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## Multi-omics approaches confirm metal ions mediate the main toxicological pathways of metal-bearing nanoparticles in lung epithelial A549 cells

Dekkers, Susan; Williams, Timothy; Zhang, Jinkang; Zhou, Jiarui; Vandebriel, Rob J.; De La Fonteyn, Liset; Gremmer, Eric; He, Shan; Guggenheim, Emily; Lynch, Iseult; Cassee, Fleming; De Jong, wim; Viant, Mark

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1	Multi-omics approaches confirm metal ions mediate the
2	main toxicological pathways of metal-bearing
3	nanoparticles in lung epithelial A549 cells
4	<u>Susan Dekkers<sup>*1</sup>, Tim D. Williams<sup>*2</sup>, Jinkang Zhang<sup>2</sup>, Jiarui (Albert) Zhou<sup>3</sup>, Rob J. Vandebriel<sup>1</sup>,</u>
5	Liset J.J. De La Fonteyne <sup>1</sup> , Eric R. Gremmer <sup>1</sup> , Shan He <sup>3</sup> , Emily J. Guggenheim <sup>4</sup> , Iseult Lynch <sup>4</sup> ,
6	Flemming R. Cassee <sup>1,5</sup> , Wim H. De Jong <sup>1</sup> , Mark R. Viant <sup>2</sup>
7	
8	<sup>1</sup> National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
9	<sup>2</sup> School of Biosciences, The University of Birmingham, Birmingham, B15 2TT, UK
10	$^3$ Centre for Systems Biology, The University of Birmingham, Birmingham, B15 2TT, UK $^4$ School of
11	Geography Earth and Environmental Sciences, The University of Birmingham, Birmingham, B15 2TT, UK
12	<sup>5</sup> Institute of Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
13	* These authors contributed equally
14	Corresponding author: Susan Dekkers, <u>Susan.Dekkers@rivm.nl</u> , Tel: +31 30 274 7596
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17	
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23

#### 24 Abstract

25 The toxicity of silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs) has been associated with their 26 dissolution or ability to release metal ions while the toxicity of cerium dioxide (CeO<sub>2</sub>) NPs has been 27 related to their ability to induce or reduce oxidative stress dependent on their surface redox state. To 28 examine the underlying biochemical mechanisms, multiple omics technologies were applied to 29 characterise the responses at the molecular level in cells exposed to various metal-based particles and 30 their corresponding metal ions. Human lung epithelial carcinoma cells (A549) were exposed to various Ag, ZnO, and CeO<sub>2</sub> NPs, Ag and ZnO micro-sized particles (MPs), Ag ions (Ag<sup>+</sup>) and zinc ions (Zn<sup>2+</sup>) over a 31 32 24h time course. Molecular responses at exposure levels that caused ~20% cytotoxicity were 33 characterised by direct infusion mass spectrometry lipidomics and polar metabolomics and by RNAseq transcriptomics. All Ag, Zn and ZnO exposures resulted in significant metabolic and transcriptional 34 35 responses and the great majority of these molecular changes were common to both ionic and NP 36 exposures and characteristic of metal ion exposure. The low toxicity CeO<sub>2</sub> NPs elicited few molecular changes, showing slight evidence of oxidative stress for only one of the four CeO<sub>2</sub> NPs tested. The 37 38 multiple omics analyses highlight the main pathways implicated in metal ions-mediated effects. These 39 results can be used to establish adverse outcome pathways as well as strategies to group nanomaterials 40 for risk assessment.

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### 42 Keywords

43 nanoparticles, nanotoxicology, transcriptomics, metabolomics, lipidomics

## 44 **1. Introduction**

Although nanomaterials are increasingly used in many different applications, detailed 45 knowledge on the underlying biochemical mechanisms by which they may induce harmful 46 effects on humans and the environment is lacking. Several possible mechanisms of action have 47 been proposed<sup>1</sup>. One of the proposed mechanisms of action is related to the release of metal 48 ions. For both silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs), toxicity is often related to 49 their dissolution or ability to release metal ions<sup>2, 3</sup>. However, studies comparing the toxicity of 50 these NPs with that of their ionic forms indicate that the toxicity of the NPs cannot always be 51 fully explained by the release of metal ions<sup>4, 5</sup>. Another proposed mechanism of action is via the 52 induction of oxidative stress through the generation of reactive oxygen species (ROS). Cerium 53 dioxide (CeO<sub>2</sub>) NPs may have oxidative as well as anti-oxidative properties, depending on their 54 redox surface state. The ability to shift valence states from  $Ce^{3+}$  to  $Ce^{4+}$  or from  $Ce^{4+}$  to  $Ce^{3+}$  at 55 the surface of the NPs is suggested to influence the ability of the CeO<sub>2</sub> NPs to either scavenge 56 or generate reactive oxygen species (ROS), respectively, subsequently increasing or decreasing 57 the ability to induce oxidative stress<sup>6, 7</sup>. However, previous studies comparing CeO<sub>2</sub> NPs with 58 59 different valence states indicate that the mechanisms by which the redox surface status of NPs influences the toxicity are not yet fully understood<sup>7</sup>. 60

