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

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Running Head – A549 metal-bearing nanoparticle multi-omics

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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  
31 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  
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  
34 transcriptomics. All Ag, Zn and ZnO exposures resulted in significant metabolic and transcriptional  
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  
37 changes, showing slight evidence of oxidative stress for only one of the four CeO<sub>2</sub> NPs tested. The  
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.

41

## 42 **Keywords**

43 nanoparticles, nanotoxicology, transcriptomics, metabolomics, lipidomics

## 44 **1. Introduction**

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

61  
62 Transcriptomics (gene transcriptional profiling), metabolomics (profiling of polar metabolites),  
63 proteomics (profiling of proteins) and lipidomics (profiling of lipids) are valuable non-hypothesis  
64 driven methods to gain insight into the mechanisms of actions or pathways leading to biological  
65 effects of NPs on living organisms, especially when these approaches are combined into a multi-

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

75  
76 In this study, toxicological, analytical and computational methods are combined to 1) identify  
77 the molecular mechanisms by which Ag, ZnO and CeO<sub>2</sub> NPs induce toxicity and 2) investigate  
78 the influence of dissolution and redox surface state on the NP toxicity using transcriptomics,  
79 metabolomics and lipidomics. A549 lung epithelial cells were exposed to nano, micro and ionic  
80 forms of Ag, Zn or ZnO and various CeO<sub>2</sub> NPs over a 24 hour time course. A549 cells were  
81 selected as they are lung epithelial cells and inhalation is considered an important route of  
82 exposure in occupational settings and for consumers using spray products<sup>13, 14</sup>. To investigate  
83 the role of dissolution and ionic release on the pathways leading to adverse effects of metal  
84 (oxide) NPs, Ag and ZnO NPs and MPs as well as Ag<sup>+</sup> and Zn<sup>2+</sup> were studied in parallel. In  
85 addition, CeO<sub>2</sub> NPs with different amounts of zirconium (Zr)-doping were studied as a means to  
86 investigate the effect of the redox surface state on the biological response. Zr-doping increases  
87 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>

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

93

94

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

96

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

98 Ag, ZnO and CeO<sub>2</sub> NPs were selected because of their expected modes of action that involve  
99 either the release of ions or their ability to generate or scavenge ROS. An overview of the  
100 physicochemical characteristics of the selected materials is given in **Table 1**. When provided as  
101 powder the micro- and nano-sized particles were dispersed using the previously published  
102 protocol by Jensen et al.<sup>15</sup>. For a final stock concentration of 2.56 mg/mL the powder was pre-  
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  
107 15 s and sonicated for 5 mins in an ultrasonic bath (Branson CPX2800, 40 kHz, 110W) to re-  
108 disperse any possible agglomerates.

109

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

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

117  
118 To determine the EC<sub>20</sub> (effective concentration resulting in 20% cytotoxicity) dose-response  
119 studies were performed. A549 cells were harvested and counted 24 hrs before exposure.  
120 Twenty thousand ( $2 \times 10^4$ ) cells were seeded in wells of 96-well plates in 100 µL supplemented  
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  
123 determined after 24 hrs of exposure by a colorimetric assay using cell proliferation reagent  
124 WST-1 (Roche, Sigma-Aldrich Chemie). All exposures were performed in triplicate. Dose-  
125 response modelling and derivations of the EC<sub>20</sub> were performed using PROAST software <sup>16</sup>  
126 version 60.1. For the four CeO<sub>2</sub> NPs and Ag MP no EC<sub>20</sub> was obtained, since the highest  
127 concentration tested (128 µg/mL) resulted in less than 20% cytotoxicity. An overview of the  
128 EC<sub>20</sub> values and confidence intervals can be found in **Electronic Supplementary Information**  
129 **(ESI) 1**. Dissolution of nanoparticles and microparticles in cell culture medium was measured  
130 using ICP-OES **(ESI 2)**.

