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Report on cell type and in vitro-in vivo correlation studies for inhalation toxicity

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Table of Content

1 Description of task

Inhalation toxicity *in vitro* focuses on conducting toxicity screening experiments exploring the effects on lung cells after exposure to airborne nanoparticles (NPs). Because chronic lung diseases affect various parts of the lung, as well as various cell types, various test systems covering different types of cells will be exposed in order to get an accurate picture of the lung´s responsiveness to a chemical challenge. The air-liquid interface (ALI) technique will be used to model cell systems of e.g. pulmonary alveoli. This method makes it possible to deposit NPs directly from the gas phase on cells and comes as close as currently possible in mimicking inhalation exposure *in vitro*. Besides, other types of cell models such as cells grown as monolayer in submerged conditions are also proposed.

This task will deal with the identification of the most suitable *in vitro* model to assess inhalation toxicity. WP5 will take advantage of the unique nature of the *in vivo* experimentation carried out in WP4 (with for example titanium oxide, cerium oxide, barium sulphate and a suite of 20-40 HARNS) to perform parallel experiments which may allow for extrapolation of results in a series of implementation steps, these being:

Step 1: Perform several *in vitro* toxicity tests covering different cell types and endpoints. A comparative toxicity study will be performed with cellular systems, at least for some of the NM studied in WP4 (NM100, NM101, NM212 and NM220 and potentially nanocellulose). Different cell types (e.g. human nasal and bronchial model, human nasal model co-culture with fibroblasts, human bronchial model co-culture with fibroblasts, human alveolar-epithelial co-culture model) and different endpoints are proposed within the task, e.g. variations in uptake mechanisms and intracellular markers as candidate predictors for effects shown after inhalation such as inflammation and genotoxicity. *In vitro* end points will follow OECD recommendations to avoid interference of NMs with testing strategies (OECD ENV/JM/MONO2009/20/Review).

Step 2: Inter-laboratory study of the Cyto-TP device. In the EU-FP7 Project "Nanodevice", the Cyto-TP was developed, a thermal precipitator capable of depositing airborne NPs on cells at air-liquid interface. The Cyto-TP is used as the exposure device in an interlaboratory study, to compare toxicity data, obtained with the same air-liquid interface model cell system. Additionally, this task will reproduce the aerosols of task WP4 "long time low-dose *in vivo* study", potentially enabling the partners of both WPs to compare results.

Step 3: Compare results of different cell types and endpoints (cell type correlation study)

a. Making use of NM and results from WP4, different cell types will be exposed to, for example, MWCNTs and nanoTiO_2 and examined for variations uptake mechanisms and intracellular markers as candidate predictors for effects shown after inhalation such as inflammation and genotoxicity.

b. Further *in vitro* studies will be considered depending on input from WP1.

Steps 1 to 3 will be addressed within D5.4.

Step 4: Compare results to *in vivo* toxicity study (*in vitro-in vivo* correlation study). This is the focus of the present deliverable (D5.5).

a. Are cell types used *in vitro* and *in vivo* comparable?

b. Are exposure times comparable between *in vivo* and *in vitro*?

c. What are the most suitable cytotoxicity end points to correlate *in vitro* and *in vivo*?

Step 5: Develop *in vitro* screening methodology to evaluate toxicity by inhalation taking into account results from steps 1-4 (above) and WP4.

a. The *in vitro* uptake of nanomaterials in human lung epithelial cells will be examined and mechanisms of cell death will be assessed as predictive markers of inhalation carcinogenesis, making use of NM and results from WP2-4 for experimentation and validation.

b. Using nanomaterials from WP2-4 (i.e MWCNTs and TiO_2) transformation assays using human normal lung epithelial cells will be developed (under D 5.6).

c. Results from further *in vitro* studies carried out through steps 1-4 will also be considered.

Task 5.4 will get input from FP projects like QNano. *In vitro* screening methodology to evaluate toxicity by inhalation can be used in the development of the risk assessment decision tree (Task 5.7) and the regulatory framework/toolbox (Task 1.4).

