separation and quantification of lipids

After quantification of the amount of cellular protein by the bicinchoninic acid method following manufacturer (PIERCE) instructions, lipids were extracted from 2 mg of cellular protein following the method of Folch *et al.* [39]. Briefly, eight volumes of chloroform/methanol/water (2:1:0.0075, v:v:v) were added and the methanol phase was re-extracted with four volumes of the same mixture. The chloroform phases were aspirated, combined, and washed with 1.5 ml of 0.88% KCl. Different species of lipids were separated using a thin-layer chromatography system composed of six sequential mobile phases as described by Ruiz and Ochoa [40]. Standard curves for all lipid classes were run in each plate. The lipid spots were quantified as detailed previously [41] using Quantity One software (Bio-Rad). Analysis was carried out at least twice per extract.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA). Paired comparisons were made using Student's t-test while the comparison of multiple treatments to a common control was performed using one-way analysis of variance (ANOVA) with Dunnett's test, and $p < 0.05$ was considered significant.

3. Results

NPs characterization

The results of NPs characterization in powder form and dispersed in the cell media are represented in Table 1. Information about the properties of the NPs in powder form was obtained from the manufacturer. NPs in the hepatocyte culture media showed agglomeration and/or aggregation. The NPs hydrodynamic size was characterized using Dynamic light scattering (DLS) which showed, in general, a bimodal distributions at concentrations 5 and 500 mg/L. The hydrodynamic size of CuO-NPs could not be obtained at 5 mg/L due to high noise to signal ratio. Generally, a stable suspension has a zeta potential value higher or lower than +/-30 mV (Malvern) and therefore none of the NPs were in stable suspension.

NPs oxidative ability and impact in cell viability

The oxidative ability of the metal and metal oxide NPs was investigated by cell-free dichlorofluorescein (DCFH) assay using 5 and 1 mg/L after 2 h exposure (Figure 1A). Our results evidenced that only ZnO-NPs and CuO-NPs at 5 mg/L had significant oxidative activity ($p<0.01$) while Ag-NPs and TiO_2-USNPs at 5 mg/L showed a significantly low fluorescent intensity ($p<0.01$), remarking their negligible oxidizing activity. The cell viability has been assessed by MTT assay after NPs exposure for 48 h. Hepatocytes exposed to low and high concentration of TiO_2-USNPs, and ZnO-NPs, and to low concentration of CuO- and Ag-NPs did not show effects in the cell viability. However, the viability of the hepatocytes exposed to high concentration of CuO-NPs and Ag-NPs significantly decreased by 50% compared to non-treated cells (Figure 1B).

Proteomic analysis of impact of NPs exposure

Two dimensional DIGE (2D-DIGE) images of the protein extracts from hepatocytes (NPs treated and untreated) were imported to REDFIN software that detected 998 spots per gel (Supplementary Figure 1) evenly distributed along the whole range of pH (3-10) but more abundant between 24-150 kDa. Comparisons between several groups control versus all treated or each treatment were taking in consideration for the statistical analysis of the data. The comparison control versus all NPs treatments revealed a total of84 spots differentially expressed ($p<0.05$, fold change ratio$\geq$1.5) (Figure 2A). In particular exposure to CuO-NPs and Ag-NPs at 5 mg/L showed the largest number of modified proteins. ZnO-NPs exposure showed similar number of differentially expressed proteins at both concentrations, underlining a concentration-independent response. The TiO_2-USNPs exposures caused the least modified protein profiles (Figure 2B). We found the highest number of unique spots at the high concentration exposure for all NPs. However, the concentration-dependent response varied among the NPs studied. The

CuO-NPs and Ag-NPs exposures duplicated and triplicated respectively, the number of differentially expressed spots from low to high concentration whereas a very low increase of concentration–dependent response was observed at TiO_2-USNPs and ZnO-NPs exposures. The impact at the protein level of the NP exposures was characterized by down-regulation. In hepatocytes exposed to Ag-NPs, most of the differentially expressed proteins were down-regulated underlining the strongest effects on the proteome. The changes in protein expression profile ($p<0.05$, fold change ratio ≥ 2) caused by exposure to the studied type and concentration of NPs were summarized in the supplementary material (Supplementary, Figure 1 and 2).