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Transcriptomics (gene transcriptional profiling), metabolomics (profiling of polar metabolites), proteomics (profiling of proteins) and lipidomics (profiling of lipids) are valuable non-hypothesis driven methods to gain insight into the mechanisms of actions or pathways leading to biological effects of NPs on living organisms, especially when these approaches are combined into a multi-

omics approach to explore a larger molecular landscape<sup>8</sup>. Computational modelling can be used 66 67 to search for molecular signatures that can contribute to the discovery of molecular key (initiating) events within adverse outcome pathways (AOPs), i.e. mechanistically based 68 molecular changes that are related to both an (upstream) molecular initiating event and 69 (downstream) key events leading to higher levels of phenotypic change<sup>9</sup>. These signatures can 70 be used in the screening, ranking and risk assessment of nanomaterials. When designing a 71 multi-omic study, it is important to generate time-resolved data to be able to follow the 72 different molecular responses within a pathway leading to a biological response<sup>10</sup>. Multi-omics 73 approaches have not yet been widely used within the field of nanotoxicology  $^{11, 12}$ . 74

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In this study, toxicological, analytical and computational methods are combined to 1) identify 76 the molecular mechanisms by which Ag, ZnO and CeO<sub>2</sub> NPs induce toxicity and 2) investigate 77 78 the influence of dissolution and redox surface state on the NP toxicity using transcriptomics, metabolomics and lipidomics. A549 lung epithelial cells were exposed to nano, micro and ionic 79 forms of Ag, Zn or ZnO and various CeO<sub>2</sub> NPs over a 24 hour time course. A549 cells were 80 selected as they are lung epithelial cells and inhalation is considered an important route of 81 exposure in occupational settings and for consumers using spray products<sup>13, 14</sup>. To investigate 82 83 the role of dissolution and ionic release on the pathways leading to adverse effects of metal (oxide) NPs, Ag and ZnO NPs and MPs as well as  $Ag^+$  and  $Zn^{2+}$  were studied in parallel. In 84 addition, CeO<sub>2</sub> NPs with different amounts of zirconium (Zr)-doping were studied as a means to 85 investigate the effect of the redox surface state on the biological response. Zr-doping increases 86 the Ce<sup>3+</sup>:Ce<sup>4+</sup>-ratio and is therefore expected to increase the antioxidant potential of the CeO<sub>2</sub> 87

NPs<sup>6, 7</sup>. Concentrations of NPs and their equivalent ions that induced approximately 20% cytotoxicity after 24 hrs exposure were chosen for this study, aiming to achieve a similar level of cellular damage in all cases. Importantly, temporal responses were investigated by sampling after different exposure times (1, 6 and 24 hrs) to characterize the development of the toxicological responses over time.

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## 95 **2. Materials and Methods**

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#### 97 **2.1** Nanomaterials: selection, dispersion and characterisation

Ag, ZnO and CeO<sub>2</sub> NPs were selected because of their expected modes of action that involve 98 either the release of ions or their ability to generate or scavenge ROS. An overview of the 99 physicochemical characteristics of the selected materials is given in **Table 1**. When provided as 100 powder the micro- and nano-sized particles were dispersed using the previously published 101 protocol by Jensen et al.<sup>15</sup>. For a final stock concentration of 2.56 mg/mL the powder was pre-102 103 wetted with 0.5 vol% ethanol and dispersed in water with 0.05% w/v bovine serum albumin 104 from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands), and sonicated for 16 minutes on ice 105 using a 400 Watt Branson Sonifier S-450D set at 10% amplitude with a 3 mm probe (Branson 106 Ultrasonics Corp., Danbury, CT, USA). When provided as dispersions, the NPs were vortexed for 15 s and sonicated for 5 mins in an ultrasonic bath (Branson CPX2800, 40 kHz, 110W) to re-107 108 disperse any possible agglomerates.

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#### 110 **2.2 Cell culture and exposures**

A549 cells were obtained from ATCC (VA, USA). The cells were cultured in tissue culture flasks in RPMI 1640 medium with Glutamax (Gibco, ThermoFisher Scientific Inc., Landsmeer, the Netherlands) supplemented with 10% Fetal Bovine Serum (FBS, Greiner BioOne BV, Alphen aan de Rijn, the Netherlands) and 1% penicillin/streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The adherent cells were harvested by a short incubation with 0.5% EDTA trypsin in Ca/Mg free Dulbecco's Phosphate Buffered Saline (Gibco).

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To determine the EC20 (effective concentration resulting in 20% cytotoxicity) dose-response 118 119 studies were performed. A549 cells were harvested and counted 24 hrs before exposure. Twenty thousand  $(2x10^4)$  cells were seeded in wells of 96-well plates in 100 µL supplemented 120 121 RPMI 1640 medium. After 24 hrs incubation a semi-confluent monolayer of cells was obtained 122 and the cells were exposed to the various materials. Cell survival (i.e. cytotoxicity) was determined after 24 hrs of exposure by a colorimetric assay using cell proliferation reagent 123 WST-1 (Roche, Sigma-Aldrich Chemie). All exposures were performed in triplicate. Dose-124 response modelling and derivations of the EC20 were performed using PROAST software <sup>16</sup> 125 version 60.1. For the four CeO<sub>2</sub> NPs and Ag MP no EC20 was obtained, since the highest 126 127 concentration tested (128 µg/mL) resulted in less than 20% cytotoxicity. An overview of the 128 EC20 values and confidence intervals can be found in Electronic Supplementary Information (ESI) 1. Dissolution of nanoparticles and microparticles in cell culture medium was measured 129 130 using ICP-OES (ESI 2).

