A multi-laboratory toxicological assessment of a panel of ten engineered nanomaterials to human health - ENPRA project - the highlights, limitations and current and future challenges

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Abstract

ENPRA was one of the earlier multi-disciplinary European Commission FP7 funded projects aiming to evaluate the risks associated with nanomaterial (NM) exposure on human health across pulmonary, cardiovascular, hepatic, renal and developmental systems. The outputs from this project have formed the basis of this review. A retrospective interpretation of the findings across a wide range of *in vitro* and *in vivo* studies was performed to identify the main highlights from the project. In particular focus was placed on informing what advances were made in the hazard assessment of NM, as well as offering some suggestions on the future of "nanotoxicology research" based on these observations, shortcomings and lessons learned from the project. A number of issues related to the hazard assessment of NM are discussed in detail and include use of appropriate NM for nanotoxicology investigations; characterization and dispersion of NM; use of appropriate doses for all related investigations; need for the correct choice of experimental models for risk assessment purposes and full understanding of the test systems and correct interpretation of data generated from *in vitro* and *in vivo* systems. It is hoped that this review may assist in providing information in the implementation of guidelines, model systems, validation of assessment methodology and integrated testing approaches for risk assessment of NM. It is vital to learn from on-going and/or completed studies to avoid unnecessary duplication and offer suggestions that might improve different aspects of experimental design.

Keywords Engineered nanomaterials, hazard assessment, human health, *in vitro*, *in vivo*, current and future challenges

Introduction

The production and use of engineered nanomaterials (NM) is constantly expanding due to exploitation of unusual properties exhibited by materials at the nano-scale, making them extremely desirable. Hence, nanotechnology has become one of the most important new technologies of the 21st century, with the global market for these products expected to grow to $64 billion by 2019 (BCC Research 2015). However, there are still numerous uncertainties regarding the potential risks posed to human health and the environment following long-term exposure to these materials. It is essential that the hazards associated with NM exposure are assessed in parallel to their benefits for society in order to better understand potential detrimental implications on human health. The toxicological data combined with exposure data might provide input into risk assessment of NM and support of safe, responsible and sustainable development of the field of nanotechnology.

In the last two decades emerging data has shown a range of adverse effects induced by engineered NM which include but not limited to oxidative stress, inflammation and genotoxicity suggesting that long-term exposure may result in a risk to human health with numerous potential manifestations including cancer, respiratory and cardiovascular diseases (Guo et al. 2012; Li et al. 2014a; Oberdorster et al. 2015; Pattan and Kaul 2014; Zhao and Castranova 2011). Furthermore, there is historical information available from the discipline of fiber and particle toxicology such as asbestos, quartz, urban particulate matter and ultrafine particles (carbon, TiO_2)) that may be used to guide experimental design of hazard investigations for NM to inform which biological responses are most important, as well as signifying what physicochemical properties of NM may drive their toxicity and should be used to identify the potential consequences for human health (Donaldson and Poland 2013a; Donaldson and Seaton 2012; Tran et al. 2011). However, there are relatively few epidemiology or human volunteer studies which have directly assessed the outcome of engineered NM exposure on health. As such, hazard assessment of NM has relied on the use of a wide range of

in vitro and *in vivo* models in order to predict the potential health outcomes (Snyder-Talkington et al. 2012). Although standard methods exist for hazard and risk analysis for chemicals, the suitability of these tools for NM needs to be evaluated and adapted, where appropriate. In addition, development of alternative testing systems that are less reliant on animal testing are required due to the vast array of NM under development and use (Snyder-Talkington et al. 2012; Stone et al. 2014). There is an urgent need to develop reliable, predictive approaches for NM risk assessment to be integrated into a coherent strategy both for the regulators and industry (Stone et al. 2014).

The overall aim of the European Commission Framework Programme (FP7) funded Risk Assessment of Engineered Nanoparticles (ENPRA - NMP4-SL-2009-228789) project (2008-2012) was to develop and validate methods for the assessment of NM hazard to human health. The project aimed to implement an integrated approach for NM risk assessment by utilization of an exposure-dose-response approach for NM (Figure 1). This approach is principled on the identification of human exposure routes including inhalation, ingestion or dermal to NM with different physicochemical characteristics which is likely to lead to distinct bio-kinetics and translocation to different body systems. The accumulation of NM in the exposure site and distal secondary target organs might result in the manifestation of effects in a dose-related manner. Thus local and systemic toxicity of NM was assessed in the project.

The objectives of the ENPRA project were to: (1) analyze the physicochemical characteristics of a panel of 10 commercially available engineered NM; (2) assess hazards of NMs by means of *in vitro* toxicology testing with cells sourced from 5 important body systems including pulmonary, cardiovascular, hepatic, renal and developmental systems and investigate 5 potential mechanisms of adverse health effects including cytotoxicity, oxidative stress, inflammation and immune responses, genotoxicity and fibrogenicity (where applicable); (3) verification of *in vitro* findings with pertinent *in vivo* testing (acute 24 hr intratracheal (IT) exposure of mice); (4) use obtained data from the

project in an exposure-dose-response relationship paradigm by means of mathematical modeling; (5) develop and implement a strategy for dissemination of findings within the project in relation to appropriate risk management actions.

The main findings from ENPRA are summarized within this review and encompass findings from studies on NM, which were prepared in a liquid vehicle and protocol developed for the project. Most of the NM tested in the project were obtained from the Joint Research Centre (JRC) repository and tested in diverse research projects to assess NM hazard, at an international level (e.g. EU funded project Nanogenotox, Inlivetox). However, it should be stated that the toxicological effects of materials investigated in laboratories not part of the ENPRA consortium are not discussed within the context of the review mainly due to the differences in applied dispersion protocols may affect data generated. From the lessons learned from the project a number of issues related to the hazard assessment of NM will receive special attention including: (1) selection of appropriate NM for nanotoxicology investigations; (2) characterization and dispersion of NM for hazard assessment; (3) use of appropriate doses for all related investigations; (4) the need for the correct choice of experimental models and end-points for risk assessment; (5) full understanding of the test systems and correct interpretation of data; (6) need for distinction and understanding of differences between data generated in *in vitro* and *in vivo* systems; (7) current problems with NM risk assessment.

The overall aim of the review is to offer recommendations based on highlights and shortcomings of the project that might help provide solutions to challenges which exist in the field of nanotoxicology, in particular with respect to hazard assessment. This is made especially relevant by the current drive (Krug 2014) to achieve a harmonized approach for testing the safety of NM and provide understanding of what this testing strategy should encompass.

Panel of NM in ENPRA

It is not possible to test the safety of all engineered NM; and consequently within hazard investigations a panel of 'representative' NM is often selected. Within ENPRA, the NM were purchased as follows: NM 101 (Hombikat UV100; titanium dioxide (TiO_2), rutile with minor anatase; 7 nm), NM 110 (BASF Z-Cote; non-functionalized zinc oxide (ZnO), 100 nm), NM 111 (BASF Z-Cote; ZnO coated with triethoxycaprylylsilane, 130 nm), NM 300 (RAS GmbH; silver (Ag) capped with polyoxylaurat Tween 20, < 20 nm), NM 400 (Nanocyl; entangled multi-walled carbon nanotube (MWCNT), diameter 30 nm) and NM 402 (Arkema Graphistrength C100; entangled MWCNT, diameter 30 nm). The above mentioned NM were sub-sampled under Good Laboratory Practice conditions and preserved under argon in the dark until use. The NRCWE samples were procured by the National Research Centre for the Working Environment (Copenhagen, Denmark). The NRCWE 001 NM (TiO_2 rutile 10 nm) was purchased from NanoAmor (Houston, USA) and used for production of NRCWE 002 (TiO_2 rutile 10 nm with a positive charge) and NRCWE 003 (TiO_2 rutile 10 nm with a negative charge) (Kermanizadeh et al. 2013a). The NRCWE 004 (TiO_2 rutile 94 nm) was procured from NaBond (Hong Kong, China).

The selection of such a diverse panel of particulate and fibrous NM was justified in order to capture a range of materials that have commercial use, which might result in human exposure and in order to investigate specific hypotheses relating to relationship between NM physicochemical properties and their toxicity. Beyond industrial relevance, the selection criteria of the tested NM were representative of materials with: (1) different chemical composition; (2) varying crystal structures with the same composition; (3) differing primary material size with the same composition; (4) varying surface charges and same core material; (5) different engineered surface coatings with the same core material; (6) materials with differing solubility; (7) NM which could be dispersed in a liquid medium for *in vitro* and *in vivo* testing. In addition, ENPRA aimed to support

the test program under the OECD Working Party on Manufactured NM. Therefore, 6 of the NM were selected from the so-called JRC NM repository (NM 101, NM 110, NM 111, NM 300, NM 400 and NM 402) (JRC nanomaterials repository 2015).

Consequently, the 5 TiO_2 samples comprised a test matrix of small and large nano-sized insoluble NM of the same chemical composition (small NM of anatase and rutile and expected differences of catalytic properties and small nano rutile covalently functionalized to have either a negative or positive surface charge). The role of surface coating was investigated by utilization of the 2 ZnO and Ag NM. The two MWCNT selected were found to have relatively similar dimensions although the manufacturer identified them as having different sizes (with NM 402 supposedly 4-fold longer than NM 400).

TiO_2, ZnO and Ag are currently amongst the most abundantly used engineered NM, as they are integrated into many different products including food additives, paints, sunscreens, pesticides, products with an anti-bacterial coating and photocatalytic cleaning products (CPI 2015). MWCNT have numerous potential applications and are on the way to becoming a "high production volume chemical". In addition, sports goods, electronics, nano-composites, epoxy paints and textiles with carbon nanotubes (CNT) incorporated are already available on the market (CPI 2015). In general, there is a lack of information regarding the level of human exposure to such materials, however some data exists. For example, dietary exposure to TiO_2 (Weir et al. 2012) and exposure to Ag utilized in textiles and wet aerosol disinfectants (Benn and Westerhoff 2008; Roberts et al. 2013) has been quantified.

NM handling and dispersion

The Ag NM utilized in the ENPRA project was supplied in de-ionized water (85%) with 7% stabilizing agent (ammonium nitrate) and 8% emulsifiers (4% each of polyoxyethylene glycerol trioleate and Tween 20). The dispersant, which is commercially available, was also tested in the

project for potential toxicological effects. Testing the toxicity of NM dispersants is critical in the discipline of nanotoxicology to distinguish between material and dispersant effects. All other materials were supplied as dry powders. It is known that the dispersion protocol used can impact NM toxicity, and thus prior to hazard studies a common approach (across laboratories) to the dispersion of ENPRA NM was developed. The NM were dispersed in MilliQ de-ionised water supplemented with 2% fetal calf serum (FCS). For ZnO, materials were wetted with 0.5% vol ethanol predominately to achieve a good suspension for the material with a hydrophobic coating (NM 111). In addition, ZnO without a hydrophobic coating (NM 110) was treated similarly for direct comparison. The NM were sonicated for 16 min without pause following the protocol developed for the project (Jacobsen et al. 2010). Following the sonication step, all samples were immediately transferred to ice and utilized within 1 hr (optimal material size distribution and stability in the suspensions).

In the *in vivo* experiments, the vehicle control mice received the dispersant of NM only. For all materials with the exception of Ag and ZnO NM, this was MilliQ water with 2% mouse serum. For Ag NM, the commercially available dispersant was utilized (Mercator nano) (Moscow, Russia). Finally, for ZnO MilliQ water with 0.5% ethanol and 2% mouse serum was used.

It has been habitually difficult to disperse and stabilize all types of NM in one physiologically relevant media. In general, when dispersed in aqueous media NM are likely to agglomerate. For example, hydrophobic materials are not readily dispersed in saline media. It is also often the case that NM may initially be well-dispersed before agglomeration and settlement to the bottom over time. Thus a number of approaches have been employed to improve dispersion of NM, including but not limited to; sonication, inclusion of solvents, stirring and addition of proteins such as lung lining fluid, serum and albumin. However the best approach to dispersion of NM in aqueous media is still widely debated as a sufficient stability is perquisite to ensure reliable dosing and correct

interpretation of data. There is often a compromise that needs to be made between generating a NM suspension that mimics real-life conditions and production of a mono-dispersed suspension of NM. Finally, a consistent approach to dispersion of NM is critical in order to enable comparisons to be made between different material-induced effects. As touched upon, it has also been demonstrated that inclusions of different biological molecules such as proteins modify NM toxicity to mammalian cells *in vitro* (Brown et al. 2014; Foucaud et al. 2007). In ENPRA, the development of the dispersion protocol allowed for NM to be dispersed in a format biologically acceptable for most bioassays and ensured stability of the dispersion for approximately 1 hr with re-dispersion easily achieved by simple vortex shaking. This dispersion protocol has been utilized in numerous European projects since including managing risks of nanomaterials (Marina 2015) and mitigation of risk and control of exposure in nanotechnology based inks and pigments (Nanomicex 2015).

NM characterization

The complete characterization of NM is crucial for interpretation and relevance of toxicological data (Silva et al. 2013). In ENPRA NM size, morphology, crystal structure, surface area, hydrodynamic diameter, zeta potential and elemental composition (purity) for NM in their 'as supplied' form and when dispersed in biological media was assessed. The phase compositions and average crystallite sizes were determined from powder. The primary and aggregate size range, shape and crystal structure of the test materials were determined by transmission electron microscopy. Finally, the surface areas and pore volumes were obtained. In addition, the hydrodynamic size distributions of NM dispersed in different biological media were determined in the 16-128 µg/ml concentration range by individual project partners. The complete list of investigated NM in the project including information provided by the suppliers and comprehensive characterization data is reproduced from previously published findings (Table 1) (Kermanizadeh et al. 2013a).

The main findings from the ENPRA project

In vitro models

All NM were investigated in two-fold serial dilutions ranging from 0.31-80 µg/cm^2 (corresponding up to 1-256 µg/ml). At the time of the initiation of the project there was little access to relevant exposure concentrations, or deposition rates on which the range could be based upon. It was envisaged that the concentration range could be useful in categorization of the relative toxicity of the different NM. Although the top concentrations in the range are high and may have little physiological or toxicological relevance in terms of most occupational or environmental exposures, they might be pertinent for medical/homeopathic exposures. In addition, the use of a wide range of concentrations allowed for the generation of values such as NOEL, BMD_{20}, LC_{50}, etc. A summary of the *in vitro* data generated in the ENPRA project is presented in part in Figures 2, 3 and 4 and Table 2 and described in more detail below. A comprehensive description of all *in vitro* experiments is provided as supplementary information.

Pulmonary system

The overall objective of the investigation was to assess the responses of lung derived cells to the panel of NM in terms of cytotoxicity, generation of reactive oxygen species (ROS) and related induction of oxidative stress, inflammatory responses and genotoxicity. The lung epithelial cells are the first target cells after inhalation either at the bronchial or alveolar level (Oberdorster et al. 2015). Alveolar macrophages were also considered due to their importance in host immune response, and known contribution to material toxicity in the lungs (Braydich-Stolle et al. 2014; Yuta et al. 2014).