Identification of differentially expressed proteins

Considering the analytical method applied, 2D-DIGE, and the results showing a general response based on down-regulation, many differentially expressed spots were under the expression level required for identification. For those spots, additional trials were performed after pooling the same spot from all the DIGE gels but unfortunately some excised and selected spots analyzed by mass spectrometry remained still unidentified. The identified proteins were selected among the proteins differentially expressed ($p<0.05$ and with fold change ≥ 1.5) and in common with at least two NPs exposures included the comparison control versus all NPs treatments (Figure 3, Table 2). Most of the identified proteins were common among all the exposures but some NPs had specific effect on the expression of unique proteins. The protein (ID25) carbamoyl-phosphatase synthase (CSP1) was the most commonly differentially expressed protein being up-regulated in CuO-NPs (5 mg/L), ZnO-NPs (5 and 1 mg/L) and Ag NP (5 mg/L). TiO_2-USNPs caused the up-regulation ATP-Synthase and ETF protein subunit alpha while CuO-NPs (1 mg/L) caused the down-regulation of ETF protein subunit beta as well as Tubulin beta-6 chain (ID497) at both concentrations. ZnO-NPs caused the down-regulation of RNA helicase (Figure 3). Approximately 50% of the identified proteins are localized in the specific organelles such as mitochondria (including matrix and membrane) while the remaining proteins belong to cytoplasm and also with the exception of alpha-enolase (ID49 and ID102) and guanine nucleotide-binding protein (G-Protein) subunit beta-2-like 1 (ID 572) which can also be from cell membranes. The only nuclear protein identified was heterogeneous nuclear ribonucleo-protein F (HNRPF) (ID222) (Table 2). The only protein with unclear subcellular localization was helicase eIF4A (ID 273) which can be both in the nucleus and in the cytoplasm.

Post-translational modifications

306 The main post-translational modification found in numerous proteins was the oxidation of methionine
307 residues which causes small change of pI from the theoretical value (Table2). It is significantly in the
308 mitochondrial ATP synthase subunit alpha (ID209), (ATPA) that showed a big difference in pI from the
309 theoretical value (Table 2). However the sequence found by mass spectrometry (the pI value was 6.1),
310 which is close to that observed by 2DE, would match with the main chain of this protein without transit
311 peptide.

312

313 *Lipidomics*

314 Details on the lipid composition of hepatocytes from control and exposed to NPs at 5 mg/L are represented
315 in Figure 4. Interestingly, a significant decrease in the percentage of sphingomyelin (SM) was found in the
316 cells exposed to Ag-NPs ($p<0.001$) but also exposed to TiO_2-USNPs ($p<0.05$) (Figure 4A). CuO-NPS
317 exposure caused a decrease in the percentage of PI and PE (Figure 4A) which made the PC/PE ratio
318 decreased (Figure 4B), a predictor of altered membrane fluidity. In the cells exposed to Ag-NPs changes
319 in the total lipid quantities were observed with a significant increase of triacylglycerol (TG) cell content
320 (Figure 4C).

321

322 **4. Discussion**

323 The application of quantitative proteomics in combination with lipidomics can be a a useful method to
324 illustrate the effects of NPs in cell lines. In this study the effects of exposure to TiO_2-USNPs, ZnO-NPs,
325 CuO-NPs and Ag-NPs for 48 h were studied on primary mouse hepatocytes. After characterization of the
326 physicochemical properties of the NPs, their cytotoxicity was assessed followed by quantitative proteomic
327 and lipidomic analysis. Based on the cellular and molecular effects on the primary mouse hepatocytes, the
328 overall ranking of the impact of the NPs exposures is as follows: TiO_2<ZnO<Ag<CuO.

329 *Cytotoxicity of NPs*

330 TiO_2-USNPs (1-3 nm) used in this study were not cytotoxic (Figure 1B) at 1 or 5 mg/L. They did not
331 produce significant ROS (Figure 1A) and the insoluble nature of TiO_2-NPs has been shown in previous
332 studies [42]. Thus effects observed upon exposure to TiO_2-USNPs can be solely due to their size and direct
333 interactions with cellular components. ZnO-NPs exposures did not affect to the cellular viability, although
334 high concentration exposures could cause cytotoxicity in *in vitro* [15, 43]. However, despite lack of
335 toxicity, these NPs produced significant ROS (Figure 1A) and based on a previous study conducted by this
336 group, ZnO-NPs and CuO-NPs had the highest capacity of ions leakage [4]. Previous studies have

illustrated the importance of Zn ions in progression of alcoholic liver disease and hepatic lipid homeostasis where it was shown that Zn supplementation reverses alcoholic steatosis by inhibiting oxidative stress [19]. Therefore the impact of ZnO-NPs exposure on the proteome could be related to the disruption of Zn homeostasis and in combination with the increase of ROS levels cause cytotoxicity. As mentioned, similar to ZnO-NPs, CuO-NPs produced ROS (figure 1A) and leaked ions. However the exposure to CuO-NPs caused the most severe effects at the cellular and molecular level with significant reduction of cell viability. The severe toxicity of CuO-NPs has been shown previously [23, 24]. Since the amount of ROS produced alone could not be the unique cytotoxic input (as shown for ZnO-NPs), it is likely that the released ions had actively contributed to the cytotoxicity. The importance of the intracellular solubility of NPs has arisen from understanding the Trojan horse-type mechanism of intracellular dissolution and its impact on the release of ions inside the cells leading to toxicity [44]. It has recently been reported that the intracellular solubility of CuO-NPs has the most critical role on the cytotoxicity [45]. Another type of NPs with great impact on the hepatocytes viability was Ag-NPs. These NPs however did not produce ROS. Previous studies have shown the uptake of the Ag-NPs despite different pattern of agglomeration as well as release of ions, both contributing to toxicity [46, 47].

Global impact of the NPs exposure to hepatocytes

The cellular impact of the NPs exposure was globally studied by combining proteomics and lipidomics. The differentially expressed proteins identified were involved in lipid metabolism, electron transport chain, structure of the cell, signaling, metabolism as well as nuclear proteins.