## **Table 1:** Physicochemical characteristics of the selected materials<sup>a</sup> and cell viability at doses applied in the omics study

Test material	Batch no	Short Description	Primary size (nm±SD)	Hydrodynamic size (nm±SD) measured with disc centrifuge	Hydrodynamic size (nm±SD) measured with DLS	Dose <sup>b</sup> (µg/mL)	lon conc. <sup>c</sup> (µg/mL)	Cell viability (%)
Ag NP- NM300K	JRC-Ag<20nm- NM03002a000855b	Ag NPs dispersed in H <sub>2</sub> O with 4% polyoxy-ethylene glycerol trioleate and 4% Tween 20.mean particle size 15 nm	< 20	n.m.	50 to 70	38.6	0.04	79
Ag MP	SIGMA- AgBulk - 2- 3.5microns- 180215a	micro-sized Ag particles powder	>1000?	n.m.	n.m.	128	0.005	95
AgNO <sub>3</sub>	-	ionic silver nitrate (AgNO <sub>3</sub> ) soluble powder	n.a.	n.a.	n.a.	8	5.08	83
ZnO NP- NM110	JRC-ZnOun-NM110- 0801b	uncoated ZnO NPs powder mean particle size 150 nm, primary particle size 42 nm	$151\pm57$	193 ± 3	275 ± 4	15	1.41	94
ZnO NP- NM111	JRC-ZnOTECS-NM111- 2995b	ZnO NPs coated with triethoxy- caprylsilane powder mean particle size 140 nm, primary particle size 34 nm	$141\pm 66$	n.m. <sup>d</sup>	$253\pm1$	10	0.989	89
ZnO MP	SIGMA – ZnO- 5 microns- 180215a	micro-sized ZnO particles powder	5000	n.m.	n.m.	30	1.46	82
ZnCl <sub>2</sub>	-	ionic zinc chloride (ZnCl <sub>2</sub> ) soluble powder	n.a. <sup>e</sup>	n.a.	n.a.	24.6	11.80	67
CeO <sub>2</sub> NP-A	PROM-CeO2-20nm- batchCE026A-a	undoped $CeO_2$ NPs dispersed in $H_2O$	4.7±1.4	39	$172\pm2$	128	<lod<sup>f</lod<sup>	88
CeO <sub>2</sub> NP-C	PROM-ZrCeO2- batchCE026C-a	27% $ZrO_2$ -doped $CeO_2$ NPs dispersed in $H_2O$	4.6±1.4	40	$297\pm4$	128	<lod< td=""><td>89</td></lod<>	89
CeO <sub>2</sub> NP-E	PROM-ZrCeO2- batchCE025E-a	78% ZrO <sub>2</sub> doped CeO <sub>2</sub> NPs dispersed in H <sub>2</sub> O	4.7±1.4	41	358±6	128	<lod< td=""><td>89</td></lod<>	89
CeO <sub>2</sub> NP- NM212	Umnicore-CeO2- NM212-RIVM-batch	uncoated CeO <sub>2</sub> NPs powder primary particles size 33 nm	28.4 ± 10.4	135 ± 4	213	128	<lod< td=""><td>87</td></lod<>	87

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<sup>a</sup> CeO<sub>2</sub> NP-NM212, ZnO NPNM110, ZnO NP-NM111 and Ag NP-NM300K were characterized within the OECD sponsorship programme <sup>17-19</sup>. The other NPs were

133 characterized within the NanoMILE project (Lynch et al., in preparation); <sup>b</sup> Exposure doses for the A549 cells, adapted for incubation in the 6-well plates, are

134 shown in bold figures; <sup>c</sup> Ion concentration measured for the NPs and MPs in cell culture medium after 24 hrs (see ESI 2 for details) and estimated using the

135 molecular weight for  $AgNO_3$  and  $ZnCl_2$ ; <sup>d</sup> n.m. = not measured; <sup>e</sup> n.a. = not applicable; <sup>f</sup> <LOD = below limit of detection.

For the omics studies,  $8x10^5$  cells per well were seeded in 6-well plates and cultured for 18 hrs, 136 137 after which the cells were exposed to the determined EC20 concentrations or to 128  $\mu$ g/mL for the particles where EC<sub>20</sub> was not reached. Because the cytotoxicity of the A549 cells was higher 138 in the 6-well plates compared to the 96-well plates, several concentrations were adapted and 139 140 applied as presented in Table 1. Omics analyses were carried out on independent replicates 141 from 14 exposure and control groups. Biological replication was, for polar metabolomics n=6, lipidomics n=6 and transcriptomics n=4. Different cell plates were exposed for t=1, t=6 and t=24 142 143 hrs to monitor changes in the molecular responses over time. At t=24 hrs, additional control 144 wells were included to measure the actual cytotoxicity and possible interference of the materials with the viability assay. After exposure, the cells (approximately 2x10<sup>6</sup> per well) were 145 146 quickly washed with PBS (phosphate buffered saline) twice at room temperature after which the 6-well plates were deep frozen by quenching on liquid nitrogen (-196 $^{\circ}$ C) and stored at -80 $^{\circ}$ C 147 148 until extraction for omics evaluation.

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#### 150 **2.3 Omics Analyses**

Brief descriptions of the methods used for omics analysis are shown. Full methodological detailsare provided in ESI 3.