The 10 different NM were assessed for their influence on a variety of *in vitro* models relevant to the respiratory system (A549 - human alveolar epithelial cells, NCI-H292 - human bronchial epithelial cells, 16HBE - human bronchial epithelial cells, LA-4 - murine lung epithelial cells, MHS - mouse alveolar macrophage cells, murine primary lung fibroblasts and co-cultures of A549 and

human primary neutrophils). Initially all materials were tested for cytotoxicity (WST-1 and lactate dehydrogenase (LDH) activity) over a wide range of concentrations. Data demonstrated that the materials could be divided into two categories: a low toxicity (TiO_2 and MWCNT) and a higher toxicity (Ag and ZnO) group. The cytotoxicity data related reliably to the ability of NM to induce oxidative stress in cells. The only exception to this was the observation of cytotoxicity induced by 1 MWCNT (NM 402) in macrophages. These findings suggest that phagocytic uptake related cell damage is not necessarily required for the MWCNT induced inflammatory stimulation, but might be related to cell death for the particular MWCNT. In contrast, MWCNT increased viability in fibroblasts which indicated that the materials stimulate cell activity or proliferation and that this effect is specific to structural cells (fibroblasts) (Vietti et al. 2013). The Ag NM generated the highest levels of ROS production followed by ZnO NM. The ability of TiO_2 to produce ROS varied between different forms, while MWCNT exhibited no or low ROS production. With respect to the pro-inflammatory effects of the NM, Ag and ZnO were again relatively potent at inducing cytokines such as tumor necrosis factor (TNF)-α by macrophages, interleukin (IL) 6 by lung epithelial cells and the major neutrophil-recruiting chemokine IL8 by lung epithelial cells. NM induced secretion of IL6, IL8 and TNF-α from pulmonary cell lines was also reported previously (Dekali et al. 2013; Lee et al. 2015; Napierska et al. 2012; Urisini et al. 2014). The impact of TiO_2 NM exposure on cytokine production was lower and/or less consistent. The MWCNT did not trigger IL8 release by bronchial epithelial cells. In addition, 10 out of 11 pro-inflammatory cytokines/chemokine (granulocyte-colony stimulating factor (GCSF), IL12, IL13, IL1β, IL4, IL6, chemokine C-X-C motif ligand 1 (CXCL1), chemokine C-C motif ligand 2 (CCL2), CCL3, CCL5 and TNF-α) investigated were not increased above control levels following exposure of alveolar epithelial or alveolar macrophages. Only GCSF levels were elevated in a concentration-dependent manner for NM 402 MWCNT from macrophages. The strongest DNA damaging effect on the lung epithelial

cells, assessed by alkaline comet assay, was observed with Ag NM, but these effects may be due to cytotoxicity as concurrently observed for these NM. For two out of 5 TiO_2 samples (NM 101 and NRCWE 004), DNA strand breakage was detected in the absence of cytotoxicity, indicating that specific NM properties may be involved. However, overall NM induced genotoxic effects were negligible in these studies.

Cardiovascular system

The cardiovascular system is considered as one of the main targets for adverse effects due to similarities of NM to ambient air particles. The latter were, implicated in increased risk of a number of undesirable health outcomes (Chen et al. 2015; Chiu et al. 2014; Delfino et al. 2005; Miller et al. 2014; Snow et al. 2014). The mechanisms underlying these observed effects are still not fully understood. Nevertheless, oxidative stress and inflammation have been highlighted as being key players in cardiovascular particle-induced adverse effects (Brocato et al. 2014; Brook and Rajagopalan 2010; Du et al. 2013). This section of investigation aimed to offer some clarification on the mechanisms involved in NM-induced cardiovascular complications.

The findings demonstrated that for all cell types (EAHY926 - human endothelial cells, HUVEC - human umbilical vein endothelial cells and THP-1 - human monocytic cells) Ag and ZnO NM were most cytotoxic. In general, TiO_2 and MWCNT showed low (or no) toxicity and did not reach LC_{50} values. The Ag and MWCNT increased levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), whereas only MWCNT elevated intracellular ROS-production in HUVEC and THP-1 cells. The inflammatory mediator TNF-α was produced by activated macrophages following exposure to TiO_2, but not the MWCNT or ZnO NM. Additionally, levels of IL6 were significantly higher after exposure to TiO_2 and Ag NM. Finally, the large rutile TiO_2 (NRCWE 004) was the most potent in the up-regulation of *Heme oxygenase 1* and *TNF-α* (Cao et al. 2014; Danielson et al. 2014).

Hepatic system

It is well-known that NM in blood eventually reach the liver in comparatively large quantities (Almeida et al. 2011; Balasubramanian et al. 2013; Kreyling et al. 2014; Sadauskas et al. 2009). For blood borne materials the phagocytic Kupffer cells (KCs) (hepatic resident macrophages) might be a key clearance system with subsequent potential for accumulation of dose in the liver (Chen et al. 2012; Liu et al. 2014). The *in vitro* hepatic section of the project concentrated on adverse effects of NM by investigating: (1) hepatocyte monocultures - cytotoxicity, inflammatory response, function markers, oxidative stress and genotoxicity; (2) primary human hepatocytes vs. hepatic cell line: suitability of both models for *in vitro* nanotoxicology; (3) co-culture of primary rat liver cells (hepatocytes and KCs); (4) 3 dimensional (3D) human liver microtissue.

The Ag NM followed by the two ZnO NM elicited the greatest cytotoxicity to hepatocyte cultures (all models). The LC_{50} was not attained in the presence of any of the other NM (up to 80 $\mu g/cm^2$). A concentration-dependent decrease in cellular total glutathione (GSH) content occurred following exposure of C3A cells to Ag, ZnO and MWCNT. Intracellular ROS levels were also measured and shown to increase significantly following exposure of C3A to low toxicity NM (MWCNT and TiO_2). All NM significantly increased IL8 production by the cell line. Meanwhile no significant change in TNF-α, IL6 or C-reactive protein was detected. The anti-oxidant Trolox in part prevented the detrimental effect of NM on cell viability and decreased NM-induced IL8 production after exposure to all but the Ag particulate. Following 4 hr exposure of C3A cells to sub-lethal levels of the NM, the greatest extent of DNA damage was induced by 2 of the TiO_2 samples (NM 101 and NRCWE 002). Concurrently, in HepG2 cell line, 2 TiO_2 NM (NM 101 and NRCWE 004) induced DNA damage at non-cytotoxic concentrations. The C3A cell line was demonstrated to be a reliable model for assessing NM-induced hepatotoxicity *in vitro* when compared to primary human and primary rat hepatocytes. Finally, multiple exposures to low concentrations of NM

resulted in enhanced cytotoxicity/tissue damage over time in a 3D human hepatocyte model (Kermanizadeh et al. 2012; 2013a; 2013b; 2014a).

Renal system

Some NMs can translocate to secondary target organs from the primary source of exposure one of which is the kidneys (Li *et al*, 2014b). The kidneys are principally responsible for removal of metabolic waste and filter approximately 1.2 L blood per min, around 25% of the cardiac output, making them a potential major target for any blood-borne materials. Proximal tubule cells are characterized by well-developed basal infolding and an apical brush border with the ability for intense pinocytic activity and variable transport and co-transport of materials. Therefore, these cells are an ideal candidate for nanotoxicological investigations.

The two ZnO and Ag NM were highly toxic to proximal tubule cells. The LC_{50} was not attained in the presence of any of the other engineered materials up to 80 $\mu g/cm^2$. All NM significantly increased IL8 and IL6 production in exposed renal cells. In addition, no significant change in TNF-α or CCL2 was detectable. There was a significant rise in ROS following 24 hr exposure with Ag and two ZnO NM (sub-lethal concentrations) while no marked change was observed with any of the other NM investigated. Finally, genotoxicity measured at sub-lethal concentrations displayed significant elevation in DNA damage following exposure to 7 of 10 NM investigated (coated ZnO and 2 of the TiO_2 (NRCWE 001 and NRCWE 003) being the exceptions) (Kermanizadeh et al. 2013c). Interestingly, in a second set of experiments DNA damaging effect were only noted with TiO_2 (NM 101) at sub-lethal concentrations in HK-2 cells. These differences might be due to varying cell batches/passages and culture media used in the different laboratories.

Developmental toxicity

The effect of the panel of the NM on the development of contracting myocardiocytes was evaluated using the embryonic stem cell test (EST - inhibition of differentiation of stem cells into

contracting cardiomyocytes). Furthermore, the effects of NM exposure on neural differentiation of ES cells was determined. Incubation of the cells for 10 days with 5 TiO_2 NM suggested that cytotoxicity execrated these materials was relatively small. It was noted that only exposure to the higher concentrations the TiO_2 NM induced an inhibition of the development of contracting cardiomyocytes. In addition, treatment with MWCNT (NM 400) did not produce a significant cytotoxic effect. The Ag NM was found to be highly cytotoxic. Moreover, the two ZnO NM were also highly cytotoxic albeit at lower levels in comparison to Ag NM. Therefore, it was concluded that any inhibition observed in the EST may be attributed to general cellular toxicity and not a specific developmental toxic effect of NM (unpublished data).

Round robin testing

In the ENPRA project, cytotoxicity assessment of the non-functionalized ZnO NM (NM 110) to A549 cells as measured via the WST-1 assay was selected for round robin testing by 9 of the partners in the consortium. The NM were dispersed utilizing the ENPRA dispersion protocol in a water vehicle with or without 2% FCS supplementation (Figures 5). The cells were cultured with complete medium with 10% FCS during the 24 hr NM exposure. The partners utilized cells with different passage numbers but the cells per well was consistent for all round robin experiments.

The data showed that there were no significant differences in cytotoxicity following dispersion in presence or absence of serum. All partners demonstrated cytotoxicity of ZnO NM with a steep concentration response at low exposure levels. However, the steepness of the curve made it difficult to interpret differences between laboratories. Only one partner did not note a 100% cytotoxicity at the high exposures concentrations (the reasons for this is not entirely clear). Three of the partners found greater mitochondrial activity at the lowest concentrations indicating that ZnO NM were less toxic to A549 cells (unpublished data). These alterations may potentially be due to differences in handling of NM between groups or variations between cell batches used by the different partners.

In vivo **data**

The *in vivo* part of the ENPRA project aimed to validate and verify *in vitro* findings discussed above in a healthy and a compromised Apolipoprotein E knockout (*ApoE*$^{-/-}$) mouse model. This section is broken down into data generated from healthy animals and the disease model separately.

Healthy mice

In these experiments female C57BL/6 mice (10 week old) were exposed to the panel of NM via IT instillation (0, 1, 4, 8, 16, 32, 64 and 128 µg/mouse) for 24 hr unless otherwise stated. For the instillation the highest dose of 128 µg/mouse was selected based on previous inhalation and instillation studies (Dybdahl et al. 2004; Saber et al. 2006). The doses reflect pulmonary deposition in mice equivalent to 1-10 working days of 8 hr at the Danish occupational exposure limit of 3.5 mg/m^3 for carbon black (Poulsen et al. 2015). In addition, the use of dose-range allowed for PROAST analysis with priority firmly based on the lower doses that would allow for comparisons and calculations of doses related to relevant physiological human exposure. In an adjacent study, the effects of NM exposure on liver following intravenous (IV) exposure of C57BL/6 mice were investigated. The animals were exposed to either a single dose of NM (128 µg/animal) or three doses of (64 µg/animal) every 24 hr, before dissection 6, 24, 48 and 72 hr after the single IV exposure or 72 hr after the triple injection regime. All experiments were ethically approved by either an independent ethical committee at the National Institute of Public Health and the Environment (RIVM) or performed under a project license and personal license issued by the UK Home Office.

Pulmonary system

NM-induced effects were measured as LDH activity and cytokine/chemokine levels (markers of cytotoxicity) and inflammation in the supernatant of the broncho-alveolar lavage fluid (BALF) of exposed animals. Elevated LDH activity and protein levels were detected after administration of 2

ZnO and 1 TiO_2 NM (NRCWE 001). Next, the total number of cells in the BALF was investigated displaying a significant dose-dependent increase in total number of neutrophils following administration of the ZnO NM. This was accompanied by an increase in total number of macrophages following exposure to high doses of ZnO NM. There was no other significant change in total cell counts following exposure to the other 7 NM. The strongest inflammatory response was noted after exposure to the 2 ZnO NM with an up-regulation of IL6, IL12, GCSF, CXCL1 and CCL2 (Gosens et al. 2015). The comet assay demonstrated dose-dependent DNA damage after treatment of NM 402 MWCNT. Finally, in an independent set of studies C57BL/6 mice exposed to 2 MWCNT and assessed after 8 week noted an induction of lung fibrosis in the exposed animals (Vietti et al. 2013).

Cardiovascular system

The acute (24 hr) toxic effects in blood parameters after IT NM exposure were investigated by analysis of changes in the number of neutrophils, lymphocytes, red blood cells and platelets, as well as the hemoglobin content. For the non-functionalized ZnO, an increase in hemoglobin content and number of red blood cells and platelets was observed, while exposure to coated ZnO NM resulted in a rise in absolute number of neutrophils and decrease in absolute lymphocyte numbers (unpublished data).

Hepatic system

Total liver weight was measured 24 hr post IT NM administration. First, a significant dose-dependent decrease in liver weight was noted after treatment with ZnO, Ag, TiO_2 (NRCWE 002 and NRCWE 003) and 1 MWCNT (NM 402). The instillation of Ag resulted in high 8-isoprostane values in the organ indicating hepatic lipid peroxidation. In addition, a significant dose-dependent reduction in GSH levels were detected after exposure to ZnO and Ag NM (Ag was the most potent in reducing GSH levels). The Ag induced GSH depletion was linked to the presence of NM/ions in

the organ itself (high resolution inductively coupled plasma mass spectrometry) (Gosens et al. 2015).

A wide array of NM (non-functionalized ZnO, 1 MWCNT (NM 400) and 1 TiO_2 (NRCWE 002)) induced a neutrophil influx into the liver as early as 6 hr post IV exposure. It was noted that neutrophils were only involved in the initial phases of the immune response against the NM. The analysis of mRNA expression in mouse livers showed alterations in levels of *C3*, *IL6*, *IL10*, *CXCL2* and *ICAM-1* most notable for the ZnO and Ag. Overall, data suggested that low doses of NM were not sufficient to produce any chronic inflammation in the hepatic tissue (Kermanizadeh et al. 2013c).

Finally, the role of KCs in the overall inflammatory response in the organ was assessed following IV exposure to Ag NM. The cytokine expression in the normal liver was measured post NM treatment in terms of IL2, IL4, TNF-α, IFN-γ and IL10 released from the organ, with significant up-regulation of TNF-α and IL10. For livers in which the macrophage population was specifically targeted and destroyed this cytokine profile was significantly decreased. The findings indicated a potentially important role for KCs in the anti-inflammatory response and suggested that tolerance to the Ag NM is favored over a fully activated immune response (Kermanizadeh et al. 2014b).

Renal system

There were no acute biological relevant changes in relative and absolute kidney weight detected after administration of the full panel of NM (unpublished data).

Disease model - *ApoE*$^{-/-}$ mice

It is widely believed that pulmonary inflammation can be a driving factor for systemic cardiovascular complications; therefore, progression rates of atherosclerotic plaques in the aorta of MWCNT exposed *ApoE* KO mice on a high fat diet was investigated. These mice are hyper-

lipidemic and susceptible to development of atherosclerotic plaques, with marked similarities to humans. Previous studies have demonstrated the progression of atherosclerosis following exposure to combustion derived particles; with fine and ultrafine (nano-sized) particulate fraction clearly drive these effects (Møller et al. 2011; Suwa et al. 2002). In contradiction, in a recent investigation MWCNT exposure resulted in local airway inflammation but failed to augment atherosclerosis in female *ApoE*$^{-/-}$ mice (Han et al. 2015). The 2 MWCNT were chosen as it had been previously shown that CNT exposure promoted plaque progression in atherosclerosis-prone *ApoE*$^{-/-}$ mice (Li et al. 2007). In this series of experiments, the animals were exposed by IT on 5 instances (128 µg/mouse) over a period of 28 days.