Impact on lipids and fatty acid metabolism

One of the common cellular responses observed was variation of the cellular lipids (i.e. CuO-NPs, Ag-NPs and TiO_2-USNPs) and differential expression of proteins involved in fatty acid and lipid metabolism was also observed. The lipidomic results showed a significant decrease of percentage of SM in the hepatocytes exposed to TiO_2-USNPs at 5 mg/L, although the PC/PE and CL/PL values indicated that the membrane fluidity was not affected (Figure 4). Lipid rafts, defined as cholesterol- and sphingolipid-enriched membrane micro-domains, might be altered by TiO_2-USNPs exposure in plasma membrane, triggering ROS release by enzymes localized in the membrane rafts. These ROS stimulate ceramide-releasing enzymes (e.g. acid sphingomyelinase) which are responsible for converting SM into phosphorylcholine and ceramide, increase the ceramide-enriched membrane platforms [48, 49]. It has been reported that carbon-based NPs treatment in lung epithelial cells led to an increase of ceramides in lipid rafts [50]. This feed-forward mechanism can justify the decrease of SM in the TiO_2-NPs exposure. The exposure to CuO-NPs caused significant increase of the ratio PC/PE and a decrease percentage of some PE and PI as well as

increase in concentration of TG. The effect of Cu on the cellular lipid droplets has been shown previously [4]. Damage of the cellular plasma membrane has been shown to be one of the primary events in heavy metal (Cu and Zn) toxicity in plants [51, 52]. Previous studies have shown heavy metal stress increased PE, decreased PI, and PG [53], although the decrease in PE values observed in our study has also been shown in other studies [54]. Cu deficiency has been shown to increase *in vivo* hepatic synthesis of fatty acids, TG, and PL in rats [55]. Therefore the decrease of this lipid class could be correlated to Cu overload. Cells exposed to Ag-NPs had decrease in SM but increase in the number of TG and total lipids. The increase in total lipids due to exposure to Ag-NPs has been observed previously [56]. Proteomic data in this study showed that mitochondrial HMG-CoA synthase was down-regulated in the cells exposed to TiO_2-USNPs at 1 mg/L and to CuO-NPs at 5 mg/L. This enzyme has a key function in regulating the ketogenesis, pathway involved in the biosynthesis of ketones bodies, metabolic fuel during starvation [57]. Another mitochondrial protein involved in lipid and fatty acid metabolism, 3-oxoacyl-coA thiolase was up-regulated in CuO-NPs, and particularly, in Ag-NPs treatment. This enzyme catalyzes the last step in mitochondrial and peroxisomal β-oxidation [58]. The increase the total lipids and TAG observed in cells exposed to Ag-NPs could have led to an increase in 3-oxoacyl-coA thiolase involved in beta oxidation and lipid metabolism.

Impact on proteins involved in electron transport chain

The differential expression of protein involved in the electron transport chain could reflect the increase in cellular energy demand upon exposure to NPs. CuO-NPs at both concentrations, TiO_2-USNPs (1 mg/L) and ZnO-NPs (5mg/L) affected these proteins. However proteins involved in this pathway were mostly affecting to one type of NPs exposure. The up-regulation of ATP synthase was only found in the hepatocytes exposed to TiO_2-USNPs. This protein is one of the most abundant proteins in the inner mitochondrial membrane which is involved in H^+ transport at the mitochondrial membrane and provides ATP [59, 60]. Another protein uniquely affected by TiO_2-USNP exposure was ETF subunit alpha which are heterodimers and function as electron shuttles between primary flavoprotein dehydrogenases involved in mitochondrial fatty acid and amino acid catabolism and the membrane-bound electron transfer flavoproteins ubiquinone oxidoreductase [61]. In cells exposed to CuO-NPs a remarkable reduction of the expression of ETFs subunit beta was detected. An imbalance of these "housekeeping" proteins can have serious repercussions especially in the oxidation of fatty acids [62]. ZnO-NPs and CuO-NPs at 5 mg/L evidenced an increase of ROS and the up-regulation of the subunit 1 of cytochrome b-c1 complex or Complex III, protein. Complex III is the major ROS production site among all mitochondrial electron transport chain complexes, and it is the only complex that generates $^{-}O2^{\cdot}$ in the mitochondrial inter-

membrane space [63, 64]. Xia et al.[65] observed mitochondrial contribution to ZnO-NPs-induced ROS production, through the ultra-structural, and thereby membrane potential changes in this organelle. They also suggest that the release of Zn ions from NPs may exert extra-mitochondrial effects contributing to ROS generation, including NO production and generation of peroxynitrite (ONOO-). We have previously shown the significant release of Zn ions from ZnO-NPs [4].