2 Description of work & main achievements

2.1 Summary

The main goal of the task 5.4 was to identify suitable *in vitro* models to assess inhalation toxicology. To do this, a comparison between *in vitro* results obtained with conventional methods (e.g. submerged) and Air Liquid Interface (ALI) exposure to aerosols has been performed in a first step. Then, in a second step, a correlation has been done with *in vivo* results obtained in respiratory studies performed in WP4.

Both *in vitro* and *in vivo*, biological endpoints (cytotoxicity, inflammation, oxidative stress, genotoxicity) were determined for each methodology of exposure for the NM under study.

Qualitative (Cf. § 2.4.1 and 2.4.3) or quantitative (Cf. § 2.4.1) comparisons were performed depending on the data provided. To perform quantitative comparisons between the *in vitro* and the *in vivo*, we paid attention to the deposited dose on cells or into the lungs and we selected dose metrics compatible between all the methodologies used. The suitability of using the deposited doses to assess dose response effects was evaluated in this task. Nevertheless, as it was not planned to express deposited doses in other metrics (e.g. surface area/cm^2; number/cm^2…), the relevancy of one metrics to the other ones was not evaluated. A particular focus on comparison of the cellular uptake under *in vivo* and *in vitro* conditions was also made, as it represents the basis for addressing the question of the *vitro-vivo* relevance on a quantitative basis (Cf. § 2.4.2).

In the deliverable, correlations were extended to an oral toxicity study (Cf. § 2.4.4)., in order to assess if the general conclusions about *in vitro-in vivo* comparisons were specific to the exposure route.

On this front, the task 5.4 focused on four specific NM, including a pair wise comparison between 2 different types of (TiO_2) as follows:

1. Comparing a NM of the same nature (TiO_2) characterized by different parameters:

TiO_2	NM100	NM101
Atomic structure	Tetragonal	Anatase
Mineral polymorph	Anatase	-
Diameter	110	6
Surface area (m^2/g)	9	316

Other core NM include

NM		NM212
CeO_2	Atomic structure	
	Mineral polymorph	Barite
	Length	33
	Diameter	33
	Thickness	
	Surface area (m^2/g)	28

And nanocelullose: NFC Fine, NFC Medium-coarse, UPM Biofibrils AS, UPM Biofibrils NS, UPM Bleached Birch Pulp

NM	**Polymorph**	**Particle type**	**Diameter (nm)**	**Length (nm)**	**Inorganic coat/ass elements**	**Organic coat/ass organics (wt5)**
NFC Fine	Cellulose	nanofiber	2-15	1000-20000	no	No
NFC Medium-coarse	Cellulose	nanofiber	3->	2000-50000	no	No
UPM Biofibrils AS	Cellulose	nanofiber	5-10	500-10000	NA	pending
UPM Biofibrils NS	Cellulose	nanofiber	7-20	2000-20000	none	None
Non-NM c						
UPM Bleached Birch Pulp	Cellulose	fiber	30000	1000000	none	None

Different end points have been addressed in the different studies and these included cytotoxicity, generation of reactive oxygen species, inflammation, genotoxicity and cellular uptake. As a result, it can be concluded that though *in vivo* experimentation remains to be the methodology of reference for the evaluation of toxicity of NM, *in vitro* methodologies may be used to rank NM according to their toxic responses and estimate initial dosage for *in vivo*

experimentation (providing a correction factor have been previously studied). The combination of both *in vitro* and *in vivo* experimentation may also be very valuable to produce a complete picture of toxicological mechanisms behind NM exposure, reducing the amount of experimental animals. Several methodological key points were also highlighted in this deliverable to provide further *in vivo-in vitro* correlations and should be considered in the future. This is a prerequisite to better understand the gap existing between the *in vivo* and the *in vitro* and to better define the limits of the *in vitro* in the 3Rs process.