Pulmonary effects

The cell counts in BALF at 24 hr after the last of 5 IT treatments of *ApoE*$^{-/-}$ mice showed an increased influx of neutrophils and a reduction in number of lymphocytes. The number of macrophages and epithelial cells were unaltered by MWCNT exposure. The influx of neutrophils was not higher in *ApoE* KO mice compared with wild type at 24 hr or day 28 after the last IT. The data indicated that multiple IT administration produced a more severe inflammatory response. The cytokine release (IL6) was higher following exposure to NM 400 in the wild type and *ApoE*$^{-/-}$ mice. The cytokine levels in BALF at day 28 after the last IT showed higher levels of IL1-β, IL6, CXCL1, CCL2, CCL4 and CCL5 in *ApoE*$^{-/-}$ mice that were exposed to NM 400 compared with NM 402. The expression of genes relating to cellular adhesion (*Vcam1*), growth factor (*Vegf*), inflammation (*Ccl2* and *Nos2*), oxidative stress (*Hmox1*) and DNA repair (*Ogg1*) were significantly up-regulated in lung tissue of *ApoE*$^{-/-}$ mice exposed to the MWCNT. In addition, exposure to NM 402 was associated with a significant up-regulation of mRNA in lungs. DNA damage as measured by the comet assay, showed increased levels of strand breaks in lung tissue, whereas there were unaltered

levels of oxidatively generated DNA lesions (potentially related to OGG1-mediated repair of oxidized purine lesions in DNA) (Cao et al. 2014).

Cardiovascular effects

Aortas from both MWCNT exposed mice displayed a 2-fold elevation in plaque area compared to vehicle exposed mice 24 hr after last exposure. Likewise, exposure to the MWCNT was associated with increased levels of CXCL1 in serum at 24 hr after the 5 IT treatments in *ApoE*$^{-/-}$ mice (Cao et al. 2014). A summary of *in vivo* data generated in the ENPRA project (sub-categorized for each target system) is presented in Table 3.

Discussion

There are currently thousands of different NM available, with more generated on a daily basis, which is far too many to be tested on a case-by-case basis due to ethical and financial constraints. Instead there is the need for development of an extensive knowledge base which can be used to predict NM toxicity. Current research aims to generate computer based mathematical models that will predict toxicity from the known physical and chemical characteristics, also known as a 'structure activity relationship', as is currently in place for pharmaceutical agents, with ENPRA one of the projects which aimed to contribute to this overall objective.

In vitro

ENPRA aimed to develop *in vitro* test models to assess the toxicity of the panel of NM. The studies in this section were broken into three phases: (1) assessment of cytotoxicity over a range of concentrations for all 10 NM in each model. This information would then enable ranking of toxicity of NM and prioritization of certain materials for *in vivo* testing (disease models and multiple dosing regimens); (2) investigation of mechanisms underlying toxicity; (3) assessment of reliability and reproducibility of the protocol to determine cell death induced by NM across different laboratories. The first phase of the *in vitro* work package identified that the panel of NMs can be divided into

categories of highly toxic (Ag and ZnO NM) and a lower toxicity group (TiO_2 and MWCNT) with a relatively similar ranking over 16 different *in vitro* systems used (Figure 2). This in itself was interesting as different partners in the consortium utilized differing culture conditions (cell types, cell numbers, exposure periods and culture media), although the dispersion protocol was standardized. An investigation into solubility of materials identified the ZnO NM to be highly soluble (50-60%) while there was less than 1% solubility for Ag NM (in water and complete cell culture medium) under the same conditions (Kermanizadeh et al. 2013a). This indicated that solubility and generation of Ag ions was less important for these particular NM in these experiments (at least in the *in vitro* models) which is not always the case as ions have been heavily implicated in toxicity of soluble NM (Aldieri et al. 2013; Hirn et al. 2014; Yuta et al. 2014). This being said, the solubility status of Ag NM *in vivo* might be more complex. It has been demonstrated that these NM are likely to be oxidized in physiological environments (Jacobsen et al. 2009). On the same theme, the formation of AgCl is another possibility depending on the locality of the Ag NM *in vivo* (i.e. intestines) (Loeschner et al. 2011; van der Zande et al. 2012).

As it has been shown that proteins, lipids and DNA are all highly susceptible to damage from ROS, combined with the fact that these species are generated by a wide range of NM - this end-point was scrutinized in the ENPRA project. The presence of high ROS levels forces the cells to activate defense mechanisms, which vary with degrees of severity leading to a wide range of outcomes from cell recovery to disease progression and eventual cell death (Kermanizadeh et al. 2015a; Møller et al. 2015a; Unfried et al. 2007). The cytotoxicity data correlated well with the ability of NM to induce oxidative stress in some cell models. The highly toxic NM were most effective at inducing production of ROS in lung and kidney cells, while measurement of anti-oxidant levels also identified ZnO NM to be efficient at initiating oxidative stress in pulmonary cells. Ag and ZnO NM also induced anti-oxidant depletion in liver cells. In addition, anti-oxidant

pre-treatment partially prevented hepatic cell death and chemokine secretion. In contrast, in endothelial cells up-regulation of cell surface adhesion molecules was not oxidative stress dependent which suggested that while oxidative stress plays a role in the regulation of inflammatory responses, it is not always the mechanism involved in NM-induced cellular responses. The assessment of oxidative stress was somewhat complicated however, as the physical (MWCNT) and highly toxic (Ag, ZnO) nature of some materials interfered with the reliability of some data generated (the interference of NM with biochemical assays is not a novel observation (Ong et al. 2014; Toutnebize et al. 2013)). Finally, MWCNT did not trigger a pro-inflammatory response in some cell types but did in others suggesting a cell specific response.

As for the assessment of the reproducibility of *in vitro* data, improvement of future round robins might entail more than simple circulation of a protocol (for better compliance of the generated data between groups). For example, the circulation of the same batch of cells and testing materials might be advantageous. It might be beneficial to inaugurate workshops with partners working on the same protocol in the same location. In addition, it seems appropriate that more than one end-point are investigated to ensure that the same data can be generated by different people when the same experimental protocols are followed.

In conclusion, some remarkable comparability was achieved across a range of cell types from the lung, blood (including immune cells), liver and kidneys in terms of their *in vitro* susceptibility to adverse effects of the ENPRA NM. In terms of sub-lethal effects, all materials were also able to produce significant responses (i.e. oxidative stress) highlighting the importance of the use of low concentrations and multiple end-points.

In vivo

It is believed some NM penetrate the lung epithelium and enter the blood stream (Kreyling et al. 2002; 2014; Semmler-Behnke et al. 2014). This is of critical importance as it offers a route of

NM/systemic adverse effects in target sites that are not initially exposed to the NM. Therefore, translocation of NM across the air-blood barrier was investigated in terms of accumulation in secondary organs utilizing Au and TiO_2 NM. The administration of the materials via IT exposure demonstrated that the amounts of NM reaching blood are strongly size dependent (Kreyling et al. 2002). The TiO_2 NM (20 nm anatase) was bio-persistent in lungs and secondary organs for up to 4 weeks (Hirn et al. 2011a; 2011b). After direct injection into the blood stream a large majority of the NM accumulated in the liver and the spleen (Choi et al. 2015; Hirn et al. 2011b; Lipka et al. 2010).

With regards to toxicity, it was noted that two ZnO NM were the most harmful in terms of acute inflammation and lung damage. The ZnO NM also produced a reduction in spleen weight of exposed animals and a concurrent increase in erythrocytes numbers and hemoglobin content. The data here, suggested a compensatory mechanism for the onset of ZnO NM induced anemia. The MWCNT exposure resulted in a lower acute inflammatory potential (compared to ZnO); however, two months after a single exposure of MWCNT a dose-dependent lung inflammation and fibrosis in wild type mice occurred. The positively charged TiO_2 NM (NRCWE 002) produced a mild inflammatory response and some lung cell damage (lesser extent compared to the ZnO NM). The uncharged TiO_2 NM (NRCWE 001) induced cell damage without inflammation, while, no adverse effects was observed for the other TiO_2 NM. Interestingly, Ag NM exerted minimal effect *in vivo*; despite being highly toxic *in vitro*. The reasons for this disparity are not fully understood, but might be related to a difference in cellular uptake and/or solubility *in vitro* and *in vivo*. Other NM-induced systemic responses included GSH depletion and alterations in hepatic gene expression. Finally, aortas of MWCNT exposed *ApoE*$^{-/-}$ mice displayed a 2-fold increase in plaque area compared to unexposed control animals.

For comparison between *in vitro* and *in vivo* data the bench mark dose (BMD) was calculated for some of the comparable end-points (dose/concentration for which a response of 10% or greater was

noted) (PROAST software). The *in vitro* models correctly predicted the magnitude of liver oxidative stress response to the Ag, ZnO and most of the TiO_2 NM (7 out of 10 NM). In contrast one TiO_2 (positively charged) induced a greater oxidative stress in rodent livers while MWCNT produced less oxidative stress compared to the predicted response from *in vitro* data.

ENPRA data in a structure-activity and PBPK modeling

The relationships between the physicochemical properties of NM and their fate and effects in the environment and biological systems provide the basis for structure-activity modeling. These models may include both qualitative and quantitative structure-activity relationships (SAR) that make qualitative (e.g. the potential for oxidative stress) or quantitative predictions (e.g. cytotoxic potency), depending upon the data and modeling approach which provides an efficient and cost-effective means of prioritizing toxicological testing and also for filling data gaps in hazard and risk assessment. The development of SAR models requires three components: (1) data sets providing a measure of the toxicity of a group of NM; (2) molecular structure and/or property data of NM that are used as descriptors and (3) a model that can relate descriptors to the toxicological effect of interest. Although the SAR paradigm is well established in the areas of drug discovery and risk assessment of small molecules, its application in modeling behavior of NM is still in development. Hence, ENPRA included exploratory analysis on the development of SAR for the panel of NM. Such an approach might hopefully in time help in identification of structural parameters of NM which are most crucial in SAR as well as informing on the most relevant end-points which need to be measured and eventual appropriate standardization of methodologies.

Furthermore, the data generated was utilized to develop a physiologically based pharmacokinetic (PBPK) model which predicts the movement of materials throughout the body after exposure and ultimately predicts organ burdens over time. Within ENPRA the PBPK model was parameterized using *in vivo* kinetics data following inhalation (obtained from data out with the ENPRA

consortium), IT and IV exposures. Each exposure route resulted in a different set of optimal parameter estimates due to the differences in the portal of entry and the rate at which materials translocate to secondary organs. Further work involved the development of a nano-specific model of occupational exposure which was set up to estimate the size-resolved concentrations after emission from a source while accounting for, agglomeration, dispersion, diffusion and deposition of the different NM. It is hoped that in time this model will allow for the predication of size-dependent exposure concentrations associated with occupational settings.

The combination of the occupational exposure model and PBPK models can allow for an estimation of personal exposure, given specified conditions and the organ burdens that would be expected given these exposure concentrations. The modeling of toxicity data could also be used to estimate the expected toxicity in specific organs (based on the predication of accumulated dose by the PBPK model) to complete the exposure-dose-response chain. This process could also be established in reverse to estimate the doses of NM required for the development of hazardous outcomes in occupational exposure settings.

Within ENPRA the models were developed and combined as outlined although the lack of additional data available at the time meant that it was not possible to test how well the model predicted for materials other than those used to parameterize the model. However, this work has continued within other EU projects since (mainly MARINA and NANoREG) where further development and validation are being undertaken which will result in greater confidence in the predictive capabilities of both models. This work could be combined with QSAR models, to adjust the parameters of the PBPK model to be material specific, or in order to investigate whether the predicted organ burdens would induce toxicity, if and when reliable QSAR models have been developed.

The main shortcomings of the ENPRA project

The main goal of ENPRA was to develop a framework for quantitative risk assessment of NM in occupational settings. This risk assessment relies on quantification of exposure and hazard associated with the NM in question. Although as a whole ENPRA was efficacious in many regards it might be argued that it failed in aspects of assessing risk associated with the panel of NM. This was fundamentally due to selection of the exposure route (IT) and the length of exposure (24 hr for the majority of the studies) in the *in vivo* settings (these choices were formed based on the finances available). The large succession of *in vitro* data and acute *in vivo* exposure utilized within ENPRA were crucial for better understanding of the toxicity of the NM and the mechanisms underlying these induced adverse effects; however, for evaluating risk; studies with low dose multiple administrations (preferably via the inhalation route) over time with a longer recovery period are needed (discussed in length in the following section). It has been suggested that inhalation exposure of NM in a rat model might be the closest representative of potential adverse health effects in humans (Klein et al. 2012; Ma-Hock et al. 2008).

In addition, the investigation of intermediate biomarkers rather than actual health outcomes might also be considered as a limitation. For instance, the predictive value of transient pulmonary inflammation and oxidative stress for health outcomes such as cancer is not known. It has been demonstrated that the concordance between chemical compounds ability to generate DNA strand breaks and tumorigenicity in rodent bioassays is 78-85% (Møller 2005). However, predictive values for cancer following exposure to NM is as yet unknown, although it has been demonstrated that a large number of NM generate DNA strand breaks in cultured cells and rodent tissue (Møller et al. 2015a; 2015b) as well as altered mRNA expression for early lung cancer detection (Guo et al. 2012).

It is important to note that the density of the NM/agglomerates was not measured in the ENPRA project. This could potentially be important in any toxicology study as the calculation of an accurate delivered dose might also require quantification of material sedimentation rather than relying exclusively on the administered dose (initial mass concentration) (further discussed below) (Liu et al. 2015).

There is also the issue of generalizability of present data to other types of NM. As an example, the ENPRA panel of materials contained two types of MWCNT and the findings might potentially be utilized for extrapolation to other types of MWCNT with the same characteristics, whereas this would be less plausible for other types of pristine CNT and not feasible for hazard identification of functionalized CNT. Similar limitations pertain to the other types of materials that were investigated within ENPRA (Figure 6). Overall, ENPRA was successful in the categorization (ranking of toxicity) and bench marking of the panel of materials if not necessarily in their risk assessment.

Current and future challenges

Different NM dissolve at different rates which no doubt influences overall toxicity, bio-availability and eventual destination and fate. The dissolution within a biologically relevant medium indicates that the material is unlikely to be bio-persistent; hence dissolution may also be a mechanism of clearance. Alternatively, if the NM is made from a toxic substance, such as the cadmium in quantum dots, then dissolution results in the release of the toxic core and further propagation of adverse effects (Tang et al. 2013). Moreover, if NM are internalized into cells, they are likely to be compartmentalized into the relatively aggressive environment of the lysosomes (pH of 4.5). This is one reason to better understand the solubility of NM and distinguish between material types. It is crucial to establish whether this solubility exerts an influence on long-term toxicity; hence, bio-persistence is fundamental for risk assessment of NM.