Impact on proteins from urea cycle

CPS1, a mitochondrial enzyme involved in ATP-dependent formation of carbamoyl phosphate from glutamine or ammonia and bicarbonate in the first step of the urea cycle. This protein was over-expressed in the cells exposed to ZnO-NPs (5 and 1 mg/L), Ag-NPs (5 mg/L) and CuO-NPs (5 mg/L). Generally, an increase of CPS1 expression has been observed in the case of liver damage or during acute hepatitis, as disorders induced by oxidative stress [66] and it is one of the main potential toxicity markers found in rat liver cells [67]. Previous studies have reported the effect of Zn in urea cycle and increased of activities of CPS1 in the liver of zinc-deficient rats[68]. It is interesting that the possible Zn ions released by the NPs in this study have caused the up regulation of CPS1.

Impact on nuclear proteins

ZnO-NPs were the only NPs that affected both RNA helicase, and hnRNP. It has been described how ultrafine NPs could affect the expression of nuclear proteins [69]. We observed that ZnO-NPs exposure specifically caused the down-regulation of the ATP-dependent RNA helicase (elF4) which plays important roles in the unwinding and remodeling of structured RNA as well as virtually all aspects of nucleic acid metabolism, and regulation, possibly enhancing the biosynthesis of altered proteins [70]. Previous study has shown that down-regulation in helicase is associated with cell cycle perturbations and in apoptosis which in this case might be an indication of oxidative stress and early stages of apoptosis experienced by the cells [71] .

Among all identified differentially expressed proteins, only one nuclear protein, the hnRNP F, was affected by NPs treatment and was down-regulated by treatment with Ag-NPs and up-regulated by ZnO-NPs, and CuO-NPs treatment. The hnRNP complexes are known to play a role in the regulation of the splicing events but they have also been shown to function in the regulation of cell proliferation. Overexpression of hnRNP F has been shown to promote cell proliferation while reverse effect was observed upon knockdown of hnRNP F [72]. Disruption in this protein therefore could lead to genotoxicity as well as disruption in cell proliferation. It is possible that the cytotoxicity observed in Ag-NPs exposed cells was due to down-regulation of this protein.

Impact on structural proteins

Another modified protein in hepatocytes exposed to ZnO-NPs or Ag-NPs (at 5 mg/L) was ß-tubulin IV (TBB4B) which was down-regulated especially for the Ag-NPs treatment. This protein is the main constituent of microtubules, key components of the cytoskeleton of eukaryotic cells and has an important role in various cellular functions such as intracellular migration and transport, cell shape maintenance, polarity, and cell signaling. Previous *in vitro* studies showed that metal and metal oxide NPs can directly bind functional groups of microtubules [73, 74]. In particular, Ag-NPs interacting with tubulin in correspondence of -SH residue may be responsible of ineffective mitotic spindle function [75][76]. Tubulin is the first non-receptor protein found to be phosphorylated by G-protein receptor kinases [77]. Interestingly both ZnO-NPs (5mg/L) and Ag-NPs (1 mg/L) induced an increase of G-protein expression involved in many cellular signaling pathways, including the ubiquitination and proteasome-mediated degradation [70]. The isotype of ß-tubulin (TBB6) was significantly up-regulated in hepatocytes exposed to CuO-NPs at 5 and 1 mg/L which can contribute to an adaptation to oxidative stress conditions and drug resistance [78]. A compensatory mechanism from the hepatocytes exposed to CuO-NPs might occur to overwhelm the structural damages in the cytoskeleton, especially in the case of the highest concentration. HSPs function in important intracellular tasks such as protein folding and transport acting as chaperones under stress to prevent protein denaturation and loss of function [79]. HSP60 is a mitochondrial expressed stress protein that can be translocated to the cytosol and, later, transported to the cell surface. The HSP60 stress response is correlated with apoptosis and exacerbation of the disease state [80]. This protein was over-expressed in the two cytotoxic NPs i.e. Ag-NPs and CuO-NPs illustrating the apoptotic response of the cells.

Impact on cellular metabolism

Mitochondrial ALDH (ID34), and Alpha-enolase (ID49 or ID102) were found up-regulated in NPs treatments and can be considered as an early cellular defense response to general stress conditions. ALDH catalyzes the oxidation of various aliphatic and aromatic aldehydes to the corresponding acids and is in cellular defenses against toxic aldehydes [81]. Also it has been shown that mitochondria-located alpha-enolase stabilizes mitochondrial membrane and its' displacement may involve in activation of the intrinsic cell death pathway [82].

5. Concluding Remarks

Characterization of the NPs, classical toxicity assays and quantitative proteomics in combination with lipidomics could provide a detailed overview of the effects of NPs on primary hepatocytes. Most proteins identified to be differentially expressed were in common for the different NPs exposures and were involved in lipid metabolism, electron transport chain, cellular structure, metabolism, signaling as well nuclear proteins. CuO-NPs produced ROS, were cytotoxic, affected the PL and caused the down-regulation of ETF protein beta. Ag-NPs did not produce ROS but were cytotoxic, affected the SM as well as increasing total cellular lipids and TG. ZnO-NPs despite producing significant ROS were not cytotoxic and did not affect the cellular lipids but affected the RNA helicase. TiO_2-USNP did not produce ROS, were not cytotoxic yet affected the SM and affected ATP-synthase as well as ETF protein alpha. This work showed that some of our gaps for understanding the NP impact at the cellular level could be filled by combining data from alterations on lipidomic profiles with proteomic profiles. This OMICs methods or any extension to other OMICs methodologies would lead to a system biology understanding of NP impact and possible adverse outcome pathway.