2.2 Background of the task

In vitro in vivo correlations

There is growing recognition that more efficient methods and strategies to assess the risk associated with inhalation of NPs are needed, suggesting an increased use of *in vitro* assays. The advantages of *in vitro* assays are numerous; they are simpler, faster, and less expensive than their *in vivo* counterparts. In addition, there are no ethical concerns. Presently, however, there is a lack of information regarding *in vitro-in vivo* correlations.

Indeed, there are several limitations inherent to *in vitro* assays systems that can make correlations difficult including; 1) comparisons of NP dose, 2) selection of cell types for simulating the lung microenvironment, 3) difficulties in simulating an aerosol exposure (from inhalation studies) in a liquid and 4) time course of effects (acute vs. chronic). Obviously, *in vitro* systems consisting of single types of cells or co-cultures, do not reflect the complexity of the human body where organs and tissues are interlinked via the circulatory system. Thus, it may not be possible to assess the kinetics of absorption, distribution, metabolism/degradation and excretion (ADME) and therefore the concentration of the NPs in different organs, although a combination of *in vitro* and *in silico* methods have been suggested to be helpful (Bachler et al., 2015). For most NPs, however, the lung is the main organ of interest, and focusing on lung allows for exposure/dose comparisons between the *in vitro* and *in vivo* situation.

In principle, the *in vitro-in vivo* correlations can be done on different levels. The comparison can be more qualitative, i.e. establishing whether the effects observed *in vivo* can be predicted from *in vitro* studies without any attempts for dose comparisons. This approach was used in one of the first attempts to systematically explore *in vitro-in vivo* correlations by Sayes and co-workers (2007), who studied the cytotoxic (LDH release) as well as inflammatory responses of rats exposed to crystalline and amorphous silica as well as to zinc oxide in nano- and micron-size. The inflammatory effects of the same substances on rat macrophages and epithelial cells exposed *in vitro* were also studied and it was concluded that no clear correlation between the *in vivo-in vitro* exposures could be observed. For example, LDH release from crystalline silica observed *in vivo* was not predicted *in vitro* and inflammatory effects observed for the fine-sized ZnO could not be detected *in vitro*. Several critical factors can contribute to such discrepancies and Rushton et al (2010) re-analyzed the results using new *in vitro* data and another dose metric, determination of the steepest slope as a measure of the response-metric (greatest response per unit dose) from *in vitro* and *in vivo* dose-response curves. Inflammatory effect per surface NP was plotted against different *in vitro* effects (such as ROS) per surface area and a good *in vivo-in vitro* correlation was found. The concept was also shown to be useful in another study testing TiO_2 NPs of different sizes (Han et al, 2012).

The *dose metric* thus appears to be a key factor in comparisons between *in vitro* and *in vivo* assays. In the case of *in vitro* studies, mass of NPs per unit volume (mass/mL) or per unit cell surface (mass/cm^2) are often used. Rather than expressing the *in vitro* dose in units of mass, Donaldson and co-workers investigated *in vivo-in vitro* correlations by expressing the dose as the surface area of the particles (BET area) per volume of liquid (cm^2/mL). Nine different NPs (CeO_2, TiO_2, carbon black, SiO_2, NiO, Co_3O_4, Cr_2O_3, CuO, and ZnO) were examined and acute pulmonary inflammation in rats exposed via instillation was compared to cytotoxicity, expression of pro-inflammatory cytokine, and haemolytic potential *in vitro*. Agreement between the assays was found to depend on the mechanism of toxicity. The *in vitro* assays predicted the *in vivo* toxicity of the ZnO and CuO NPs, which dissolve and release ions, whereas the inflammatory

response caused by other mechanisms (with CeO_2, NiO, Co_3O_4) was more difficult to predict *in vitro* (Cho et al, 2011). In another study, lung toxicity of five materials (size in parenthesis) was investigated: SiO_2 (10), CeO_2 (23), CeO_2 (88), TiO_2 (10), and TiO_2 (200). CD-1 mice were exposed by oropharyngeal aspiration at a dose of 100 µg. In addition, mouse lung tissue slices and alveolar macrophages were also exposed to the same materials. The *in vivo* results showed a pro-inflammatory response for the SiO_2 (10), CeO_2 (23) and TiO_2 (10) and similar pattern was observed in the *in vitro* assays when using $\mu g/cm^2$ as dose metric, but not when µg/mL was used (Kim et al., 2014).