A better comprehension of the physicochemical characteristics of NM would also cut out the need for unnecessary work and expenses. As an example, the composition and properties of the 2 MWCNT were similar in the ENPRA project - with removal of the redundant materials and generation of a smaller panel of NM would have allowed for better understanding of the mechanisms underlying the adverse effects without detracting from the overall impact of the project (in terms of categorization of materials). In the future, full characterization of NM before finalization of the material list to be investigated for their nanotoxicological effects would be extremely beneficial.

The need for characterization of NM is further highlighted by the fact that the pattern and efficiency of inhaled materials deposition and translocation is largely dependent on the aerodynamic and/or physical diameter of the material in question. Several studies noted that the pattern of distribution tends to be size and charge dependent (Hirn et al. 2011; Semmler-Behnke et al. 2008) with markedly greater bio-distribution and accumulation of NM in comparison to larger materials of the same chemical composition (Han et al. 2014; Kreyling et al. 2009; Liu et al. 2012; Semmler-Behnke et al. 2008; Zhang et al. 2014). In addition, it has been suggested that smaller NM might bind lung lining layer proteins and lipids and use them as transporting vehicles across membranes, whereas micro-particles would be excluded from this transport mechanism (Lynch et al. 2008).

The quality of the nanotoxicology research (in particular with regard to the characterization of materials used) has been heavily criticized recently with suggestions at the need for international harmonization of the physicochemical characteristics of NM (Krug 2014). Although characterization of materials is essential in any nanotoxicological study - the necessity for its regulation seems excessive. The characterization of any material is always dependent on the toxicological context of the investigation and may differ from study to study. Crucially, the responsibility falls on individual researchers (and to a lesser extent of their peers in the review

process) to ensure the quality of the research remains as high as possible. This does not infer that the field would not benefit from some degree of stringency; for example the standardization of the most widely used methodologies would be beneficial and ensure that any differences in observed responses are more likely to be due to materials and not the experimental variations. The comet assay for genotoxicity testing is a good example of an assay that is widely used in nanotoxicology with clear existing guidelines for *in vivo* testing (Møller et al. 2015b). Overall Europe seems on the right track on this particular issue as there are number of EU-funded projects in progress which aim to develop and implement best practice and quality in all aspects of nanosafety research i.e. "Quality nano" and "FutureNanoNeeds".

Another issue which is often troublesome and on occasion overlooked in certain toxicological studies is the ability of some NM to interfere with assays with potential for artifacts and false positive or false negative data being generated. Therefore utilization of two assays for the relevant end-point would be highly beneficial. This was evident in the ENPRA project with the Ag and MWCNT interference with numerous assays such as DCFH-DA and LDH.

Due to expansion of the field of nanotoxicology and the escalating number of the published studies on the topic it is becoming increasing difficult to publish negative data. This has been highlighted recently (Krug 2014), but due to its importance it is worth emphasizing again. NM are not equally harmful - from a toxicological point of view it is illogical that only harmful effects are published; while no toxic/adverse effects at relevant dose ranges are largely ignored. In order for the field of nanotoxicology to progress it is crucial that both positive and negative outcomes are regarded with equal prominence in well-designed experiments. This is also important for safety by design and legislative purposes.

In order to build a solid knowledge base, it is crucial to identify the hazards associated with NM exposure in both *in vitro* and *in vivo* settings. However, it is often difficult to make direct

comparisons between *in vitro* and *in vivo* responses, (often *in vitro* findings can only act as an indicator of possible *in vivo* effects). One of the key reasons for this is that in reality, comparisons between the systems are rarely like for like (i.e. *in vivo* equivalence for *in vitro* toxicity). An example of such difference is that soluble NM remain trapped in an *in vitro* system, whereas, soluble material constituents may be removed *in vivo*. Furthermore, utilization of a single cell type can never be a representative model of a whole organ or systemic effects that can only be observed *in vivo*. Another major stumbling block in the development of reliable *in vitro* toxicological screening methods for NM is the need for accurate dosimetry (Han et al. 2012). In an *in vitro* cytotoxicity study, NM are suspended in a liquid medium. Typically investigations of biological responses to NM exposure is dependent on administered dose metrics based on NM properties as measured in the dry powder form and not taken account the material-medium interactions (Oberdorster et al. 2005; Rushton et al. 2010). It has been demonstrated that NM form agglomerates in an exposure medium (Cohen et al. 2013; Deloid et al. 2014; Demokritou et al. 2012) which will affect the amount of material that reaches the cells. In a typical *in vitro* experiment, delivery of NM in liquid suspension to cells in culture is determined by two fundamental transport mechanisms namely diffusion and sedimentation. Accurate dosimetry requires characterization of agglomerate properties in liquid suspension, particularly their effective diameter and density (Cohen et al. 2013; Deloid et al. 2014; Teeguarden et al. 2007). It is also necessary to note that for partially soluble NM, changes in agglomerate diameter and effective density may result from mass loss due to dissolution. Finally, with the complication associated with differences in the so-called "protein corona" and other types of surface modifications to the NM that will take place during transit through the body (interaction of NM with countless proteins and other molecules), may influence the effects attributed to NM and make *in vitro* and *in vivo* comparisons even more difficult. Attempting to reproduce an exact protein corona *in vitro* test systems can be problematic; however,

knowledge about the route of exposure might improve the preparation and dispersion of the NM (Johnston et al. 2012). This was not performed as part of ENPRA but reported elsewhere (NeuroNano 2015).

On a similar theme, it is imperative that attention is paid in selection of biologically relevant doses for studies of both bio-kinetics and toxicological end-points. The selection of higher effective dose might lead to enhanced toxicity at the port of entry and potentially translocation to secondary organs, which might not necessarily result a relevant biological response (Donaldson et al. 2013). As more and improved exposure assessments for aerosolized NM in occupational and other settings become available, the measured aerosol concentrations and particle size distributions might be used to estimate deposited and retained mass doses of NM in pulmonary alveolar region of humans and animals. Currently, a number of numerical models are available such as the widely used and well-accepted multiple-path particle dosimetry model (MPPD) (Asgharian et al. 2007; Oberdoester et al. 2015), which can be employed for this purpose. This approach was utilized to determine human lung surface mass concentrations for a given NM exposure scenario (Gangwel et al. 2011). Moreover, a similar method was proposed for comparing *in vivo* toxicity of aerosolized NM exposures with *in vitro* data which incorporated characterization of NM transformations in liquid suspension and their influence on NM kinetics and mass transport using the ISDD (*in vitro* sedimentation, diffusion and dosimetry) model which estimated the *in vitro* equivalent dose before conversion to administered mass concentration using this model (Demokritou et al. 2012a). Although these approaches are not entirely accurate it is critical that attempts are made in order to mimic the whole-animal exposure scenario. This being said, it is important to distinguish between proof of concept studies and those focusing on NM hazard assessment. In order to understand the mechanisms underlying toxicity the use of higher dose/concentration might be required and need to be judged on a case to case basis and justified within the context of the study.

The *in vivo* exposures by IT as employed in ENPRA have some major disadvantages compared to the gold-standard inhalation exposure. Using IT, the deposition patterns of materials in the lung result in some heavily localized exposed areas; while some regions might remain completely unexposed (Landsiedel et al. 2014). In addition, the high dose rate in IT has shown to generate more severe inflammation and oxidative stress in lung tissue of rats that were exposed to the same deposited dose by low dose-rate inhalation administration (Baisch et al. 2014). These differences indicate that adverse effects observed following an IT exposure might not necessarily be detected following an inhalation study. Moreover, in instillation and aspiration exposures, NM are prepared as suspensions. This might introduce further non-physiological observations as these NM have altered surface characteristics, agglomeration and deposition patterns differing from their airborne counterparts (Balasubramanian et al. 2013). Therefore, in ENPRA, the short term instillation studies only serve as a screening tool or provide mechanistic understanding in the case of multiple administrations of MWCNT with respect to a fibrotic response or the *ApoE*$^{-/-}$ disease model. For risk assessment, chronic low dose multiple exposures via inhalation are needed. Therefore, inhalation studies should be designed and executed with intermittent dosing and long recovery periods to allow for the development of patterns in the accumulation and clearance at the portal of entry and manifestation of toxicity (Kermanizadeh et al. 2015b; Pauluhn 2014). It needs to be acknowledged however, that realistic inhalation studies cannot be routinely conducted due to their expense and technical difficulty and the large numbers of NM which are widely available.

Current issues with risk assessment of NM

Ordinarily, exposure risks of materials are assessed through consideration of exposure data and hazard information within risk assessments but this is currently challenging for NM due to a lack of relevant exposure and hazard data within the available literature. In addition, industrial research is often only available internally. Risk assessments for REACH usually require exposure data and

establishment of derived no effect levels (DNELs) that are extrapolated from animal investigations to the human situation using uncertainty factors (Aschberger et al. 2011). This risk assessment with respect to the life cycle of NM is often difficult for numerous reasons. Firstly, NM are often transformed, e.g. by agglomeration or development of coatings, which may impact their biological effect after uptake (Cai and Yao 2014; Kuhn et al. 2014). In addition, the information regarding the level of exposure to NM is often disintegrated with data from most studies unsuitable for the estimation of the exposed dose (as discussed above). Besides, to estimate the impact of a health risk, an accurate representation of the number of potential exposed population is needed. This number strongly relates to the individuals (in the manufacture and/or use) exposed to the nano-based products - with accurate information on this situation also lacking. The assessment of hazard in vulnerable groups (i.e. infants, elderly and individuals with pre-existing medical conditions) is another area which is currently largely non-existent. As exemplified, most of the parameters of the NM risk assessment are currently far from ideal. Hence to date, only a few health-based exposure limit values have been proposed for NM use (NIOSH 2011; 2013).

Due to these current limitations, there might be a need to find some alternative approaches as interim measurements until the above issues are remedied (Johnston et al. 2012). An alternative approach could be the Weight of Evidence (WoE) approach. WoE is an adaptable approach that allows usage and integration of different types of available data and associated uncertainties as well as expert judgment to assess risk and/or hazard. The assessment is based on different pieces of information (called the lines of evidence (LoE)) used to evaluate the assessment endpoint. LoE pertain to an important aspect of the environment or human health (Smith et al. 2002) and serve to predict hazard or risk. The WoE approach integrates information provided by multiple LoE and relates them to a single measure for decision making. In the ENPRA project, the WoE approach was applied to the panel of NM by the integration and combination of physicochemical properties of

NM and toxicity data for said materials, in order to assess potential risk to human health (unpublished data). There are some apparent advantages for use of mathematical models for risk assessment of NM. For example, such a model allows for specific exposure scenarios predictions influenced by various factors such as the general room size and ventilation.

Conclusions

A large body of toxicology data is now being generated for NM, using a wide variety of models, protocols and end-points in which ENPRA was useful. It is clear that not all NM are equally toxic and these disparities are to a large extent based upon physical and chemical properties. Data generated will hopefully in time provide the opportunity for a variety of different analysis to be conducted, ranging from the relatively simple questions, such as the toxic effects of a particular NM type, to relatively complex issues such as the generation of *in silico* models to allow SAR with the overall aim of having the knowledge base to enable prediction of toxicity of new NM. However, this is in reality still years away, and thus in the shorter term there is the need for further relevant models to try to predict risk relevant low doses and appropriate route of exposure of materials, dispersed using relevant physiologically methods. It remains to be established whether chronic NM exposure might lead to accumulation of sufficiently high doses that might initiate and contribute to the progression of disease in humans.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Aldieri, E., Fenoglio, I., Cesano, F., Gazzano, E., Gulion, G., Scarano, D., Attanasio, A., Mazzucco, G., Ghigo, D., Fubini, B. 2013. The role of iron impurities in the toxic effects exerted by short multiwalled carbon nanotubes (MWCNT) in murine alveolar macrophages. *J Toxicol Environ Health A* 76: 1056-1071.

Almeida, J. P., Chen, A. L., Foster, A., Drezek, R. 2011. *In vivo* biodistribution of nanoparticles. *Nanomedicine* 6: 815-835.

Aschberger, K., Micheletti, C., Sokull-Kluttgen, B., Christensen, F. M. 2011. Analysis of currently available data for characterising the risk of engineered nanomaterials to the environment and human health - lessons learned from four case studies. *Environ Int* 37: 1143-1156.

Asgharian, B., Price, O. T. 2007. Deposition of ultrafine (nano) particles in the human lung. *Inhal Toxicol* 19: 1045-1054.

Baisch, B. L., Corson, N. M., Wade-Mercer, P., Gelein, R., Kennell, A. J., Oberdorster, G., Elder, A. 2014. Equivalent titanium dioxide nanoparticle deposition by intratracheal instillation and whole body inhalation: The effect of dose rate on acute respiratory tract inflammation. *Part Fibre Toxicol* 11: 5.

Balasubramanian, S. K., Poh, K. W., Ong, C. N., Kreyling, W. G., Ong, W. Y., Yu, L. E. 2013. The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. *Biomaterials* 34: 5439-5452.

BCC Research. 2015. http://www.bccresearch.com/market-research/nanotechnology/nanotechnology-market-assessment-report-nan031f.html (accessed on 12-1-2015).

Benn, T. M., Westerhoff, P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environ Sci Technol* 42: 4133-4139.

Brocato, J., Sun, H., Shamy, M., Kluz, T., Alghamdi, M. A., Khoder, M. I., Chen, L.C., Costa, M. 2014. Particulate matter from Saudi Arabia induces genes involved in inflammation, metabolic syndrome and atherosclerosis. *J Toxicol Environ Health A* 77: 751-766.

Braydich-Stolle, L. K., Breitner, E. K., Comfort, K. K., Schlager, J. J., Hussain, S. M. 2014. Dynamic characteristics of silver nanoparticles in physiological fluids: Toxicological implications. *Langmuir* 30: 15309-15316.

Brook, R. D., Rajagopalan, S. 2010. Particulate matter air pollution and atherosclerosis. *Curr Atherosclerosis Rep* 12: 291-300.

Brown, D. M., Johnston, H., Gubbins, E., Stone, V. 2014. Serum enhanced cytokine responses of macrophages to silica and iron oxide particles and nanomaterials: a comparison of serum of lung fluid and albumin dispersions. *J Appl Toxicol* 34: 1177-1187.

Brunauer, P. H., Emmet, H., Teller, E. 1938. Adsorption of gases in multi-molecular layers. *J Am Chem Soc* 60: 309-315.

Cai, H., Yao, P. 2014. Gold nanoparticles with different amino acid surfaces: Serum albumin adsorption, intracellular uptake and cytotoxicity. *Colloids Surfaces B-Biointerfaces* 123: 900-906.

Cao, Y., Jacobsen, N. R., Danielsen, P. H., Lenz, A. G., Stoeger, T., Loft, S., Wallin, H., Roursgaard, M., Mikkelsen, L., Møller, P. 2014. Vascular effects of multiwalled carbon nanotubes in dyslipidemic *ApoE*$^{-/-}$ mice and cultured endothelial cells. *Toxicol Sci* 138: 104-116.

Chen, S. H., Li, F. C., Souris, J. S., Yang, C. S., Tseng, F. G., Lee, H. S., Chen, C. T., Dong, C. Y., Lo, L. W. 2012. Visualizing dynamics of sub-hepatic distribution of nanoparticles using intravital multiphoton fluorescence microscopy. *ACS Nano* 6: 4122-4131.

Chen, Y. C., Weng, Y. H., Chiu, Y. W., Yang C. Y. 2015. Short-term effects of coarse particulate matter on hospital admissions for cardiovascular diseases: A case-crossover study in a tropical City. *J Toxicol Environ Health A* 78: 1241-1253.