6. Supplementary material

S1: Supplementary Figure 1: Representative 2D-DIGE proteins from hepatocytes exposed to NPs. A total of 998 spots were detected by REDFIN software.

S2: Supplementary Figure 2: Proteins up- and down-regulated by NPs along with fold change (F.C.).

S3: Table lipidomics: TG, triacylglycerol; CE, cholesteryl ester; FC, free cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin. Total lipid quantities correspond to the summation of all measured lipid species, which are expressed as the percentage of the summation. Total phospholipid quantities correspond to the summation of PC, PE, SM, PS and PI and total cholesterol to the summation of FC and CE. Data are expressed as the mean ± SEM and correspond to the results obtained using 5 mg/L concentration of NPs in the culture medium. Control vs. treated: $*P \leq 0.05$, $***P \leq 0.001$.

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Figures

Figure 1: A) Oxidative potential assay. Fluorescence intensity [arbitrary units (a.u.)] of the NPs after incubation with DCFH for 2 h at 37°C. Values are the mean ± SEM from three experiments. For each treatment, two concentrations were used 1 and 5 *** $p < 0.001$. B) MTT assay for estimation of cell viability, expressed as absorbance at 560 nm. *$p< 0.01$ and *$p<0.001$.

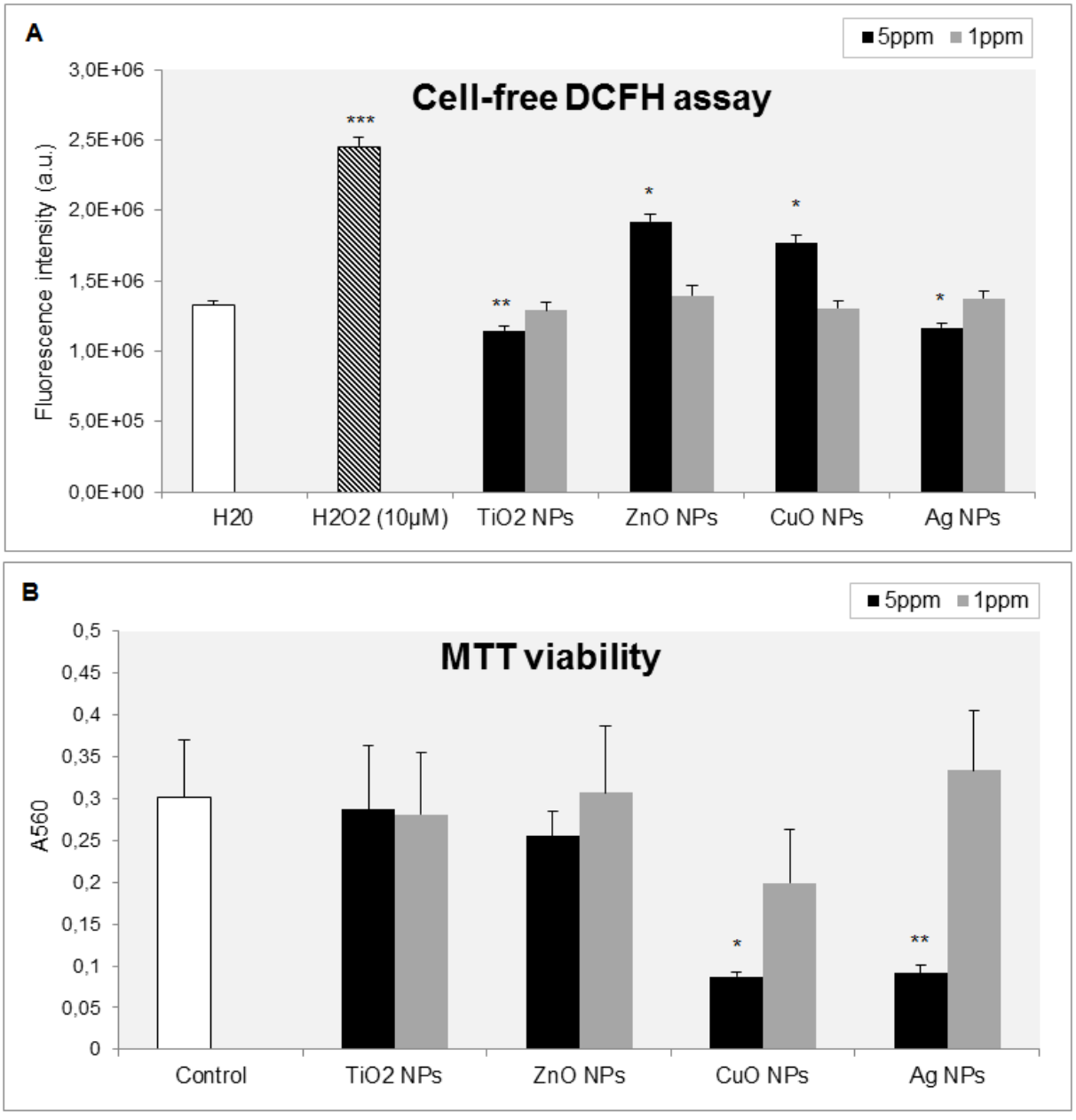


Figure 2: A) Differentially expressed proteins comparing control (untreated hepatocytes) versus each NPs exposure and B) Venn diagram representing differentially proteins among the exposures. The protein expression modification was considered significant for $p<0.05$ and fold change ratio≥ 1.5.