The in *vitro-in vivo* correlations can also be more advanced and probably the most extensive and sophisticated correlation test was recently published by Wiemann et al (2016). This study on 18 different nanomaterials tested whether a macrophage assay correctly could distinguish between active and passive nanomaterials that had been tested in short-term inhalation studies (STIS). The activity of the materials were based on a no-observed-adverse-effect concentrations (NOAECs) in the STIS studies and the test materials were categorized as active if NOAEC was below 10 mg/m^3, otherwise the material was passive. A surface area-based dose metric was used and a threshold value of 6000 mm^2/mL was set. This value was based on *in vivo* findings and should reflect the highest "non-overload" dose. The *in vitro* effects analyzed were lactate dehydrogenase, glucuronidase, and TNFα, assessed after 16 h, as well H_2O_2 assessed after 1.5 h. The test materials were assessed as active if ≥2 of the 4 *in vitro* parameters were positive under the threshold value. An overall assay accuracy of 95 % was achieved with all "active" materials correctly assessed as active and only one false positive (Wiemann et al., 2016).

The studies discussed above suggest that dose considerations are important. The need to consider the *cellular target dose* when comparing the toxicity of different NPs in different systems has been highlighted in recent years (Teeguarden et al, 2007). This dose is seldom considered, which is likely to contribute significantly to the poor correspondence between *in vitro* and *in vivo* responses. Teeguarden and co-workers tested the hypothesis that this correspondence could be improved in the case of nanomaterials by expressing the *in vitro* and *in vivo* dose in the same manner, i.e. as the amount of material associated with cells (target cell dose). Mice were exposed through the nose-only to an aerosol (19.9 mg/m^3) of iron oxide NPs for four hours, target cell doses were calculated and markers for inflammation analyzed. The dose was normalized by dividing the deposited mass in a given region by the corresponding surface area. The dose was also calculated by dividing the total dose deposited in a given region by the number of macrophages present. In parallel, epithelial cells and macrophages were exposed *in vitro* to the same material in liquid suspension for four hours, and the levels of inflammatory markers, as well as the cellular dose determined. These investigators found that more NPs per cell were required to induce inflammation in the alveolar epithelial cells *in vitro* than *in vivo*. However, in the case of macrophages the correspondence between target cell dose that triggered inflammatory processes *in vitro* (8-35 pg/cell) and *in vivo* (1-100 pg/cell) was good. This study thus highlights the possibility a making quantitative in *vitro-in vivo* correlations.

Indeed Donaldson and colleagues (2008) have reported similar findings for so-called low-solubility, low-toxicity particles (in this case TiO_2 and $BaSO_4$) of both nano- and larger sizes. Their hypothesis was that the cellular dose in the proximal alveolar region (PAR) of the lung that initiates inflammation can be predicted from *in vitro* studies. The PAR region includes the terminal bronchioli as well as 400-600 µm section immediately beyond the junction between the bronchiole and alveolous. Deposition of particles within this region is likely to be high due to the transition from a relatively narrow airway with airflow to an exponential increase in volume and surface area with no airflow. In that study, the surface area of the PAR region in rats, as well as the particle dose in that region leading to inflammation in the rats, were calculated. This revealed a value of approximately 1 cm^2 (particle area)/cm^2 (lung PAR area) as the critical threshold for the onset of inflammation. Interestingly, a similar threshold of 1 cm^2/cm^2 for the onset of inflammation *in vitro* (IL-8 release following exposure of A549) was clear. These investigators concluded that in the case of low-solubility, low-toxicity particles, the threshold dose for stimulation of IL-8 gene expression *in vitro* predicts the threshold dose for stimulation of neutrophil influx into the lungs *in vivo* (Donaldson et al, 2008).