Chiu, H. F., Chang, C. C., Yang, C. Y. 2014. Relationship between hemorrhagic stroke hospitalization and exposure to fine particulate air pollution in Taipei, Taiwan. *J Toxicol Environ Health A* 77: 1154-1163.

Choi, J., Kim, H., Kim, P., Jo, E., Kim, H. M., Lee, M. Y., Jin, S. M., Park, K. 2015. Toxicity of zinc oxide nanoparticles in rats treated by two different routes: single intravenous injection and single oral administration. *J Toxicol Environ Health A* 78: 226-243.

Cohen, J., Deloid, G., Pyrgiotakis, G., Demokritou, P. 2013. Interactions of engineered nanomaterials in physiological media and implications for *in vitro* dosimetry. *Nanotoxicology* 7: 417-431.

CPI. 2015. http://www.nanotechproject.org/cpi/ (accessed on 18-3-2015).

Danielsen, P. H., Cao, Y., Roursgaard, M., Møller, P., Loft, S. 2014. Endothelial cell activation, oxidative stress and inflammation induced by a panel of metal-based nanomaterials. *Nanotoxicology* 18: 1-12.

Dekali, S., Divetain, A., Kortulewski, T., Vanbaelinghem, J., Gamez, C., Rogerieux, F., Lacroix, G., Rat, P. 2013. Cell cooperation and role of the $P2X_7$ receptor in pulmonary inflammation induced by nanoparticles. *Nanotoxicology* 7: 1302-1314.

Delfino, R. J., Sioutas, C., Malik, S. 2005. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. *Environ Health Persp* 113: 934-946.

Deloid, G., Cohen, J., Darrah, T., Derk, R., Wang, L., Pyrgiotakis, G., Wohlleben, W., Demokritou, P. 2014. Estimating effective density of engineered nanomaterials for *in vitro* dosimetry. *Nat Commun* 5: 3514.

Demokritou, P., Gass, S., Pyrgiotakis, G., Cohen, J. M., Goldsmith, W., McKinney, W., Frazer, D., Ma, J., Schwegler-Berry, D., Brain, J., Castranova, V. 2012. An *in vivo* and *in vitro* toxicological characterization of realistic nanoscale CeO_2 inhalation exposures. *Nanotoxicology* 7: 1338-1350.

Donaldson, K., Poland, C. A. 2013a. Nanotoxicity: challenging the myth of nano-specific toxicity. *Curr Opin Biotechnol* 24: 724-734.

Donaldson, K., Poland, C. A., Murphy, F. A., MacFarlane, M., Chernova, T., Schinwald, A. 2013b. Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences. *Adv Drug Deliv Rev* 65: 2078-2086.

Donaldson, K., Seaton, A. 2012. A short history of toxicology of inhaled particles. *Part Fibre Toxicol* 9: 13.

Du, Z. J., Zhao, D. L., Jing, L., Cui, G. Q., Jin, M. H., Li, Y., Liu, X. M., Liu, Y., Du, H. Y., Guo, C. X., Zhou, X., Sun, Z. 2013. Cardiovascular toxicity of different sizes amorphous silica nanoparticles in rats after intratracheal instillation. *Cardiovasc Toxicol* 13: 194-207.

Dybdahl, M., Risom, L., Bornholdt, J., Autrup, H., Loft, S., Wallin, H. 2004. Inflammatory and genotoxic effects of diesel particles *in vitro* and *in vivo*. *Mutation Research* 562: 119-131.

ENPRA. 2015. http://www.enpra.eu/ (accessed on 18-1-2015).

Foucaud, L., Wilson, M. R., Brown, D. M., Stone, V. 2007. Measurement of reactive species production by nanoparticles prepared in biologically relevant media. *Toxicol Lett* 174: 1-9.

Gangwal, S., Brown, J. S., Wang, A., Houck, K. A., Dix, D. J., Kavlock, R. J., Hubal, E. A. 2011. Informing selection of nanomaterial concentrations for ToxCast *in vitro* testing based on occupational exposure potential. *Environ Health Persp* 119: 1539-1546.

Gosens, I., Kermanizadeh, A., Jacobsen, N. R., Lenz, A. G., Bokkers, B., de Jong, W. H., Krystek, P., Tran, L., Stone, V., Wallin, H., Stoeger, T., Cassee, F. R. 2015. Comparative hazard identification of zinc oxide and silver nanomaterials in mice. *Plos One* 10: e0126934.

Guo, N. L., Wan, Y. W., Denvir, J., Porter, D. W., Pacurari, M., Wolfarth, M. G., Castranova, G., Qian, Y. 2012. Multiwalled carbon nanotube-induced gene signatures in the mouse lung: potential predictive value for human lung cancer risk and prognosis. *J Toxicol Environ Health A* 75: 1129-1153.

Han, S. G., Lee, J. S., Ahn, K., Kim, Y. S., Kim, J. K., Lee, J. H., Shin, J. H., Jeon, K. S., Cho, W. S., Song, N. W., Gulumian, M., Shin, B. S., Yu, I. J. 2014. Size-dependent clearance of gold nanoparticles from lungs of Sprague-Dawley rats after short-term inhalation exposure. *Arch Toxicol* 89: 1083-1094.

Han, S. G., Howatt, D., Daugherty, A., Gairola, G. 2015. Pulmonary and atherogenic effects of multi-walled carbon nanotubes (MWCNT) in apolipoprotein-E-deficient mice. *J Toxicol Environ Health A* 78: 244-253.

Han, X., Corson, N., Wade-Mercer, P., Gelein, R., Jiang, J., Sahu, M., Biswas, P., Finkelstein, J. N., Elder, A., Oberdorster, G. 2012. Assessing the relevance of *in vitro* studies in nanotoxicology by examining correlations between *in vitro* and *in vivo* data. *Toxicology* 297: 1-9.

Hirn, S., Haberl, N., Loza, K., Epple, M., Kreyling, W. G., Rothen-Rutishauser, B., Rehberg, M., Krombach, F. 2014. Proinflammatory and cytotoxic response to nanoparticles in precision-cut lung slices. *Beilstein J Nanotechnol* 5: 2440-2449.

Hirn, S., Semmler-Behnke, M., Schleh, C., Schffler, M., Wenk, A., Gibson, N., Holzwarth, U., Abbas, K., Kreyling, W. G. 2011a. Quantitative biokinetics study in healthy adult rats over 28 days after inhalation of 20 nm titanium dioxide (TiO_2) anatase nanoparticles aerosols. *Am J Respir Crit Care Med* 183: meeting abstract A2279.

Hirn, S., Semmler-Behnke, M., Schleh, C., Wenk, A., Lipka, J., Schaffler, M., Takenaka, S., Moller, W., Schimid, G., Simon, U., Kreyling, W. G. 2011b. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. *Eur J Pharmacol Biopharmacol* 77: 407-416.

Jacobsen, N. R., Møller, P., Jensen, K. A., Vogel, U., Ladefoged, O., Loft, S., Wallin, H. 2009. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in *ApoE*$^{-/-}$ mice. *Part Fibre Toxicol* 6: 2.

Jacobsen, N. R., Pojano, G., Wallin, H., Jensen, K. A. 2010. Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal report - National Research Centre for the Working Environment 6: 1-10.

Johnston, H., Brown, D., Kermanizadeh, A., Gubbins, E., Stone, V. 2012. Investigating the relationship between nanomaterial hazard and physicochemical properties: Informing the exploitation of nanomaterials with therapeutic and diagnosis applications. *J Control Release* 164: 307-313.

Johnston, H., Pojana, G., Zuin, S., Jacobsen, N. R., Møller, P., Loft, S., Semmler-Behnke, M., McGuiness, C., Balharry, D., Marcomini, A., Wallin, H., Kreyling, W., Donaldson, K., Tran, L., Stone, V. 2013. Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges. *Crit Rev Toxicol* 43: 1-20.

JRC nanomaterials repository. 2015. https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository (accessed on 11-3-2015).

Kermanizadeh, A., Balharry, D., Wallin, H., Loft, S., Møller, P. 2015b. Nanomaterial translocation - the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs - a review. *Crit Rev Toxicol* DOI:10.3109/10408444.2015.1058747.

Kermanizadeh, A., Brown, D. M., Hutchison, G., Stone, V. 2013c. Engineered nanomaterial impact in the liver following exposure via an intravenous route - the role of polymorphonuclear leukocytes and gene expression in the organ. *J Nanomed Nanotechnol* 4: 157.

Kermanizadeh, A., Chauché, C., Balharry, D., Brown, D. M., Kanase, N., Boczkowski, J., Lanone, S., Stone, V. 2014b. The role of Kupffer cells in the hepatic response to silver nanoparticles. *Nanotoxicology* 8: 149-154.

Kermanizadeh, A., Chauché, C., Brown, D. M., Møller, P., Loft, S. 2015a. The role of intracellular redox imbalance in nanomaterial induced cellular damage and genotoxicity - a review. *Environ Mol Mutagen* 56: 111-124.

Kermanizadeh, A., Gaiser, B. K., Hutchison, G. R., Stone, V. 2012. An *in vitro* liver model - assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanoparticles. *Part Fibre Toxicol* 9: 28.

Kermanizadeh, A., Gaiser, B. K., Ward, M. B., Stone, V. 2013b. Primary human hepatocytes vs. hepatic cell line - assessing their suitability for *in vitro* nanotoxicology. *Nanotoxicology* 7: 1255-1271.

Kermanizadeh, A., Løhr, M., Roursgaard, M., Messner, S., Gunness, P., Kelm, J. M., Møller, P., Stone, V., Loft, S. 2014a. Hepatic toxicology following single and multiple exposure of engineered nanomaterials utilising a novel primary human 3D liver microtissue model. *Part Fibre Toxicol* 11: 56.

Kermanizadeh, A., Pojana, G., Gaiser, B. K., Birkedal, R., Bilaničová, D., Wallin, H., Jensen, K. A., Sellergren, B., Hutchison, G. R., Marcomini, A., Stone, V. 2013a. *In vitro* assessment of engineered nanomaterials using C3A cells: Cytotoxicity, pro-inflammatory cytokines and function markers. *Nanotoxicology* 7: 301-313.

Kermanizadeh, A., Vranic, S., Boland, S., Moreau, K., Squiban, A. B., Gaiser, B. K., Andrzejczuk, L. A., Stone, V. 2013c. An *in vitro* assessment of panel of engineered nanomaterials using a human renal cell line: Cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. *BMC Nephrol* 14: 96.

Klein, C. L., Wiench, K., Wiemann, M., Ma-Hock, L., Ravanzwaay, B. V., Landsiedel, R. 2012. Hazard identification of inhaled nanomaterials: Making use of short-term inhalation studies. *Arch Toxicol* 86: 1137-1151.

Kreyling, W. G., Semmler, M., Erbe, F., Mayer, P., Takenaka, S., Schulz, H., Oberdorster, G., Ziesenis, A. 2002. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A* 65: 1513-1530.

Kreyling, W. G., Hirn, S., Möller, W., Schleh, C., Wenk, A., Celik, G., Lipka, J., Schaffler, M., Haberl, N., Johnston, B. D., Sperling, R., Schmid, G., Simon, U., Parak, W. J., Semmler-Behnke, M. 2014. Air-blood barrier translocation of tracheally instilled gold nanoparticles inversely depends on particle size. *ACS Nano* 8: 222-233.

Kreyling, W. G., Semmler-Behnke, M., Seitz, J., Scymczak, W., Wenk, A., Mayer, P., Takenaka, S., Oberdorster, G. 2009. Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. *Inhal Toxicol* 21: 55-60.

Krug, H. F. 2014. Nanosafety research - Are we on the right track? *Angewandte Chem* 53: 12304-12319.

Kuhn, D. A., Vanhecke, D., Michen, B., Blank, F., Gehr, P., Petri-Fink, A., Rothen-Rutishauser, B. 2014. Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages. *Beilstein J Nanotechnol* 5: 1625-1636.

Landsiedel, R., Fabian, R., Ma-Hock, L., van Ravenzwaay, B., Wohlleben, W., Wiench, K., Oesch, F. 2012. Toxico/biokinetics of nanomaterials. *Arch Toxicol* 86: 1021-1060.

Landsiedel, R., Ma-Hock, L., Hoffmann, T., Wiemann, M., Struss, V., Truemann, S., Wohlleben, W., Groters, S., Wiench, K., van Ravenzwaay, B. 2014. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. *Part Fibre Toxicol* 11: 16.

Lee, Y. G., Jeong, J., Raftis, J., Cho, W. S. 2015. Determination of adsorption affinity of nanoparticles for interleukin-8 secreted from A549 cells by in vitro cell-free and cell-based assays. *J Toxicol Environ Health A* 78: 185-195.

Li, D. S., Johanson, G., Emond, C., Carlander, U., Philbert, M., Jolliet, O. 2014b. Physiologically based pharmacokinetic modeling of polyethylene glycol-coated polyacrylamide nanoparticles in rats. *Nanotoxicology* 8: 128-137.

Li, Y., Zhang, Y., Yan, B. 2014a. Nanotoxicity overview: Nano-threat to susceptible populations. *Int J Mol Sci* 15: 3671-3697.

Li, Z., Hulderman, T., Salmen, R., Chapman, R., Leonard, S. S., Young, S. H., Shvedova, A., Luster, M. I., Simeonova, P. P. 2007. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. *Environ Health Persp* 115: 377-382.

Lipka, J., Semmler-Behnke, M., Sperling, R. A., Wenk, A., Takenaka, S., Schleh, C., Kissel, T., Parak, W. J., Kreyling, W. G. 2010. Biodistribution of PEG-modified gold nanoparticles following intratracheal instillation and intravenous injection. *Biomaterials* 31: 6574-6581.

Liu, H., Liu, T., Li, L., Hao, N., Tan, L., Meng, X., Ren, J., Chen, D., Tang, F. 2012. Size dependent cellular uptake, *in vivo* fate and light-heat conversion efficiency of gold nanoshells on silica nanorattles. *Nanoscale* 4: 3523-3529.

Liu, R., Liu H. H., Ji, Z., Chang, C. H., Xia, T., Nel, A. E., Cohen, Y. 2015. Evaluation of toxicity ranking of metal oxide nanoparticles via an in vitro dosimetry model. *ACS Nano* 9: 9303-9013.

Liu, X. S., Li, H., Chen, Y. J., Jin, Q., Ren, K. F., Ji, J. 2014. Mixed-charge nanoparticles for long circulation, low reticuloendothelial system clearance and high tumor accumulation. *Adv Healthcare Mater* 3: 1439-1447.

Loeschner, K., Hadrup, N., Qvortrup, K., Larsen, A., Gao, X., Vogel, U., Mortensen, A., Lam, H. R., Larsen, E. H. 2011. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. *Part Fibre Toxicol* 8: 18.

Lynch, I., Dawson, K. A. 2008. Protein-nanoparticle interactions. *Nano Today* 3: 40-47.

Ma-Hock, L., Burkhardt, S., Strauss, V., Gamer, A. O., Wiench, K., Ravenzwaay, B. V., Landsiedel, R. 2008. Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance. *Inhal Toxicol* 21: 102-118.

Marina. 2015. http://www.marina-fp7.eu/ (accessed on 18-5-2015).