A

Control vs Treated	Spots number
TiO_2 NPs (5ppm)	32
TiO_2 NPs (1ppm)	23
ZnO NPs (5ppm)	51
ZnO NPs (1ppm)	55
CuO NPs (5ppm)	103
CuO NPs (1ppm)	35
Ag (5ppm)	84
Ag (1ppm)	41

B

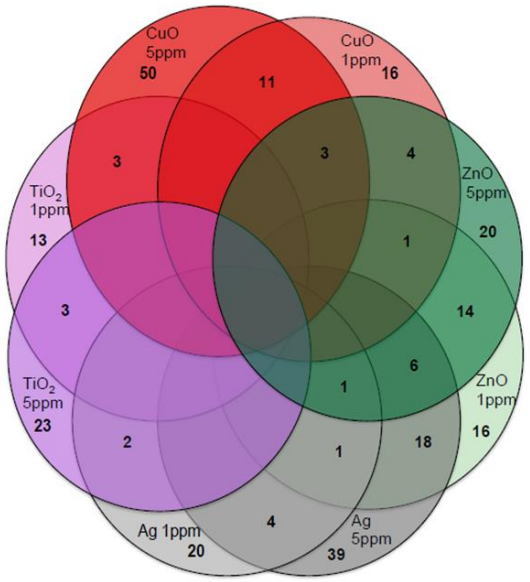


Figure 3: A) Representative 2D-DIGE with identified proteins and correspondent ID spot number. B) The protein expressions of the identified ID spots are illustrated as mean ± SEM based on fold change ratio value for the differentially expressed proteins and classified according to biological functions.