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#### 154 2.3.1 Extraction of metabolites and lipids

155 Cells were harvested then vortexed in methanol:chloroform:water (v/v/v at 1:1:0.9) and the 156 phases separated by centrifugation. The polar phase was dried in a speed vac concentrator 157 (Thermo Savant, Holbrook, NY) for 4 hr. The non-polar phase was dried under a stream of
158 nitrogen for 5 mins. All dried samples were then frozen at -80°C until analysis.

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#### 160 **2.3.2 Direct infusion mass spectrometry (DIMS)**

The DIMS analysis method used was similar to previous studies<sup>20, 21</sup>. Dried extracts were resuspended in 80:20 (v/v) methanol:water with 0.25% formic acid (for positive ion mode analysis of polar extracts) or 80 μL 2:1 methanol:chloroform with 5 mM ammonium acetate (for negative ion mode analysis of lipids). Samples were analysed (in quadruplicate) using direct infusion mass spectrometry (Q Exactive, Thermo Fisher Scientific, Germany) in positive ion mode (for polar metabolomics) or negative ion mode (for lipidomics), utilising a Triversa nanoelectrospray ion source (Advion Biosciences, Ithaca, NY, USA).

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#### 169 2.3.3 Metabolomics Data Processing

Mass spectra were recorded using the selected ion monitoring (SIM) stitching approach from 170 m/z 50-620 (for polar metabolomics) or from m/z 50-1020 (for lipidomics) and then processed 171 using custom-written Matlab scripts as previously reported<sup>22, 23</sup>. The resulting matrices of peak 172 intensities (termed "DIMS dataset") were probabilistic-quotient normalised (PQN) and 173 174 intensity-drift corrected using a Quality Control-Robust Spline Correction (QC-RSC) algorithm. 175 Finally, the missing values were imputed using the k-nearest neighbours (KNN) algorithm. For multivariate analysis, generalized log (Glog) transformation of the DIMS dataset was 176 performed. 177

178

#### 179 **2.4 RNA seq gene expression profiling**

#### 180 **2.4.1 Sequencing**

Total RNA was extracted from A549 cells using a micro RNeasy Kit (Qiagen, Crawley, UK). All 181 182 RNA libraries were produced using the Biomek FxP (Beckman Coulter A31842) with Ultra 183 Directional RNA Library Prep Kit (New England Biolabs E7420L) and NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), using provided protocols and 184 500ng of total RNA. Multiplex library clustering and sequencing was performed upon the 185 186 HiSeq2500 (Illumina) with HiSeq Rapid Cluster Kit v2 (Illumina GD-402-4002) at 12pM library 187 concentration with 10% PhiX Control v3 spiked in (Illumina FC-110-3001). The sequencing run was carried out using HiSeq Rapid SBS Kit v2 (Illumina FC-402-4021). 188

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#### 190 2.4.2 RNA seq Data Processing

191 The binary base call (BCL) files were converted to FASTQ format (containing a biological sequence and its corresponding quality scores) using Illumina bcl2fastq conversion software 192 193 (v1.8.4). Sequences were then trimmed using Trimmomatic (v0.36). Five low quality samples were identified and removed accordingly. The FASTQ files were aligned to the GENCODE human 194 transcript sequences (release 25, GRCh38.p7) using Bowtie2 (v2.3.0). The resulting Sequence 195 196 Alignment Map (SAM) data were converted into Browser Extensible Data (BED) format using 197 SAMtools (v1.3.1) and bamToBed (v2.19.1). Finally, the RNA read counts were extracted from the BED files with a Python script. To provide gene-level analysis, the RNA reads were collapsed 198 199 to the counts of their coding genes. The gene annotation information was retrieved from the 200 Ensembl database (release 87).

201 2.5 Omics Data Analysis

Putative metabolite annotations were added using MI-Pack<sup>24</sup>. ANOVA, t-tests and principal 202 components analyses (PCA) were performed in Genespring (v7.3.1. Agilent) using multiple 203 testing corrections<sup>25</sup>.  $DESeq2^{26}$  was used for differential gene expression analysis with a q<0.05 204 cut-off. Combined gene and metabolite pathway over-representation analyses were performed 205 with IMPaLA<sup>27</sup>, using gene identifiers and Human Metabolite Database (HMDB) identifiers <sup>28</sup> for 206 207 each peak identified as significantly altered as input lists. Comparative pathway analyses were 208 performed with Ingenuity Pathway Analysis (IPA; Qiagen) on combined sets of genes, lipids and polar metabolite identifiers. Raw transcriptomic data and experimental details are archived at 209 210 ArrayExpress (accession number: E-MTAB-5734).

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#### 213 **3. Results**

214 In total, 259 polar metabolomic, 250 lipidomic and 156 transcriptomic samples passed the quality control metrics. Four RNAseq samples were removed prior to further analysis due to 215 anomalously low counts. The full results of univariate analyses comparing each exposure group 216 217 with its time-matched control group are shown in ESI 4. Comparisons were made with time-218 matched controls since gene expression and metabolite profiles varied significantly with time between the control groups sampled at 1h, 6h and 24h. Figure 1 illustrates the overall numbers 219 of significantly changing (q<0.05) genes and metabolites in comparison with time-matched 220 221 controls. The numbers of molecular (transcript and metabolic) changes at 24h correlated 222 significantly with cytotoxicity (Table 1) for all silver (Ag MP, Ag NP and Ag<sup>+</sup>) exposures ( $r^2$ =0.98;