Miller, M. 2014. The role of oxidative stress in cardiovascular actions of particulate air pollution. *Biochem Soc Trans* 42: 1006-1011.

Møller, P. 2005. Genotoxicity of environmental agents assessed by the alkaline comet assay. *Basic Clin Pharmacol Toxicol* 96: 1-42.

Møller, P., Hemmingsen, J. G., Jensen, D. M., Danielsen, P. H., Karottki, D. G., Jantzen, K., Roursgaard, M., Cao, Y., Kermanizadeh, A., Klingberg, H., Christophersen, D. V., Hersoug, L. G., Loft, S. 2015b. Application of the comet assay in particle toxicology: Air pollution and engineered nanomaterials exposure. *Mutagenesis* 30: 67-83.

Møller, P., Jensen, D. M., Christophersen, D. V., Kermanizadeh, A., Jacobsen, N. R., Hemmingsen, J. G., Danielsen, P. H., Karottki, D. G., Roursgaard, M., Cao, Y., Jantzen, K., Hersoug, L. G., Loft, S. 2015a. Measurement of oxidative damage to DNA in nanomaterial exposed cells and animals. *Environ Mol Mutagen* 56: 97-110.

Møller, P., Mikkelsen, L., Vesterdal, L. K., Folkmann, J. K., Forchhammer, L., Roursgaard, M., Danielsen, P. H., Loft, S. 2011. Hazard identification of particulate matter on vasomotor dysfunction and progression of atherosclerosis. *Crit Rev Toxicol* 41: 339-368.

Nanogenotox report. 2015. http://www.nanogenotox.eu/files/PDF/Deliverables/d4.1_summary%20report.pdf (accessed on 11-3-2015).

Nanomicex. 2015. http://nanomicex.eu/ (accessed on 18-5-2015).

Napierska, D., Thomassen, L. C. J., Vanaudenaerde, B., Luyts, K., Lison, D., Martens, J. A., Nemery, B., Hoet, P. H. M. 2012. Cytokine production by co-culture exposed to monodisperse amorphous silica nanoparticles: the role of size and surface area. *Toxicol Lett* 211: 98-104.

NeuroNano. 2015. http://www.neuronano.eu/ (accessed on 16-3-2015).

NIOSH Current intelligence bulletin 63. 2011. Occupational exposure to titanium dioxide. Department of Health and Human Services, Public Health.

NIOSH Current intelligence bulletin 65. 2013. Occupational exposure to carbon nanotubes and nanofibers. Department of Health and Human Services, Public Health.

Oberdorster, E., Oberdorster, J. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ Health Presp* 113: 823-839.

Oberdorster, G., Castranova, V., Asgharian, B., Sayre, P. 2015. Inhalation Exposure to Carbon Nanotubes (CNT) and Carbon Nanofibers (CNF): Methodology and Dosimetry. *J Toxicol Environ Health B* 18: 121-212.

Ong, K. J., MacCormack, T. J., Clark, R. J., Ede, R. J., Ede, J. D., Ortega, V. A., Felix, L. C., Dang, M. K., Ma, G., Fenniri, H., Veinot, J. G., Goss, G. G. 2014. Widespread nanoparticle-assay interference: implications for nanotoxicity testing. *Plos One* 11: 9.

Pattan, G., Kaul, G. 2014. Health hazards associated with nanomaterials. *Toxicol Indust Health* 30: 499-519.

Pauluhn, J. 2011. Poorly soluble particulates: Searching for a unifying denominator of nanoparticles and fine particles for DNEL estimation. *Toxicology* 279: 176-188.

Poulsen, S. S., Saber, A. T., Williams, A., Williams, A., Andersen, O., Købler, C., Atluri, R., Pozzebon, M. E., Mucelli, S. P., Simion, M., Rickerby, D., Mortensen, A., Jackson, P., Kviovska, Z. O., Mølhave, K., Jacobsen, N. R., Jensen, K. A., Yauk, C. L., Wallin, H., Halappanavar, S., Vogel, U. 2015. MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs. *Toxicol App Pharamacol* 284: 16-32.

Roberts, J. R., McKinney, W., Kan, H., Krajnak, K., Frazer, D. G., Thomas, T. A., Waugh, S., Kenyon, A., MacCuspie, R. I., Hackley, V. A., Castronova, V. 2013. Pulmonary and cardiovascular responses of rats to inhalation of silver nanoparticles. *J Toxicol Environ Health A* 76: 651-668.

Rushton, E. K.., Jiang, J., Leonard, S. S., Eberly, S., Castranova, V., Biswas, P., Elder, A., Han, X., Gelein, R., Finklestein, J., Oberdorster, G. 2010. Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. *J Toxicol Environ Health A* 73: 445-461.

Saber, A. T., Jacobsen, N. R., Bornholdt, J., Kjaer, S. L., Dybdahl, M., Risom, L., Loft, S., Vogel, U., Wallin, H. 2006. Cytokine expression in mice exposed to diesel exhaust particles by inhalation. Role of tumor necrosis factor. *Part Fibre Toxicol* 3: 4.

Sadauskas, E., Jacobson, N. R., Danscher, G., Soltenberg, M., Larsen, A., Kreyling, W. G., Wallin, H. 2009. Bio-disruption of gold nanoparticles in mouse lung following intratracheal instillation. *Chem Central J* 3: 16-23.

Semmler-Behnke, M., Kreyling, W. G., Lipka, J., Fertsch, S., Wenk, A., Takenaka, S., Schmid, G., Brandau, W. 2008. Biodistribution of 1.4- and 18-nm gold particles in rats. *Small* 4: 2108-2111.

Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schaffler, M., Tian, F., Schmid, G., Oberdorster, G., Kreyling, W. G. 2014. Size dependent translocation and fetal accumulation of gold nanoparticles from maternal blood in the rat. *Part Fibre Toxicol* 11: 33.

Silva, R. M., Teesy, C., Franzi, L., Weir, A., Westerhoff, P., Evans, J. E., Pinkerton, K. E. 2013. Biological response to nano-scale titanium dioxide (TiO_2): role of particle dose, shape, and retention. *J Toxicol Environ Health A* 76: 953-972.

Smith, E. P., Lipkovich, I., Ye, K. Y. 2002. Weight-of-evidence approach (WOE): Quantitative estimation of probability of impairment for individual and multiple lines of evidence. *Human Ecol Risk Assess* 8: 1585-1596.

Snow, S. J., De Vizcaya-Ruiz, A., Osornio-Vargas, A., Thomas, R. F., Schladweilder, M. C., McGee, J., Kodavanti, U. P. 2014. The effect of composition, size, and solubility on acute pulmonary injury in rats following exposure to Mexico City ambient particulate matter samples. *J Toxicol Environ Health A* 77: 1164-1182.

Snyder-Talkington, B. N., Qian, Y., Castranova, V., Guo, N. L. 2012. New perspectives for in vitro risk assessment of multiwalled carbon nanotubes: application of coculture and bioinformatics. *J Toxicol Environ Health B* 15: 468-492.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M. G., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzar, O., Roncato, D., Saber, A. T., Wallin, H., Scott-Fordsmand, J. J. 2014. ITS-NANO - Priortising nanosafety research to develop a stakeholder driven intelligent testing strtegy. *Part Fibre Toxicol* 11: 9.

Suwa, T., Hogg, J. C., Quinlan, K. B., Ohgami, A., Vincent, R., van Eeden, S. F. 2002. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol* 20: 935-942.

Tang, S., Allagadda, V., Chibli, H., Nadeau, J. L., Mayer, G. D. 2013. Comparison of cytotoxicity and expression of metal regulatory genes in zebrafish (*Danio rerio*) liver cells exposed to cadmium sulphate, zinc sulphate and quantum dots. *Metallomics* 5: 1411-1422.

Teeguarden, J. G., Hinderliter, P. M., Orr, G., Thrall, B. D., Pounds, J. G. 2007. Particokinetics *in vitro*: Dosimetry considerations for *in vitro* nanoparticle toxicity assessments. *Toxicol Sci* 95: 300-312.

Tournebize, J., Sapin-Minet, A., Bartosz, G., Leroy, P., Boudier, A. 2013. Pitfalls of assays devoted to evaluation of oxidative stress induced by inorganic nanoparticles. *Talanta* 116: 753-763.

Tran, C. L., Tantra, R., Donaldson, K., Stone, V., Hankin, S. M., Ross, B., Aitken, R. J., Jones, A. D. 2011. A hypothetical model for predicting of toxicity of high aspect ratio nanoparticles (HARN). *J Nanopart Res* 13: 6683-6698.

Unfried, K., Albrecht, C., Klotz, L. O., von Mikecz, A., Grether-Beck, S., Schins, R. P. F. 2007. Cellular responses to nanoparticles: target structures and mechanisms. *Nanotoxicology* 1: 52-71.

Urisini, C. L., Cavallo, D., Fresegna, A. M., Ciervo, A., Maiello, R., Tassone, P., Buresti, G., Casciardi, S., Iavicoli, S. 2014. Evaluation of cytotoxic, genotoxic and inflammatory response in human alveolar and bronchial epithelial cells exposed to titanium dioxide nanoparticles. *J Appl Toxicol* 34: 1209-1219.

van der Zande, M., Vandebriel, R. J., Van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C. S., Gremmer, E. R., Mast, J., Peters, R. J., Hollman, P. C. H., Hendriksen, P. J. M., Marvin, H. J. P., Peijnenburg, A. A. C. M., Bouwmeester, H. 2012. Distribution, elimination and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. *ACS Nano* 6: 7427-7442.

Vietti, G., Ibouraadaten, S., Palmai-Pallag, M., Yakoub, Y., Bailly, C., Fenoglio, I., Marbaix, E., Lison, D., van den Brule, S. 2013. Towards predicting the lung fibrogenic activity of nanomaterials: Experimental validation of an *in vitro* fibroblast proliferation assay. *Part Fibre Toxicol* 10: 52.

Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K., von Goetz, N. 2012. Titanium dioxide nanoparticles in food and personal care products. *Environ Sci Technol* 46: 2242-2250.

Yuta, A., Takamitsu, M., Hirano, S. 2014. Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages. *Toxicology* 328: 84-92.

Zhang, J., Nie, X., Ji, Y., Liu, Y., Wu, X., Chen, C., Fang, X. 2014. Quantitative biokinetics and systemic translocation of various gold nanostructures are highly dependent on their size and shape. *J Nanosci Nanotechnol* 14: 4124-4138.

Zhao, J., Castranova, V. 2011. Toxicology of nanomaterials used in nanomedicine. *J Toxicol Environ Health B* 14: 593-632.

Figure legends

Figure 1 Exposure-dose-response paradigm for nanomaterials (adapted from the ENPRA website)

Figure 2 A simplified ranking system for the cytotoxicity of the ENPRA panel of NM in 16 different *in vitro* test models from five body systems. The NM with the highest cytotoxicity is ranked as 10 for each model and is presented on the outer edge of the circle while the least toxic NM is given the value of 1 and can be found towards the center of the circle. The data was generated by the consortium partners using differing culture conditions (i.e. cell numbers, exposure periods, culture media and detection assays).

Figure 3 The clustering of the ENPRA panel of NM into high (red), moderate (yellow) and low toxicity (green) groups from *in vitro* models across four body systems. The ranking is based on 24 hr NM exposure to concentrations of up to 80 $\mu g/cm^2$.
ZnO NMs - NM 110 and NM 111
Ag NMs - NM 300
MWCNTs - NM 400 and NM 402
TiO_2 NMs - NM 101, NRCWE 001, NRCWE 002, NRCWE 003 and NRCWE 004

Figure 4 The clustering of the ENPRA NM based on in their ability for intracellular ROS generation (high (red), moderate (yellow) and low/no (green)) in *in vitro* models across four body systems.
ZnO NMs - NM 110 and NM 111
Ag NMs - NM 300
MWCNTs - NM 400 and NM 402
TiO_2 NMs - NM 101, NRCWE 001, NRCWE 002, NRCWE 003 and NRCWE 004

Figure 5 A549 cell cytotoxicity as measured via the WST-1 assay following 24 hr treatment with non-functionalized ZnO **a)** dispersed in a water vehicle or **b)** water vehicle supplemented with 2% FCS. The P numbers represent the different partners within the ENPRA consortium.

Figure 6 The hazard assessment of the ENPRA panel of materials in the larger toxicological context. The numbers are generalized estimations of nanomaterial numbers that are continuously manufactured. The diagram highlights the difficulty and limitations in the selection of a representative panel of materials and how this corresponds to the bigger picture.

NM code	NM type	Phase	Average Size (nm) (Supplier information)	XRD Size (nm)	TEM Size	Primary characteristics by TEM analysis	Surface area (BET) [m^2/g]	Known coating/ charge	CAS number
NM 101	TiO_2	Anatase €	7	9	4-8 /50-100	Two structures found; type 1 show agglomerates in the 50 - 1500 nm range	322	none	13463-67-7
NM 110	ZnO	Zincite	100	70-100	20-250 / 50-350	2 euhedral morphologies: 1. aspect ratio close to 1 (20 - 250 nm range and few particles of approx. 400 nm) 2). ratio 2 to 7.5 (50-350 nm). A small quantity of particles with irregular morphologies observed.	14	none	1314-13-2, *EINECS* 215-222-5
NM 111	ZnO	Zincite	130	58-93	20-200 / 10-450	Similar to NM 110, but with different size distributions. 1. Particles with aspect ratio close to 1 (~ 90% in the 20-200 nm range); 2. particles with aspect ratio 2 to 8.5 (~ 90% in the 10-450 nm ratio).	18	Trie-othoxy-capry-lsilane 130	1314-13-2, 2943-75-1 *EINECS* 215-222-5, 220-941-2
NM 300	Ag	Ag	< 20	7[$] 14[£] <18 / 15 / > 100[#]	8-47 (av.: 17.5)	Mainly euhedral NM; minor fractions have either elongated (aspect ratio up to ~ 5) or sub-spherical morphology	NA	Polyoxylaurat Tween 20	7440-22-4
NM 400	MWCNT	-	D: 30 L: 5000	-	D: 5-35 L: 700-3000	Irregular entangled kinked and mostly bent MWCNT (10-20 walls). Some CNTs were capped and some cases multiple caps were found due to overgrowth. Fe/Co catalysts (6-9 nm, average 7.5 nm) were found inside the tubes.	298	none	7782-42-5, EINECS 231-955-3
NM 402	MWCNT	-	D: 30	-	D: 6-20	Entangled irregular, mostly	225	none	7782-42-5, EINECS

			L: 5000		L: 700-4000	bent MWCNT (6-14 walls). Some tubes were capped by unknown material. Some nano-onions (5-10 nm) and amorphous carbon structures mixed with Fe (5-20 nm). Residual catalyst was observed. Individual catalyst particles up to 150 nm were also detected.			231-955-3
NRCWE 001	TiO_2	Rutile[§]	10	10	80-400	Irregular euhedral particles detected by TEM	99	none	13463-67-7
NRCWE 002	TiO_2	Rutile	10	10	80-400	Irregular euhedral particles detected by TEM	84	Positively charged	-
NRCWE 003	TiO_2	Rutile	10	10	80-400	Irregular euhedral particles detected by TEM	84	Negatively charged	-
NRCWE 004	TiO_2	Rutile	94	App. 100	1-4/ 10-100/ 100-200/ 1000-2000	Five different particle types were identified: 1. irregular spheres, 1-4 nm (av. diameter); 2. irregular euhedral particles, 10-100 nm (longest dimension); 3. fractal-like structures in long chains, 100-200 nm (longest dimension); 4. big irregular polyhedral particles, 1-2µm (longest dimension); 5. large irregular particles with jagged boundaries, 1-2 µm (longest dimension).		none	13463-67-7