131 **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)	Ion 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.	<b>8</b>	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 ± 57	193 ± 3	275 ± 4	<b>15</b>	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 ± 66	n.m. <sup>d</sup>	253 ± 1	<b>10</b>	0.989	89
ZnO MP	SIGMA – ZnO- 5 microns- 180215a	micro-sized ZnO particles powder	5000	n.m.	n.m.	<b>30</b>	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 <sub>2</sub> NPs dispersed in H <sub>2</sub> O	4.7 ± 1.4	39	172 ± 2	128	<LOD <sup>f</sup>	88
CeO <sub>2</sub> NP-C	PROM-ZrCeO2-batchCE026C-a	27% ZrO <sub>2</sub> -doped CeO <sub>2</sub> NPs dispersed in H <sub>2</sub> O	4.6 ± 1.4	40	297 ± 4	128	<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	89
CeO <sub>2</sub> NP-NM212	Umicore-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	87

132 <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<sub>3</sub> and ZnCl<sub>2</sub>; <sup>d</sup> n.m. = not measured; <sup>e</sup> n.a. = not applicable; <sup>f</sup> <LOD = below limit of detection.

136 For the omics studies,  $8 \times 10^5$  cells per well were seeded in 6-well plates and cultured for 18 hrs,  
137 after which the cells were exposed to the determined EC<sub>20</sub> concentrations or to 128 µg/mL for  
138 the particles where EC<sub>20</sub> was not reached. Because the cytotoxicity of the A549 cells was higher  
139 in the 6-well plates compared to the 96-well plates, several concentrations were adapted and  
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,  
142 lipidomics n=6 and transcriptomics n=4. Different cell plates were exposed for t=1, t=6 and t=24  
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  
145 materials with the viability assay. After exposure, the cells (approximately  $2 \times 10^6$  per well) were  
146 quickly washed with PBS (phosphate buffered saline) twice at room temperature after which  
147 the 6-well plates were deep frozen by quenching on liquid nitrogen (-196°C) and stored at -80°C  
148 until extraction for omics evaluation.

149

## 150 **2.3 Omics Analyses**

151 Brief descriptions of the methods used for omics analysis are shown. Full methodological details  
152 are provided in **ESI 3**.

153

### 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.

159

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

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

168

### 169 **2.3.3 Metabolomics Data Processing**

170 Mass spectra were recorded using the selected ion monitoring (SIM) stitching approach from  
171 m/z 50-620 (for polar metabolomics) or from m/z 50-1020 (for lipidomics) and then processed  
172 using custom-written Matlab scripts as previously reported<sup>22, 23</sup>. The resulting matrices of peak  
173 intensities (termed "DIMS dataset") were probabilistic-quotient normalised (PQN) and  
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  
176 multivariate analysis, generalized log (Glog) transformation of the DIMS dataset was  
177 performed.

178

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

### 180 **2.4.1 Sequencing**

181 Total RNA was extracted from A549 cells using a micro RNeasy Kit (Qiagen, Crawley, UK). All  
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  
184 for Illumina Dual Index Primers (New England Biolabs E7600S), using provided protocols and  
185 500ng of total RNA. Multiplex library clustering and sequencing was performed upon the  
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  
188 was carried out using HiSeq Rapid SBS Kit v2 (Illumina FC-402-4021).

189

### 190 **2.4.2 RNA seq Data Processing**

191 The binary base call (BCL) files were converted to FASTQ format (containing a biological  
192 sequence and its corresponding quality scores) using Illumina bcl2fastq conversion software  
193 (v1.8.4). Sequences were then trimmed using Trimmomatic (v0.36). Five low quality samples  
194 were identified and removed accordingly. The FASTQ files were aligned to the GENCODE human  
195 transcript sequences (release 25, GRCh38.p7) using Bowtie2 (v2.3.0). The resulting Sequence  
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  
198 the BED files with a Python script. To provide gene-level analysis, the RNA reads were collapsed  
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