p<0.01) and all zinc (ZnO MP, ZnO NP and  $Zn^{2+}$ ) exposures ( $r^2$ =0.85; p<0.03) but not for the CeO<sub>2</sub> 223 224 NP exposures. All silver exposures resulted in significantly more changes than CeO<sub>2</sub> exposures, and all zinc exposures led to the most numerous alterations. Silver exposures ranked from the 225 largest to the smallest effect in the order Ag<sup>+</sup>, Ag NP and then Ag MP, whereas zinc exposures 226 ranked highest for Zn<sup>2+</sup>, followed by ZnO MP, ZnO NP-NM110 and ZnO NP-NM111. These rank 227 orders matched the orders of ionic concentrations determined by dissolution analysis (Table 1). 228 CeO<sub>2</sub> NP exposures resulted in few gene expression or metabolic changes, and ionic dissolution 229 230 was below the limit of detection. Of these few changes, CeO<sub>2</sub> NP-A (undoped CeO<sub>2</sub> NPs) was the only CeO<sub>2</sub> NP that increased metabolites putatively identified as cysteine at 1 hr (3.5 fold; 231 q<0.032) and  $\gamma$ -glutamylcysteine at 6h (1.5 fold; q<0.02). 232

233

PCA scores plots of transcriptomic and polar metabolomic data after 6h of exposure are shown in **Figure 2**, with PCAs for all other timepoints, as well as for the lipidomics data, shown in **ESI 5**. For silver there was grouping of replicate samples and separation from the controls was apparent for all exposure groups. For zinc, replicate samples grouped tightly and clearly diverged from the control group along PC1, with the degree of divergence corresponding with the number of molecular changes outlined above (see also Figure 1). There was little or no apparent grouping of the CeO<sub>2</sub> NP samples or divergence from the control group.

IMPaLA pathway over-representation analysis results are shown in full in **ESI 6**, while overrepresentation of selected pathways is illustrated in **Figure 3**. All silver and zinc exposures at 6h and 24h resulted in significant enrichment of the terms 'Response to metal ions' and (Metallothioneins bind metals'. Terms relating to the heat shock response were enriched in the same groups at 6h but not at 24h, except for the Ag<sup>+</sup> and Zn<sup>2+</sup> exposures where they persisted. Other enriched terms, including 'Translation', 'Nonsense-Mediated Decay', 'Apoptosis' and 'Immune System' were highlighted with all zinc exposures and either Ag<sup>+</sup> or Ag NP exposure at 6h and sometimes also at 24 h. Zn<sup>2+</sup>, ZnO MP and ZnO NP-NM110 repressed molecules related to DNA repair. CeO<sub>2</sub> exposures elicited few molecular changes and showed no enriched pathway annotations, apart from 'HIF-1 alpha transcription network' induced at 24h with CeO<sub>2</sub> NM212 and 'ID signalling pathway' repressed at 6 and 24h with CeO<sub>2</sub> NP-A,- C and E.

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Ingenuity Pathway Analysis (IPA) Comparison Analyses were used to compare molecular 253 254 pathway responses to the various silver and zinc exposures. Figure 4 shows the top 20 canonical pathways and the top 20 'diseases and bio-functions' associated with the silver and 255 256 zinc exposures, ordered by function or process from IPA's Pathway Activity Analysis function, 257 representing predicted pathway activation or inhibition. Particularly prominent for silver was the Nrf2-mediated oxidative stress response pathway, predicted to be activated by Ag NP at 6h 258 and by Ag<sup>+</sup> at all timepoints. The Nrf2 pathway was also predicted to be activated by all Zn 259 exposures at 6h, but repressed at 24h. The canonical pathway comparison of silver exposures 260 was otherwise dominated by modulation of several molecular signalling pathways, particularly 261 with Ag<sup>+</sup> at 6h. All Zn exposures resulted in very similar profiles of predicted pathway activation, 262 highlighting co-ordinated induction of signalling pathways at 6h, followed by repression at 24h, 263 except for Zn<sup>2+</sup> for which these pathways were predicted to still be activated at 24h. Data from 264  $CeO_2$  NP exposures were not used due to the low numbers of responsive molecules. 265

To identify candidate nano-specific responses, t-tests were performed comparing, for silver, the 266 267 Ag NP group versus respectively the control,  $Ag^+$  and Ag MP groups. Molecules were only selected if statistically significantly (FDR<0.05) changed in all comparisons. A similar procedure 268 was followed for ZnO NP-NM110 and ZnO NP-NM111. The results of these comparisons and 269 270 IMPaLA pathway analyses using these data are shown in Figure 5 and ESI 7. For Ag NPs, 17.6% 271 of transcriptional and 22% of metabolic changes were assessed as candidate nano-specific. The 272 induced molecules associated with several pathways, particularly those concerned with amino 273 acid metabolism, while the decreasing molecules associated with glycolysis and galactose 274 metabolism and reduced transcription relating to phase II xenobiotic metabolism. ZnO NP-275 NM110 elicited only 12 (0.15%) candidate nano-specific changes and ZnO NP-NM111 elicited 22 276 (0.77%), mostly reduction in metabolites associated with galactose metabolism.