Table 1. The main physical and chemical properties of ENPRA NMs (adapted and reproduced from Kermanizadeh et al. 2013a)

CAS = Chemical abstracts service
[€] = 1% rutile found in one of two samples analyzed
[$] = wet XRD in capillary tube
[£] = dried samples
[#] = sample with deposits
[§] = 6% anatase was observed in one of two samples analyzed

	Cytotoxicity of NMs ranked from high to low	Inflammatory markers	ROS production	Anti-oxidant depletion	Genotoxicity	Fibrosis
Pulmonary system						
A549	NM 300 > NM 110 = NM 111 > NM 101 > NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 > NM 400 = NM 402	NM 110 and NM 111 (Other 8 NM)	NM 110, NM 111, NRCWE 002, NRCWE 004 and NM 101 (Other 5 NM)	NM 110 and NM 111 (Other 8 NM)	NM 101 and NRCWE 004 (Other 8 NM)	NT
NCI-H292	NM 110 > NM 111 > NM 300 > NRCWE 001 > NRCWE 004 = NM 101 > NRCWE 002 > NRCWE 003 > NM 400 = NM 402	NM 300, NRCWE 001 and NRCWE 004 (Other 7 NM)	High ROS generation for NM 110, NM 111 and NM 300. Lower levels for NM 101, NRCWE 002, NRCWE 004, NM 400 and NM 402 (NRCWE 001 and NRCWE 003)	NT	NT	NT
16HBE	NM 110 > NM 111 > NM 300 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402	NT	NT	NT	NT	NT
LA-4	NM 110 = NM 111 = NM 300 > NM 101 > NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402	NRCWE 001, NRCWE 004, NM 110, NM 111, NM 300, NM 400 and NM 402 up-regulation of differing cytokines (NM 101, NRCWE 002 and NRCWE 003)	NM 110, NM 111 and NM 300 (Other 7 NM)	NT	NT	NT
MHS	NM 110 = NM 111 = NM 300 > NM 402 > NM 400 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004	NM 110, NM 111, NM 300 and NM 402 (Other 6 NM)	NM 110, NM 111 and NM 300 (Other 7 NM)	NT	NT	NT
Murine primary lung fibroblasts	NM 100 = NM 110 > NM 300 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 > NM 400 = NM 402	NT	NT	NT	NT	NM 400 and NM 402 Other 8 NMs not tested
A549 and primary neutrophils co-culture*	NM 110 > NM 101 Other 8 NMs not tested	NT	NM 101 and NM 110 Other 8 NMs not tested	NT	No effect	NT

	Cytotoxicity of NMs ranked from high to low	Inflammatory markers	ROS production	Anti-oxidant depletion	Genotoxicity	Fibrosis
Cardiovascular system						
EAHY926	NM 300 > NM 110 = NM 111 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402	NT	NT	NT	NT	NT
HUVEC	NM 111 > NM 110 > NM 300 > NM 402 > NM 400 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 004 > NRCWE 003	All NMs up-regulated ICAM-1 and VCAM-1 at different concentrations	NM 400, NM 402 and NRCWE 002 (Other 7 NM)	NT	NT	NT
THP-1	NM 300 > NM 110 > NM 111 >NM 402 = NM 400 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004	NRCWE 001, NRCWE 002 and NRCWE 004 (Other 7 NM)	NM 400, NM 402 and NRCWE 002 (Other 7 NM)	NT	NT	NT
Hepatic system						
C3A	NM 300 > NM 110 > NM 111 > NRCWE 002 > NRCWE 001 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402 > NM 101	IL8 up-regulation by all NMs at different concentrations	NRCWE 001, NRCWE 002, NRCWE 003, NRCWE 004, NM 400 and NM 402 (Other 4 NM)	NM 110, NM 111, NM 300 , NM 400 and NM 402 (Other 5 NM)	Low genotoxicity for nine of the NMs (NRCWE 003 being the exception) - most evident for NM 101 and NRCWE 002	NT
HepG2	NM 300 > NM 110 > NM 400 = NM 402 > NM 111 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004	NT	NT	NT	NM 101 and NRCWE 004 (Other 8 NM)	NT
Human primary hepatocytes	NM 300 > NM 110 > NM 111 > NRCWE 002 > NRCWE 001 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402 > NM 101	IL8 up-regulation by all NMs at differing concentrations	NT	NT	NT	NT
Co-culture of primary rat hepatocytes and KCs	NM 300 > NM 110 > NM 111 > NM 400 = NM 402 > NRCWE 002 > NRCWE 001 = NRCWE 003 = NRCWE 004 = NM 101	Increased TNF-α, IL6 and IL10 post NM treatment	NT	NT	NT	NT
3D human liver tissue$^{\phi}$	NM 110 > NM 300 > NM 400 > NRCWE 002 Other 6 NMs not tested	Up-regulation of IL8 and IL10	Repeated exposure to NM 110 and NM 300 (NM 400 and NRCWE 002)	NT	Most significant genotoxicity following repeated exposure of 4 NMs	NT

	Cytotoxicity of NMs ranked from high to low	Inflammatory markers	ROS production	Anti-oxidant depletion	Genotoxicity	Fibrosis
Renal system						
HK-2	NM 110 > NM 111 > NM 300 > NM 400 > NM 402 > NRCWE 002 > NRCWE 001 = NRCWE 004 > NRCWE 003 > NM 101	Up-regulation of IL6 and IL8	NM 110, NM 111 and NM 300 (Other 7 NM)	NT	Laboratory 1 - small yet significant genotoxicity for seven of NMs (NM 111, NRCWE 001 and NRCWE 003 being the exception) Laboratory 2 - genotoxic effects only with NM 101 (Other 9 NM)	NT
Developmental system						
D3ϵ	NM 300 > NM 110 > NM 111 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 = NM 400 = NM 402	NT	NT	NT	NT	NT
Contracting cardio-myocytesϵ	NM 300 > NM 110 > NM 111 > NM 101 = NRCWE 001 = NRCWE 002 = NRCWE 003 = NRCWE 004 > NM 400 = NM 402	NT	NT	NT	NT	NT

Table 2. The summary of the *in vitro* data generated in the FP7 funded ENPRA project

NT = Not tested
* = Only NM 101 and NM 110 utilized in the test system
Φ = Only NM 110, NM 300, NM 400 and NRCWE 002 used in the system - repeated exposure resulted in more significant accumulative adverse effects
ϵ = 10 day exposure
(Red) = No significant response noted for the particular NM in the specified model
ZnO NM - NM 110 and NM 111
Ag NM - NM 300
MWCNT - NM 400 and NM 402
TiO_2 NM - NM 101, NRCWE 001, NRCWE 002, NRCWE 003 and NRCWE 004

Cytotoxicity of NMs ranked from high to low	Inflammatory markers	ROS production	Anti-oxidant depletion	Genotoxicity	Fibrosis
Pulmonary system					
NM 110 = NM 111 = NRCWE 001 (Other 7 NM)	NM 110, NM 111 (Other 8 NM)	NT	NT	NM 402 (Other 9 NM)	NM 400 and NM 402
Cardiovascular system					
NT	NM 110 and NM 111 (Other 8 NM)	NT	NT	NT	NT
Hepatic system					
NT	NT	NM 300 (Other 9 NM)	NM 110, NM 111 and NM 300 (Other 7 NM)	No effect	NT

Table 3a. The summary of the *in vivo* data generated in the ENPRA project (healthy animals)

NT = Not tested
(Red) = No significant response for the particular NM in the specified model

Cytotoxicity of NMs ranked from high to low	Inflammatory markers	ROS production	Anti-oxidant depletion	Genotoxicity	Fibrosis
Pulmonary system*					
NT	NM 400 and NM 402	NM 400 and NM 402	NT	NM 400 and NM 402	NT

Table 3b. The summary of the *in vivo* data generated in the ENPRA project (*ApoE*$^{-/-}$ mice)

NT = Not tested
* = only NM 400 and NM 402 tested

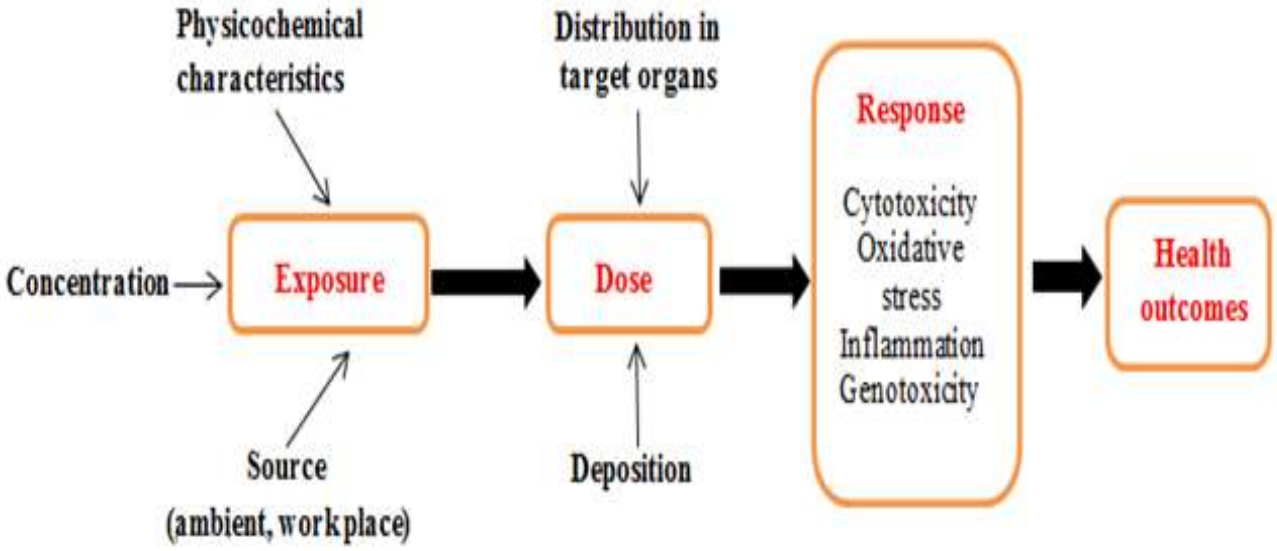
Physicochemical characteristics
Distribution in target organs
Response
Cytotoxicity
Oxidative stress
Inflammation
Genotoxicity
Concentration
Exposure
Dose
Health outcomes
Source (ambient, workplace)
Deposition

Figure 1

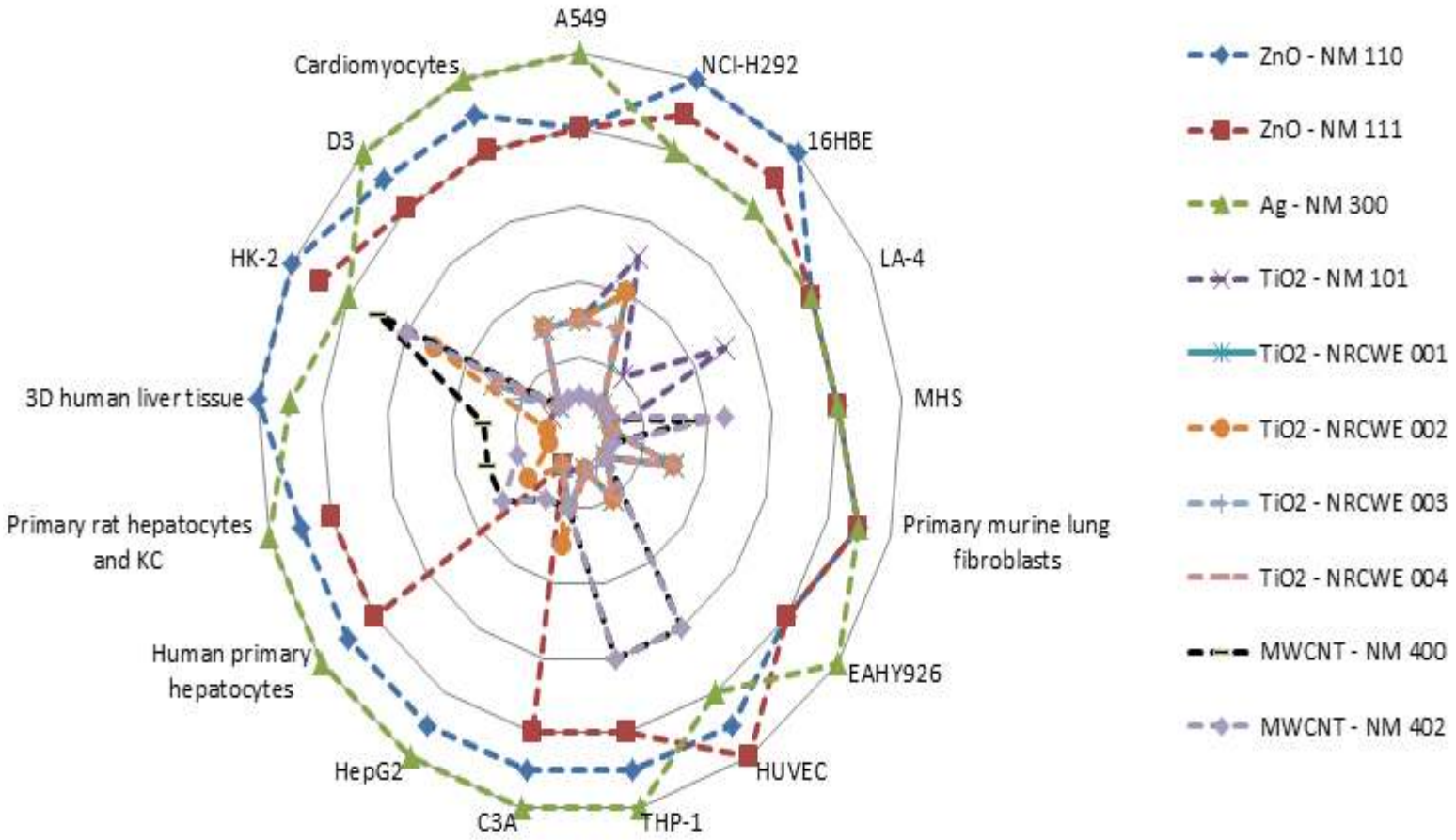
A549
NCI-H292
16HBE
LA-4
MHS
Primary murine lung fibroblasts
EAHY926
HUVEC
THP-1
C3A
HepG2
Human primary hepatocytes
Primary rat hepatocytes and KC
3D human liver tissue
HK-2
D3
Cardiomyocytes
ZnO - NM 110
ZnO - NM 111
Ag - NM 300
TiO2 - NM 101
TiO2 - NRCWE 001
TiO2 - NRCWE 002
TiO2 - NRCWE 003
TiO2 - NRCWE 004
MWCNT - NM 400
MWCNT - NM 402