202 Putative metabolite annotations were added using MI-Pack<sup>24</sup>. ANOVA, t-tests and principal  
203 components analyses (PCA) were performed in Genespring (v7.3.1. Agilent) using multiple  
204 testing corrections<sup>25</sup>. DESeq2<sup>26</sup> was used for differential gene expression analysis with a  $q < 0.05$   
205 cut-off. Combined gene and metabolite pathway over-representation analyses were performed  
206 with IMPaLA<sup>27</sup>, using gene identifiers and Human Metabolite Database (HMDB) identifiers<sup>28</sup> for  
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  
209 polar metabolite identifiers. Raw transcriptomic data and experimental details are archived at  
210 ArrayExpress (accession number: E-MTAB-5734).

211

212

## 213 3. Results

214 In total, 259 polar metabolomic, 250 lipidomic and 156 transcriptomic samples passed the  
215 quality control metrics. Four RNAseq samples were removed prior to further analysis due to  
216 anomalously low counts. The full results of univariate analyses comparing each exposure group  
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  
219 between the control groups sampled at 1h, 6h and 24h. **Figure 1** illustrates the overall numbers  
220 of significantly changing ( $q < 0.05$ ) genes and metabolites in comparison with time-matched  
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 ( $r^2 = 0.98$ ;

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

233  
234 PCA scores plots of transcriptomic and polar metabolomic data after 6h of exposure are shown  
235 in **Figure 2**, with PCAs for all other timepoints, as well as for the lipidomics data, shown in **ESI 5**.  
236 For silver there was grouping of replicate samples and separation from the controls was  
237 apparent for all exposure groups. For zinc, replicate samples grouped tightly and clearly  
238 diverged from the control group along PC1, with the degree of divergence corresponding with  
239 the number of molecular changes outlined above (see also Figure 1). There was little or no  
240 apparent grouping of the CeO<sub>2</sub> NP samples or divergence from the control group.

241 IMPaLA pathway over-representation analysis results are shown in full in **ESI 6**, while over-  
242 representation of selected pathways is illustrated in **Figure 3**. All silver and zinc exposures at 6h  
243 and 24h resulted in significant enrichment of the terms 'Response to metal ions' and  
244 'Metallothioneins bind metals'. Terms relating to the heat shock response were enriched in the

245 same groups at 6h but not at 24h, except for the Ag<sup>+</sup> and Zn<sup>2+</sup> exposures where they persisted.  
246 Other enriched terms, including 'Translation', 'Nonsense-Mediated Decay', 'Apoptosis' and  
247 'Immune System' were highlighted with all zinc exposures and either Ag<sup>+</sup> or Ag NP exposure at  
248 6h and sometimes also at 24 h. Zn<sup>2+</sup>, ZnO MP and ZnO NP-NM110 repressed molecules related  
249 to DNA repair. CeO<sub>2</sub> exposures elicited few molecular changes and showed no enriched  
250 pathway annotations, apart from 'HIF-1 alpha transcription network' induced at 24h with CeO<sub>2</sub>  
251 NM212 and 'ID signalling pathway' repressed at 6 and 24h with CeO<sub>2</sub> NP-A,- C and E.

252  
253 Ingenuity Pathway Analysis (IPA) Comparison Analyses were used to compare molecular  
254 pathway responses to the various silver and zinc exposures. **Figure 4** shows the top 20  
255 canonical pathways and the top 20 'diseases and bio-functions' associated with the silver and  
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  
258 the Nrf2-mediated oxidative stress response pathway, predicted to be activated by Ag NP at 6h  
259 and by Ag<sup>+</sup> at all timepoints. The Nrf2 pathway was also predicted to be activated by all Zn  
260 exposures at 6h, but repressed at 24h. The canonical pathway comparison of silver exposures  
261 was otherwise dominated by modulation of several molecular signalling pathways, particularly  
262 with Ag<sup>+</sup> at 6h. All Zn exposures resulted in very similar profiles of predicted pathway activation,  
263 highlighting co-ordinated induction of signalling pathways at 6h, followed by repression at 24h,  
264 except for Zn<sup>2+</sup> for which these pathways were predicted to still be activated at 24h. Data from  
265 CeO<sub>2</sub> NP exposures were not used due to the low numbers of responsive molecules.