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## 279 **4. Discussion**

280 In our study molecular changes were sought that were unique to the nanomaterial exposures, 281 not appearing in response to the ionic or micro-sized particle exposures at any timepoint. These 282 were termed 'candidate nano-specific responses' as only three timepoints were examined for 283 each exposure, raising the possibility of these responses having occurred at an unexamined 284 timepoint in the non-NP exposures. For silver there was evidence for candidate nano-specific changes supported by both the transcriptomics and metabolomics data (Figure 5 and ESI 7). 285 286 These were related to increases in amino-acid transport, reduced glycolysis and galactose 287 metabolism and reduced glucuronidation and xenobiotic metabolism. Potentially these changes

could result in lower capacity to detoxify organic xenobiotics and it could be instructive to 288 compare modulation of organic xenobiotic toxicity in co-exposures with Ag NP or Ag<sup>+</sup>. However, 289 since no dispersant control (for Ag NP-NM300K) was included in the omics study, some of these 290 nano-specific changes may be caused by the dispersant (water with 4% polyoxyethylene 291 292 glycerol trioleate and 4% Tween 20), instead of the Ag NPs. Although previous studies with the same Ag NPs and its dispersant indicated that the dispersant was not cytotoxic to A549 cells up 293 to 256  $\mu$ g/mL, <sup>29, 30</sup> DNA damage was observed in the absence of cytotoxicity <sup>31</sup> in A549 cells. 294 295 For zinc there was very little evidence of candidate nano-specific responses, limited to metabolomics changes related in pathway analyses to a reduction in galactose metabolism. 296 Although candidate nano-specific responses were found for Ag NPs, by far the majority (>78%) 297 of responses to the Ag NPs were also seen with  $Ag^+$  and Ag MP, as was found by NMR 298 metabolomics in HaCaT cells<sup>32</sup>, implying that nano-specific toxicity is likely a minor component 299 compared with that elicited by silver ions. 300

301

All silver and zinc exposures induced transcription of genes responsive to metal ions at 6h and 302 24h. Metallothionein induction was particularly notable, with MT1A, MT1B, MT1F, MT1G, 303 MT1X, and MT2A highly and significantly induced with all Ag and Zn exposures but MT1H and 304 MT1E induced only with Zn. Several of these MT transcript inductions exceeded 1000-fold, 305 including *MT1B* with Ag NPs and Ag<sup>+</sup> at 6h and *MT1H* with ZnO NM-110, Zn<sup>2+</sup> and Zn MP at 6h 306 and 24h. Additionally the zinc transporter SLC30A1 (ZnT-1), responsible for export of zinc ions, 307 was induced by both Zn and Ag exposures. Ag ions have been shown to release Zn ions in 308 fibroblasts<sup>33</sup>. 309

Metallothioneins have long been considered biomarkers for metal ion exposure and oxidative 310 stress <sup>34</sup> and metal-based NP studies frequently report their induction<sup>35, 36</sup>. Metallothionein 311 induction may be viewed as an adaptive response enabling cells to bind and sequester metal 312 ions and for Ag MP exposure this response appeared effective, resulting in only 5% cytotoxicity 313 314 (Table 1) and few significant alterations in other biological pathways (Figure 3; ESI 6). However with the other Ag and Zn exposures resulting in higher cytotoxicity, it was apparent that this 315 capacity was exceeded, leading to stress responses and cellular damage. The heat shock 316 response was activated in all 6h and 24h Zn exposures and with Ag<sup>2+</sup> and Ag NPs; transcripts 317 encoding the molecular chaperones HSPA1A, HSPA1L, HSPA6, HSPA7, HSPB1, HSPH1, 318 HSP90AA1, HSP90AB1, DNAJB1 and DNAJB6 were induced, implying a response to protein 319 damage. 320

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322 Oxidative stress is a well-established outcome following NP exposure, including Ag, ZnO and CeO<sub>2</sub>, NPs<sup>37, 38</sup>. IPA highlighted the Nrf2 mediated oxidative stress response as activated by Ag<sup>+</sup> 323 and all 6h Zn exposures. Nrf-2 (NFE2L2) transcript was significantly but not highly induced (<1.6-324 fold) with Zn<sup>2+</sup> and Zn MP and several key antioxidant enzyme transcripts were either mildly but 325 significantly induced (SOD1, TXN, GLRX, GCLM, GSS) at 6h or mildly repressed (CAT, GCLC, GSR, 326 PRDX1). Aldehyde oxidase AOX1 was induced over 2-fold, as were heme oxygenase (HMOX1), 327 several chaperones mentioned above and transcription factors FOS, JUN and ATF4. 328 Transcription of KEAP1, a repressor of Nrf-2 signalling, was significantly repressed with ZnO 329 NM-110, Zn<sup>2+</sup> and Zn MP at 6h. Nrf2 pathway induction has previously been found for Ag NPs<sup>39</sup> 330 and for ionic Ag and Zn<sup>40</sup>. Several polar metabolite peaks that were putatively annotated as 331

glutathione (GSH) followed a similar profile of a significant increase with Ag<sup>+</sup> at 1h but a significant decrease with all zinc exposures at 6h and 24h. GSH is the major intracellular antioxidant and its depletion implies vulnerability to further oxidative damage. GSH has previously been found to decrease with ZnO NP treatment in mouse livers and kidneys<sup>41, 42</sup>.