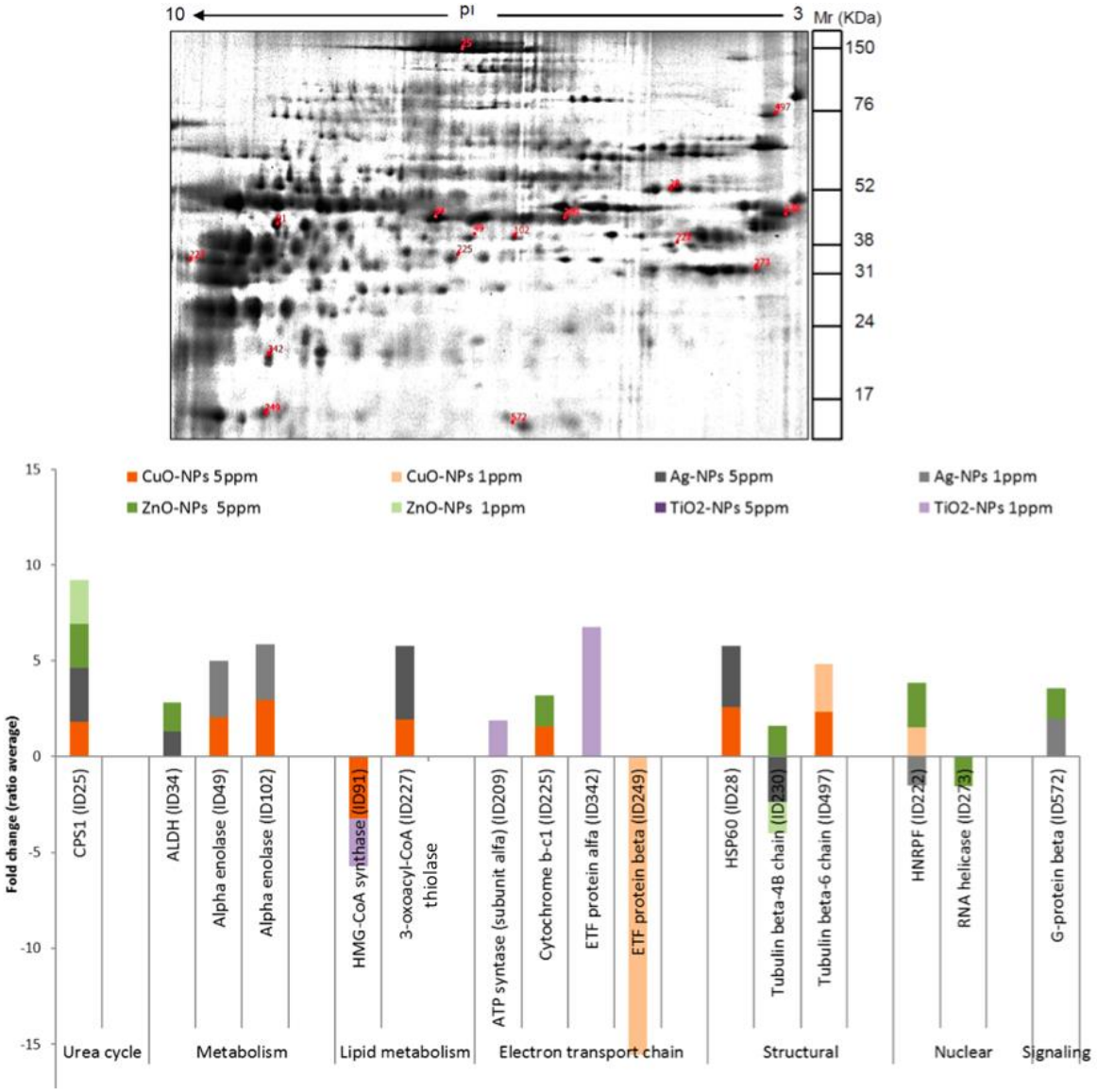
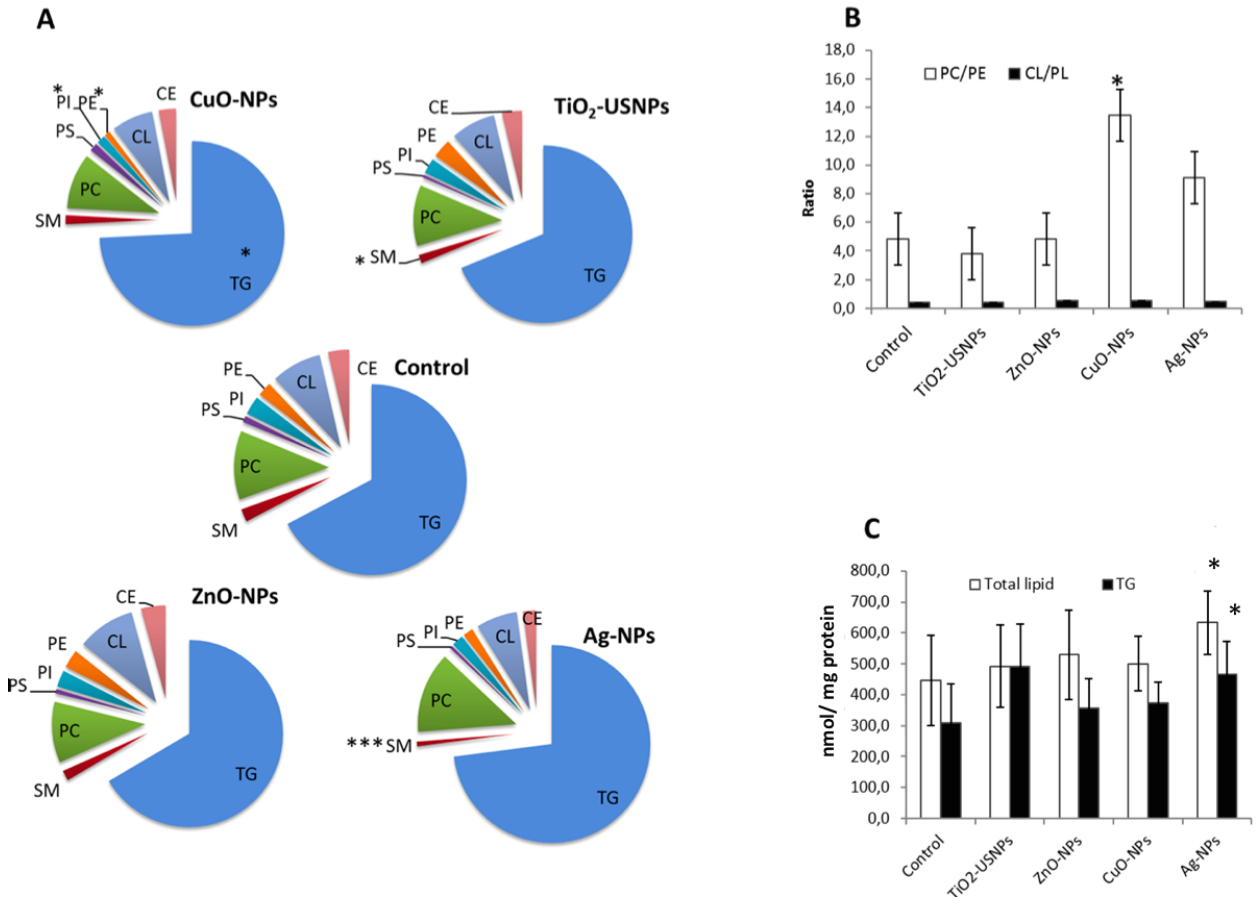


Figure 4: Distribution of total lipid content in control and exposures to NPs. A) Pie charts from percentages of lipid species; B) Ratio phosphatidylcholine/phosphatidylethanolamine and cholesterol/ phospholipid; C) Total lipid and total triacylglycerol in nmol/ mg protein TG, triacylglycerol; CL, cholesterol, CE, cholesteryl ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin. Total lipid value corresponds to the summation of all measured lipid species, which are expressed as the percentage of the summation. Total phospholipid (PL) value corresponds to the summation of PC, PE, SM, PS and PI and total CL to the summation of FC and CE. Data are expressed as the mean ± SEM and correspond to the results obtained using 5 mg/L concentration of NPs in the culture medium. Control vs. treated: *P ≤ 0.05, ***P ≤ 0.001.