266 To identify candidate nano-specific responses, t-tests were performed comparing, for silver, the  
267 Ag NP group versus respectively the control, Ag<sup>+</sup> and Ag MP groups. Molecules were only  
268 selected if statistically significantly (FDR<0.05) changed in all comparisons. A similar procedure  
269 was followed for ZnO NP-NM110 and ZnO NP-NM111. The results of these comparisons and  
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.

277

278

## 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  
285 changes supported by both the transcriptomics and metabolomics data (**Figure 5 and ESI 7**).  
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

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

301

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

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

321  
322 Oxidative stress is a well-established outcome following NP exposure, including Ag, ZnO and  
323 CeO<sub>2</sub>, NPs<sup>37, 38</sup>. IPA highlighted the Nrf2 mediated oxidative stress response as activated by Ag<sup>+</sup>  
324 and all 6h Zn exposures. Nrf-2 (*NFE2L2*) transcript was significantly but not highly induced (<1.6-  
325 fold) with Zn<sup>2+</sup> and Zn MP and several key antioxidant enzyme transcripts were either mildly but  
326 significantly induced (*SOD1*, *TXN*, *GLRX*, *GCLM*, *GSS*) at 6h or mildly repressed (*CAT*, *GCLC*, *GSR*,  
327 *PRDX1*). Aldehyde oxidase *AOX1* was induced over 2-fold, as were heme oxygenase (*HMOX1*),  
328 several chaperones mentioned above and transcription factors *FOS*, *JUN* and *ATF4*.  
329 Transcription of *KEAP1*, a repressor of Nrf-2 signalling, was significantly repressed with ZnO  
330 NM-110, Zn<sup>2+</sup> and Zn MP at 6h. Nrf2 pathway induction has previously been found for Ag NPs<sup>39</sup>  
331 and for ionic Ag and Zn<sup>40</sup>. Several polar metabolite peaks that were putatively annotated as

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

336

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

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>.

356  
357 These molecular alterations illustrate a progression from adaptive changes, such as  
358 metallothionein induction, to depletion of antioxidants, such as glutathione, repressed DNA  
359 repair and induction of apoptosis. Several of these molecular changes have been proposed as  
360 key events (KE) in the Adverse Outcome Pathway (AOP) paradigm<sup>51</sup>. Examples include increase  
361 in oxidative stress, activation of Nrf-2, depletion of glutathione, repression of DNA repair and  
362 increased apoptosis. It is however apparent that many additional pathways, genes and  
363 metabolites were altered during the exposures to Ag and Zn (**ESI 5 and 6**) and that using a cell  
364 line model one can only examine those events leading up to cell death.

365  
366 For ZnO NPs and MPs, responses indicative of damage tended to peak at 6h, with a reduction  
367 by 24h, for example acute phase signalling (**Figure 5**), while with Zn<sup>2+</sup> these changes persisted  
368 to 24h, consistent with the greater cytotoxicity caused by this treatment. The responses to Ag  
369 and Zn clearly demonstrated the importance of measuring molecular responses over time in  
370 order to robustly assess molecular toxicity. At 1h there were relatively few molecular pathway  
371 changes (**Figure 3**), at 6h molecular responses indicative of toxicity had developed, but at 24h  
372 for several exposures (Ag<sup>+</sup>, Ag MP, ZnO NP-NM110, ZnO NP-NM111 and Zn MP) the responses  
373 had declined, or even reversed (**Figure 4**) whereas these persisted with the more cytotoxic  
374 ZnCl<sub>2</sub> exposure. This time-dependence of molecular response can be explained by adaptive  
375 changes, such as induction of metallothioneins that ameliorate cellular damage by sequestering