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A metabolite peak putatively annotated as cysteine was particularly highly increased with all Ag 337 exposures, and amino acid concentrations were significantly altered with most exposures. This 338 339 may represent an adaptive reorganisation of amino acid synthesis and transport. Expression of transcripts involved in translation, including those encoding ribosomal subunits, was increased 340 with Ag NP, ZnO MP and  $Zn^{2+}$  exposure. Interestingly nonsense mediated decay pathway 341 transcripts were induced in the same exposures, implying an increase in mRNAs with premature 342 stop codons. Potentially this could be due to an increased rate of DNA damage, as transcription 343 344 of DNA repair genes was reduced, including those of the base excision repair, mismatch repair, nucleotide excision repair and double-strand break repair pathways, particularly with Zn<sup>2+</sup>, Zn 345 MP and ZnO NP-NM110. DNA damage has previously been described for Ag<sup>+</sup>, Ag NPs<sup>43, 44</sup>, Zn<sup>2+</sup> 346 and Zn NPs<sup>45</sup>. Transcription of the stress-inducible AP-1 transcription factor genes was 347 increased by Zn<sup>2+</sup> and ZnO NP exposure, and by Ag<sup>+</sup>, including induction of FOS, FOSB, FOSL1, 348 JUN and JUNB. Cell cycle gene transcription was significantly repressed with both Ag and Zn 349 exposures. These effects have previously been seen with Ag NPs<sup>43, 46, 47</sup>. There was an induction 350 of transcription associated with apoptosis and with immune signalling with Ag<sup>+</sup> and all Zn 351 exposures at 6h, persisting to 24h with Zn<sup>2+</sup>. Both ionic and NP Ag and Zn can increase 352 apoptosis in A549 cells<sup>48, 49</sup>. By 24h Zn<sup>2+</sup> exposure, both transcripts and metabolites of the TCA 353

354 cycle were significantly reduced, indicating major disruption of cellular respiration pathways,
 355 consistent with the bioenergetic disruption reported for ZnO NP exposure of A549 cells<sup>50</sup>.

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These molecular alterations illustrate a progression from adaptive changes, such as 357 358 metallothionein induction, to depletion of antioxidants, such as glutathione, repressed DNA repair and induction of apoptosis. Several of these molecular changes have been proposed as 359 key events (KE) in the Adverse Outcome Pathway (AOP) paradigm<sup>51</sup>. Examples include increase 360 361 in oxidative stress, activation of Nrf-2, depletion of glutathione, repression of DNA repair and increased apoptosis. It is however apparent that many additional pathways, genes and 362 363 metabolites were altered during the exposures to Ag and Zn (ESI 5 and 6) and that using a cell line model one can only examine those events leading up to cell death. 364

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366 For ZnO NPs and MPs, responses indicative of damage tended to peak at 6h, with a reduction by 24h, for example acute phase signalling (Figure 5), while with Zn<sup>2+</sup> these changes persisted 367 to 24h, consistent with the greater cytotoxicity caused by this treatment. The responses to Ag 368 and Zn clearly demonstrated the importance of measuring molecular responses over time in 369 order to robustly assess molecular toxicity. At 1h there were relatively few molecular pathway 370 371 changes (Figure 3), at 6h molecular responses indicative of toxicity had developed, but at 24h for several exposures (Ag<sup>+</sup>, Ag MP, ZnO NP-NM110, ZnO NP-NM111 and Zn MP) the responses 372 had declined, or even reversed (Figure 4) whereas these persisted with the more cytotoxic 373 ZnCl<sub>2</sub> exposure. This time-dependence of molecular response can be explained by adaptive 374 375 changes, such as induction of metallothioneins that ameliorate cellular damage by sequestering

the metal ions, as illustrated by the Ag MP exposure. There may also be a time dependency in 376 377 exposure to the metal ions, due to different uptake rates and dissolution kinetics of the different micro- and nano-sized particles resulting in different intracellular concentrations, or 378 different intracellular compartmentalisation of the metal ions. In A549 cells and phagocytic 379 murine macrophages, Ag NPs were associated with lysosomes<sup>52</sup>, whereas ionic  $Ag^+$  was bound 380 to metallothioneins<sup>53</sup>. ZnO NPs showed intracellular dissolution in lysosomes of macrophages<sup>54</sup> 381 and extracellular dissolution with only ions entering hepatocytes<sup>55</sup>. Additionally NP dissolution 382 can also occur within the NP preparations<sup>56</sup> and in cell culture medium (ESI 2). The dynamic 383 molecular responses detected could therefore reflect changing intracellular doses of metal ions. 384 For future studies it is therefore recommended to obtain additional supporting data to estimate 385 the intracellular doses, including time-resolved data on the bioavailable ion concentrations 386 after exposure to the salts and cellular uptake rates of MPs, NPs and ions. 387

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CeO<sub>2</sub> NPs led to relatively few significant alterations of transcription or metabolism compared 389 with silver and zinc, reflecting both a decrease in molecular alterations with increasing EC20 390 concentrations (effective concentrations resulting in 20% cytotoxicity) and their low solubility 391 (Table 1). A similar mild metabolomic and transcriptomic response to CeO<sub>2</sub> NPs was found by 392 Taylor et al.<sup>12</sup> in algae. Among the few significant alterations induced by the CeO<sub>2</sub> NPs, only 393 CeO<sub>2</sub> NP-A (undoped CeO<sub>2</sub> NP) exposure significantly increased metabolites putatively 394 395 identified as cysteine and  $\gamma$ -glutamylcysteine, potentially representing an adaptive response to oxidative stress by increased uptake and synthesis of these glutathione precursors. Because 396 these changes were not observed after exposure to CeO<sub>2</sub> NP-C (27% Zr-doped CeO<sub>2</sub> NPs) or 397

CeO<sub>2</sub> NP–E (78% Zr-doped CeO<sub>2</sub> NPs), this finding might indicate that modification of the surface redox state by Zr-doping increases the ability to scavenge ROS, resulting in a decreased induction of oxidative stress of the CeO<sub>2</sub> NPs. Since all CeO<sub>2</sub> NPs showed very low cytotoxicity and exposure to only one of the four CeO<sub>2</sub> NPs showed any slight evidence of molecular response to oxidative stress, the actual occurrence of oxidative stress, ROS or damage related to ROS was not further investigated.