TABLES

Table 1: Characterization of Nanoparticles (NPs). NPs properties in powder form and dispersed in hepatocytes media. Ag-NPs: Zeta-potential values are not showed (-) due to several aggregations. SEM images of the largest NPs (i.e. CuO- and ZnO-NPs while TEM pictures for the other NPs were taken. Information about NPs properties from the powder (or liquid form for Ag-NPs) was provided from the manufacturing companies.

			Powder		Suspension		
NPs	**Purity (%)**	**Crystal structure**	**Size (nm)**	**Specific surface area (M^2/g)**	**Concentration ppm**	**Size (nm)**	**Z-potential (mV)**
TiO_2	**99+**	**Rutile**	**1-3**	**470**	**5**	**$6.6e^4$**	**-0.5±0.1**
					500	**$1034e^5$**	**-0.9±0.6**
ZnO	**79.8**	**Hexagonal**	**<100**	**15-25**	**5**	**440.7±110.7**	**-4.6±1.0**
		Wurtzite			**500**	**747.4±3.9**	**-8.2±0.4**
CuO	**77.3**	**Monoclinic**	**<50**	**29**	**5**	**-**	**4.0±5.6**
		Crystals			**500**	**939.6±10.6**	**-7.4±2.7**
Ag	**99+**	**Spheres**	**10**	**60**	**5**	**85.4±5.6**	**-8.5±2.5**
					500		

Table 2: List of identified proteins by nano-LC-MS/MS after selection from the differentially expressed proteins ($p<0.05$ and with fold change ≥ 1.5) and in common with at least two NPs exposures included the comparison control versus all NPs treatments.

Spot no	Accession no	ID protein	Theor. /Obs. pI	Theor. Mr (Da)	Obs. Mr (Da)	Mascot score	SC (%)	Peptide sequence (if only one peptide)	Functional pathway	Subcellular location
25	gi|124248512	Carbamoyl-phosphate synthase	6.48/~6	165711	~150000	2125	48		Urea cycle	Mithocondrion
28	gi|183396771	60 kDa heat shock protein	5.91/~4.8	61088	52000-76000	1677	57		Chaperone	Mithocondrion matrix
34	gi|1352250	Aldehyde dehydrogenase	7.53/~6.2	57015	38000-52000	309	13		Alcohol metabolism; Aldehydes oxidation	Mitochondrion matrix
49 102	gi|13637776	Alpha-enolase	6.37/~5.8	47453	38000-52000	943 267	67 32		Carbohydrate degradation, glycolysis	Cytoplasm; Cell membrane
91	gi|61252474	Hydroxy-methylglutaryl-CoA synthase	8.65/~7	57300	38000-52000	310	31		Lipid synthesis	Mithocondrion
209	gi|416677	ATP synthase subunit alpha	9.22/ ~5.8	59830	38000-52000	176	12		ATP synthesis, Transport	Mithocondrion
222	gi|81918016	Heterogeneous nuclear ribonucleo-protein F	5.31/~5.2	46043	~38000	116	9	K.ITGEAFVQFAQFASQELAEK.A	Nucleotide binding; single-stranded RNA binding	Nucleus
225	gi|341941780	Cytochrome b-c1 complex subunit 1	5.81/~4.8	53446	31000-38000	115	26		Mitochondrial electron transport	Mitochondrion inner membrane
227	gi|342187137	Mitochondrial 3-oxoacyl-CoA thiolase	8.33/~9	42260	31000-38000	1355	74		Lipid metabolism	Mithocondrion
230	gi|55977481	Tubulin beta-4B chain	4.79/~4.2	50255	38000-52000	2337	64		Structural molecule activity	Cytoplasm, cytoskeleton
249	gi|92090596	Electron transfer flavoprotein subunit beta	8.24/ ~8.2	27834	17000-24000	480	45		Electron carrier activity	Mitochondrion matrix
273	gi|46397464	ATP-dependent RNA helicase eIF4A-1	5.32/~5.8	46353	~31000	64	4	K.TATFAISILQQIELDLK.A	Helicase	-
342	gi|146345417	Electron transfer flavoprotein subunit alpha	8.62/~7	35330	17000-24000	2282	66		Electron carrier activity	Mitochondrion matrix
497	gi|68775966	Tubulin beta-6 chain)	4.79/~4.2	50255	52000-76000	45	12	K.GHYTEGAELVDSVLDVVR.K	Structural molecule activity	Cytoplasm, cytoskeleton
572	gi|54037181	Guanine nucleotide-binding protein subunit beta-2-like 1	7.60/ ~5.8	35511	12000-17000	68	24		Developmental protein	Cell membrane, cell projection cytoplasm, cytoskeleton, nucleus