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

388

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

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

404  
405 CeO<sub>2</sub> NP-A, -C and -E exposures all resulted in repression of ID family gene expression (**Figure**  
406 **4**). Verstraelen et al.<sup>57</sup> similarly found repression of *ID2* transcription in A549 cells treated with  
407 CeO<sub>2</sub> NPs. The ID, or Inhibitor of DNA binding, genes are binding partners of bHLH transcription  
408 factors and are involved in regulation of a wide variety of biological processes, including  
409 metastasis and vascularisation<sup>58</sup>. CeO<sub>2</sub> NP-NM212 elicited a different molecular response from  
410 the other CeO<sub>2</sub> NPs, activating genes of the HIF1-alpha transcription factor network (**Figure 4**)  
411 by 24h. This response was also shared by the 6h Ag and Zn exposures, with additional induction  
412 of heme oxygenase (*HMOX1*). HIF1-alpha responsive genes are also commonly induced by  
413 several metal ions and particles<sup>59</sup> and in cancer cells by ROS<sup>60</sup>, leading to angiogenesis via VEGF.  
414 This finding is consistent with CeO<sub>2</sub> and Ag NPs inducing angiogenesis<sup>61, 62</sup>.

415

416

## 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

420 erroneous conclusions in the absence of internal dose measurements. The majority of  
421 molecular responses of A549 cells to the Ag and Zn NPs, such as metallothionein induction,  
422 depletion of antioxidants, repressed DNA repair and induction of apoptosis, are similar to their  
423 responses to Ag and Zn ions, respectively, confirming that the modes of action of these NPs are  
424 largely mediated by dissolved metal ions rather than by the physical aspects of the NPs. Low  
425 toxicity CeO<sub>2</sub> NPs elicited only minor molecular responses. Of the four CeO<sub>2</sub> NPs tested, only  
426 CeO<sub>2</sub> NP-A elicited any molecular changes indicative of oxidative stress.

427

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## 614 **Figure Legend**

615 **Figure 1:** Number of genes significantly differentially expressed (black bars) and metabolite  
616 peaks significantly altered in concentration (clear bars) ( $q < 0.05$ ) in A549 cells after silver, zinc, or  
617  $\text{CeO}_2$  NPs, MPs and/or ionic exposures for 1, 6 or 24 h.

618  
619 **Figure 2:** Principal components analysis scores plots of transcriptomics and metabolomics data  
620 from A549 cells exposed to silver, zinc, or  $\text{CeO}_2$  for 6 hrs. For silver, control samples are shown  
621 in black, Ag NP treated in red, Ag MP in cyan,  $\text{Ag}^+$  in blue. For zinc, control samples are shown in  
622 black, ZnO NP-NM110 in red, ZnO NP-NM111 in cyan,  $\text{Zn}^{2+}$  in blue and ZnO MP in pink. For  
623  $\text{CeO}_2$ , control samples are shown in black,  $\text{CeO}_2$  NP-A treated in red,  $\text{CeO}_2$  NP-C in cyan,  $\text{CeO}_2$   
624 NP-E in blue and  $\text{CeO}_2$  NP-NM212 in pink. PCAs for all timepoints are shown in **ESI 5**.

625  
626 **Figure 3:** Heatmap illustrating selected pathway annotation terms significantly differentially  
627 represented ( $q < 0.05$ ) by IMPaLA among genes and metabolites induced (red) or repressed  
628 (green) in comparison with time matched control groups after exposure of A549 cells to silver,  
629 zinc, or  $\text{CeO}_2$  (NPs, MPs or ions) for 1, 6 or 24h. Full data are shown in **ESI 6**.

630  
631 **Figure 4:** The top 20 canonical pathways and the top 20 'diseases and bio-functions' associated  
632 with the silver and zinc exposures in A549 cells, ordered by function or process from Ingenuity  
633 Pathway Analysis (IPA) Pathway Activity Analysis function, representing predicted pathway  
634 activation (orange) or inhibition (blue) with maximum colour intensity set to z-score  $\geq 2$ .

635

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

640

## 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