Figure 2

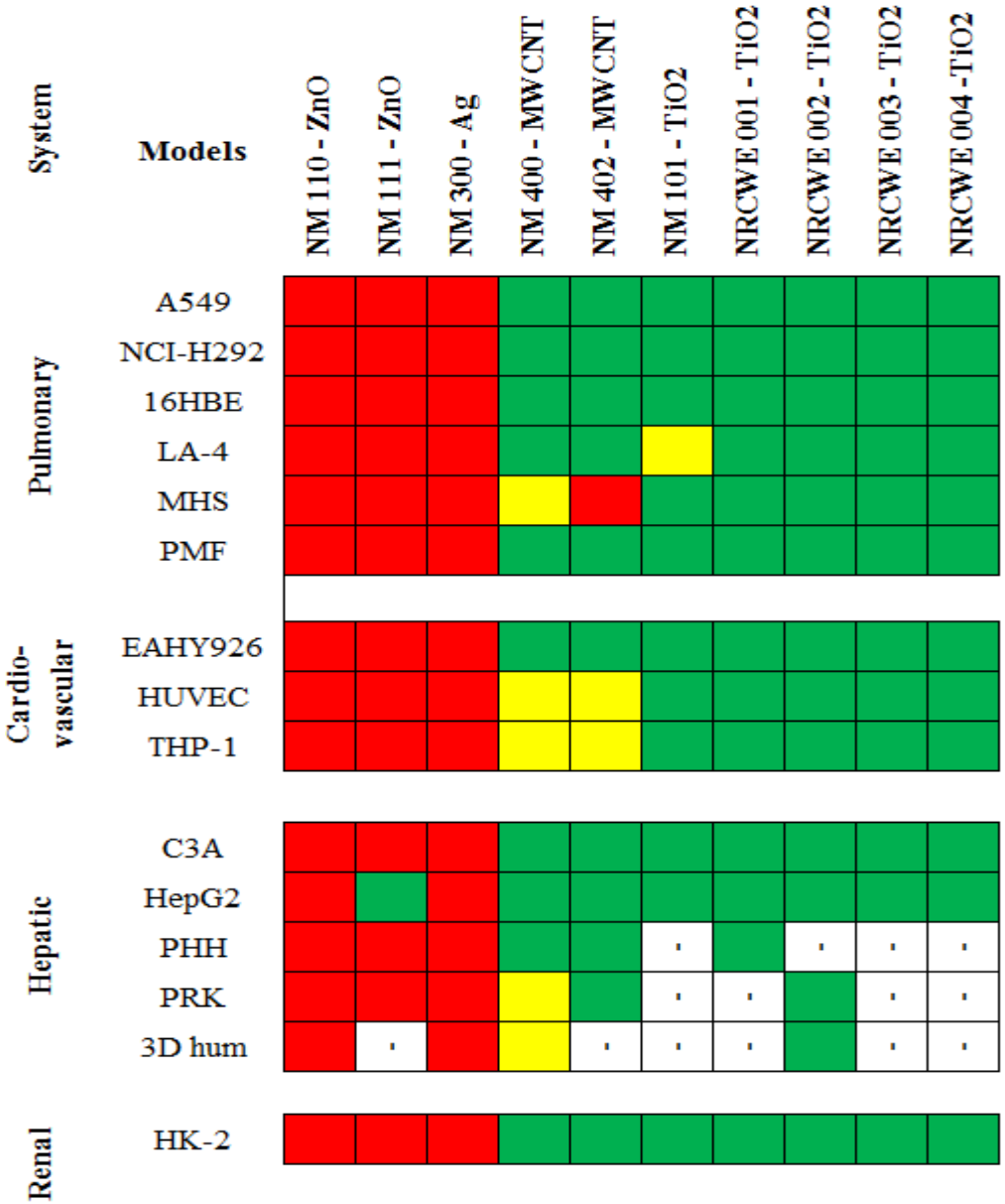
System
Models
NM 110 - ZnO
NM 111 - ZnO
NM 300 - Ag
NM 400 - MWCNT
NM 402 - MWCNT
NM 101 - TiO2
NRCWE 001 - TiO2
NRCWE 002 - TiO2
NRCWE 003 - TiO2
NRCWE 004 -TiO2
Pulmonary
A549
NCI-H292
16HBE
LA-4
MHS
PMF
Cardio-vascular
EAHY926
HUVEC
THP-1
Hepatic
C3A
HepG2
PHH
PRK
3D hum
Renal
HK-2

Figure 3

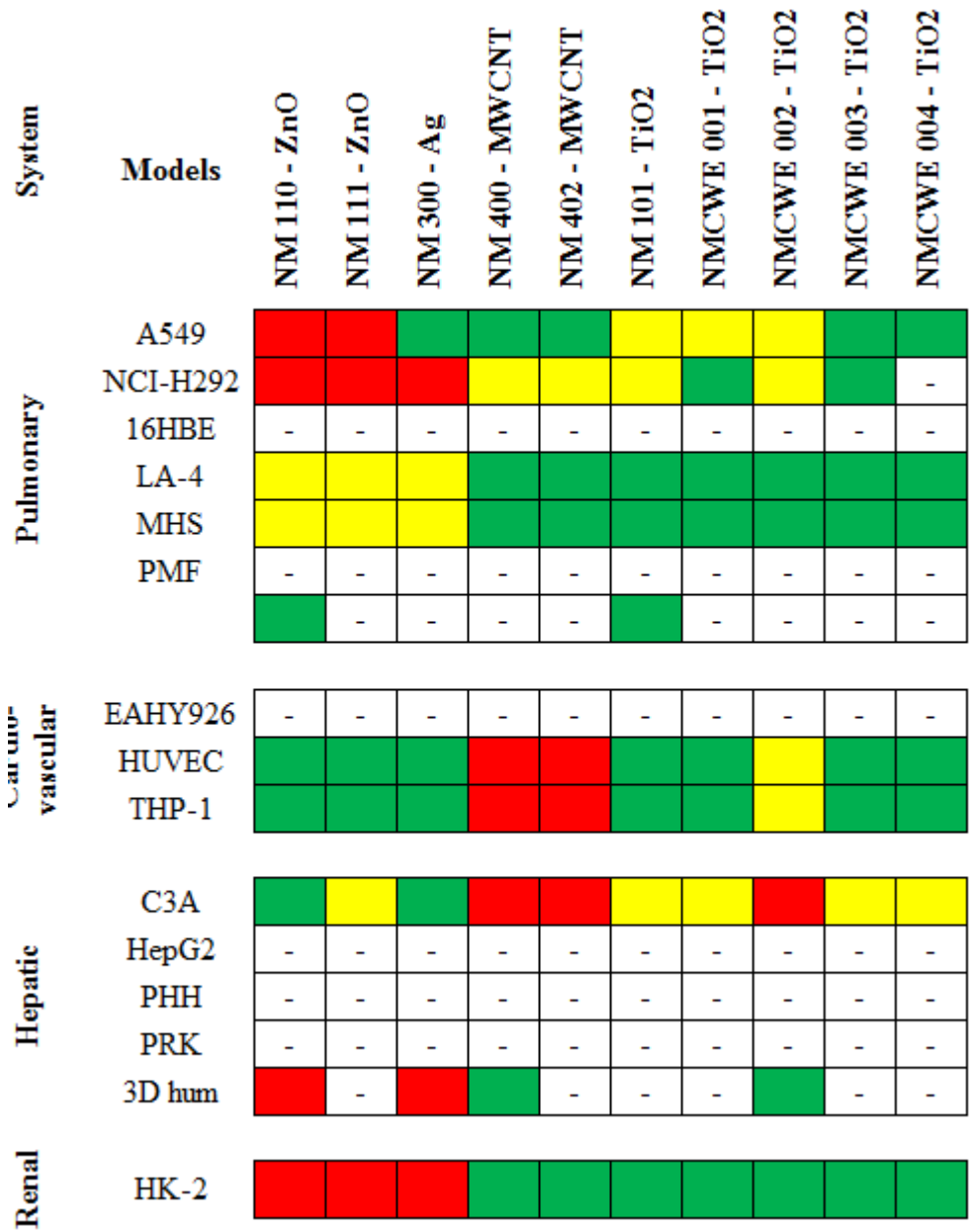
System
Models
NM 110 - ZnO
NM 111 - ZnO
NM 300 - Ag
NM 400 - MWCNT
NM 402 - MWCNT
NM 101 - TiO2
NMCWE 001 - TiO2
NMCWE 002 - TiO2
NMCWE 003 - TiO2
NMCWE 004 - TiO2
Pulmonary
A549
NCI-H292
16HBE
LA-4
MHS
PMF
EAHY926
HUVEC
THP-1
Hepatic
C3A
HepG2
PHH
PRK
3D hum
Renal
HK-2

Figure 4

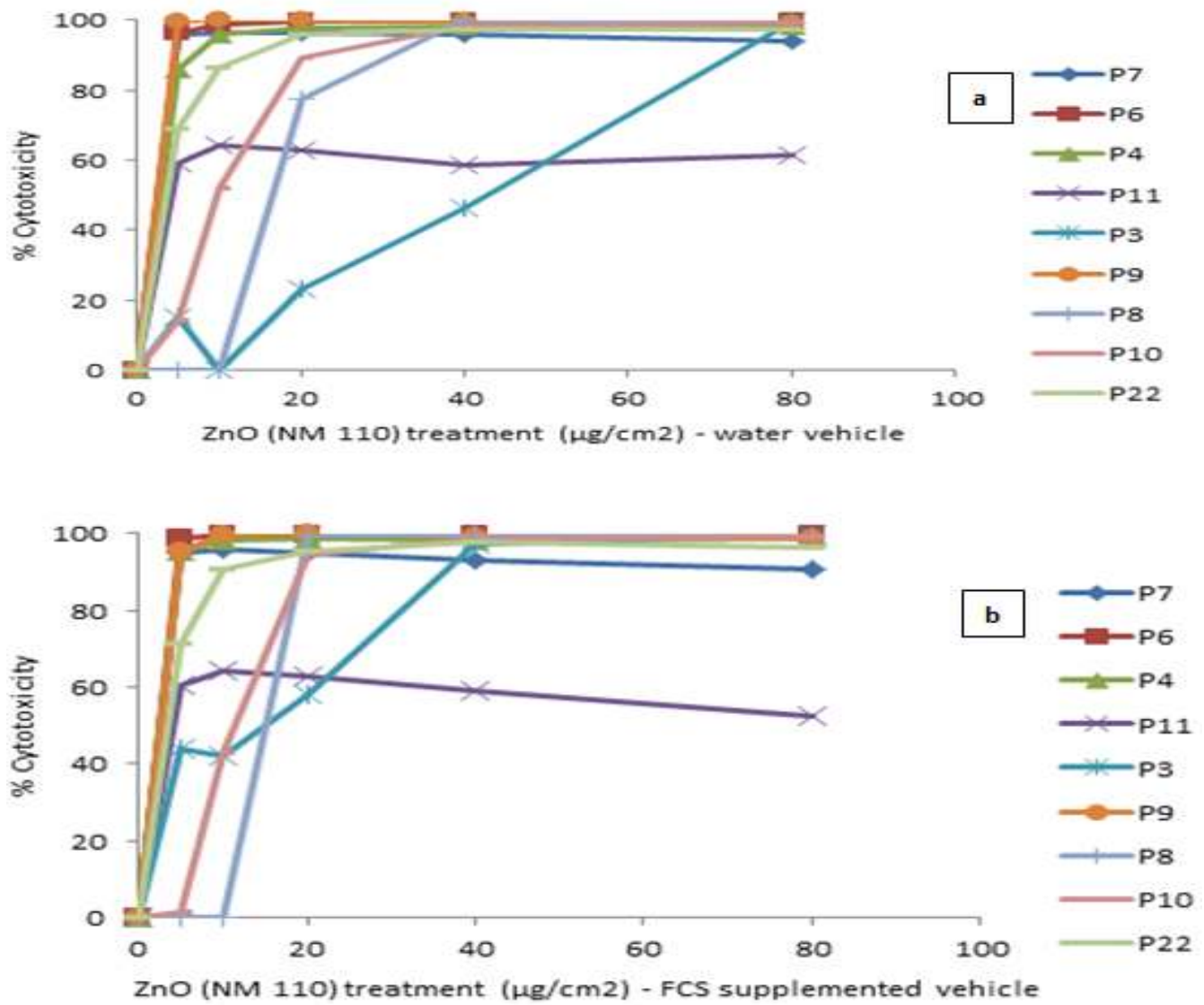
100
80
60
40
20
0
% Cytotoxicity
0
20
40
60
80
100
ZnO (NM 110) treatment (µg/cm2) - water vehicle
a
P7
P6
P4
P11
P3
P9
P8
P10
P22
100
80
60
40
20
0
% Cytotoxicity
0
20
40
60
80
100
ZnO (NM 110) treatment (µg/cm2) - FCS supplemented vehicle
b
P7
P6
P4
P11
P3
P9
P8
P10
P22

Figure 5

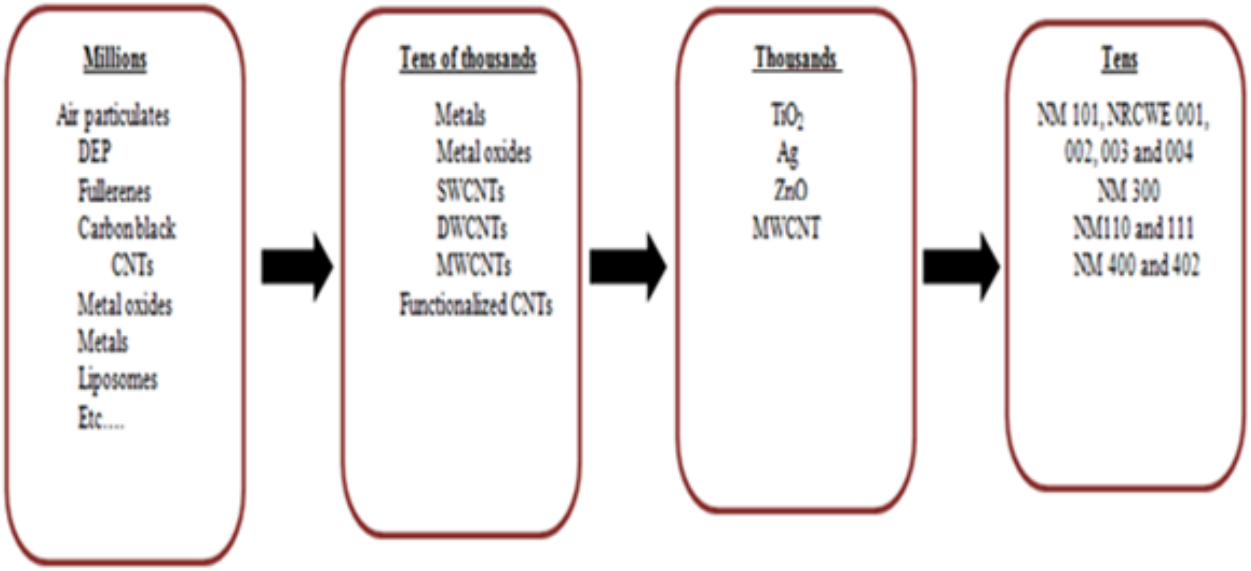
Millions
Air particulates
DEP
Fullerenes
Carbon black
CNTs
Metal oxides
Metals
Liposomes
Etc....
Tens of thousands
Metals
Metal oxides
SWCNTs
DWCNTs
MWCNTs
Functionalized CNTs
Thousands
TiO_2
Ag
ZnO
MWCNT
Tens
NM 101, NRCWE 001, 002, 003 and 004
NM 300
NM110 and 111
NM 400 and 402

Figure 6