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CeO<sub>2</sub> NP-A, -C and -E exposures all resulted in repression of ID family gene expression (Figure 405 4). Verstraelen et al. <sup>57</sup> similarly found repression of *ID2* transcription in A549 cells treated with 406 CeO<sub>2</sub> NPs. The ID, or Inhibitor of DNA binding, genes are binding partners of bHLH transcription 407 factors and are involved in regulation of a wide variety of biological processes, including 408 metastasis and vascularisation<sup>58</sup>. CeO<sub>2</sub> NP-NM212 elicited a different molecular response from 409 410 the other CeO<sub>2</sub> NPs, activating genes of the HIF1-alpha transcription factor network (Figure 4) by 24h. This response was also shared by the 6h Ag and Zn exposures, with additional induction 411 of heme oxygenase (HMOX1). HIF1-alpha responsive genes are also commonly induced by 412 several metal ions and particles<sup>59</sup> and in cancer cells by ROS<sup>60</sup>, leading to angiogenesis via VEGF. 413 This finding is consistent with  $CeO_2$  and Ag NPs inducing angiogenesis<sup>61, 62</sup>. 414

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## 417 **5. Conclusions**

418 A time series experiment was used to determine the similarity of A549 cellular responses 419 following exposure to NPs and ions, as focussing on a single timepoint would have led to erroneous conclusions in the absence of internal dose measurements. The majority of molecular responses of A549 cells to the Ag and Zn NPs, such as metallothionein induction, depletion of antioxidants, repressed DNA repair and induction of apoptosis, are similar to their responses to Ag and Zn ions, respectively, confirming that the modes of action of these NPs are largely mediated by dissolved metal ions rather than by the physical aspects of the NPs. Low toxicity CeO<sub>2</sub> NPs elicited only minor molecular responses. Of the four CeO<sub>2</sub> NPs tested, only CeO<sub>2</sub> NP-A elicited any molecular changes indicative of oxidative stress.

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- 613

## 614 Figure Legend

Figure 1: Number of genes significantly differentially expressed (black bars) and metabolite
peaks significantly altered in concentration (clear bars) (q<0.05) in A549 cells after silver, zinc, or</li>
CeO<sub>2</sub> NPs, MPs and/or ionic exposures for 1, 6 or 24 h.

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Figure 2: Principal components analysis scores plots of transcriptomics and metabolomics data
from A549 cells exposed to silver, zinc, or CeO<sub>2</sub> for 6 hrs. For silver, control samples are shown
in black, Ag NP treated in red, Ag MP in cyan, Ag<sup>+</sup> in blue. For zinc, control samples are shown in
black, ZnO NP-NM110 in red, ZnO NP-NM111 in cyan, Zn<sup>2+</sup> in blue and ZnO MP in pink. For
CeO<sub>2</sub>, control samples are shown in black, CeO<sub>2</sub> NP-A treated in red, CeO<sub>2</sub> NP-C in cyan, CeO<sub>2</sub>
NP-E in blue and CeO<sub>2</sub> NP-NM212 in pink. PCAs for all timepoints are shown in ESI 5.

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**Figure 3:** Heatmap illustrating selected pathway annotation terms significantly differentially represented (q<0.05) by IMPaLA among genes and metabolites induced (red) or repressed (green) in comparison with time matched control groups after exposure of A549 cells to silver, zinc, or CeO<sub>2</sub> (NPs, MPs or ions) for 1, 6 or 24h. Full data are shown in **ESI 6**.

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Figure 4: The top 20 canonical pathways and the top 20 'diseases and bio-functions' associated with the silver and zinc exposures in A549 cells, ordered by function or process from Ingenuity Pathway Analysis (IPA) Pathway Activity Analysis function, representing predicted pathway activation (orange) or inhibition (blue) with maximum colour intensity set to z-score  $\geq$ 2.

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Figure 5: Heatmap illustrating selected pathway annotation terms significantly differentially represented (q<0.05) by IMPaLA among candidate nano-specific transcripts and metabolites induced (red) or repressed (green) in comparison with control, microparticle and ionic exposures over all timepoints. Full data are shown in **ESI 7**.

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## 641 Supporting Information

- 642 ESI 1: Cytotoxicity of tested particles to A549 cells
- 643 ESI 2: Dissolution of NPs and MPs in cell culture medium using ICP-OES
- 644 ESI 3: Methods
- 645 ESI 4: Univariate analyses of omics data
- 646 ESI 5: PCA scores plots of omics data
- 647 ESI 6: IMPaLA pathway analysis of omics data
- 648 ESI 7: Candidate nano-specific molecular responses