As discussed in the previous section it is important to quantify the cellular target dose for comparison of *in vitro* and *in vivo* assays. One obvious reason is that the nominal concentration in the medium can differ substantially from this dose. In contrast to soluble chemicals, NPs can settle, diffuse and aggregate in various ways depending on their size, density and surface chemistry, as well as the properties of the culture medium and this can affect the particle dose reaching the cells at the bottom of the culture dish.

Cellular fate is also a highly relevant factor when trying to assess *in vivo in vitro* correlations. As discussed above, dose metrics represent one main factor to take into account when performing data extrapolation. A second point relates to the dose which actually has an effect on cells (effective dose) (Cohen et al., 2015). Different methodologies have been applied within the NANoREG project to try to get a clearer view of which is the final dose which actually has an effect on cells (the reader is referred to D5.04) and one such exercise is shown in this deliverable for case study 2 (uptake study).

Assessing cell or lung dose

The cellular dose can be measured quantitatively using e.g. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Atomic absorption spectroscopy (AAS). ICP-MS, an analytical technique used to quantify elements, combines a high-temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer. The ICP source first converts the elements in the sample to ions and these ions are then separated and detected by the mass spectrometer. AAS is based on the absorption of light by free atoms in the gaseous state for quantitative determination of elements. Using such methods it is difficult to distinguish between NPs taken up by the cells and those simply attached to the cell membrane. One the other hand, even in the absence of uptake, NPs in contact with the cell membrane can exert effects, by interfering e.g. with receptors and ion channels. Another limitation of these analytical procedures is their inability in general to distinguish between the NPs itself and ions released from the NPs.

An alternative approach is to estimate the *delivered dose* by modelling. Hinderliter and co-workers (2010) introduced the "*In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model" that incorporates both Stokes Law (sedimentation) and the Stokes–Einstein equation (diffusion) to estimate the movement of particles to the cells. One critical aspect here is the effective density and diameter of particle agglomerates in suspension (Hinderliter et al., 2010). The nominal concentration in the medium expressed on the basis of mass (µg/mL) and target cell doses expressed as the mass, particle surface area or particle number basis can differ by several orders of magnitude. As a consequence, *in vitro* hazard assessments that utilize only the nominal mass as an exposure metric can result in extensive error in cases where the number or surface area of the particles in the cells, i.e., target cell dose, determine the response. At the same time, although the ISDD model estimates the dose delivered (per unit time), once in contact with cells the cellular uptake is often an active process that can be influenced by the type of cells as well as e.g. cell density. Obviously, lower toxicity may be observed at higher cell density, since the dose per cell will be lower.

To determine the dose deposited *in vivo*, analytical approaches such as magnetic particle detection (MPD) and ICP-MS of the whole lung, can be utilized. However, assessing the regional distribution of particle deposition is much more challenging. For this purpose electron or optical microscopy can be performed on tissue sections, but this provides only semi-quantitative data and requires considerable resources (even for assessing small regions of the lung).

The difficulty of evaluating regional, tissue and cellular levels of particles experimentally has led to the development of computational models designed to accomplish this for rats and humans (ICRP, 1994, Asgharian et al., 2001) and, more recently, also for mice (Asgharian et al, 2014). The *multipath particle deposition model (MPPD)*, now widely used in the research and regulatory science communities, calculates the deposition and clearance of monodisperse and polydisperse aerosols containing particles ranging in size from ultrafine/nano-sized ($<$100 nm) to coarse particles (20 µm) in the respiratory tracts of rats and human adults and children (deposition only). Such models provide an inexpensive and rapid estimate of the internal tissue dose, which often is the missing link between external measures of exposure and response.

It should be noted, however, that current lung dosimetry models assume uniform deposition on all bronchial airway surfaces, i.e. that all epithelial cells receive the same average dose, an assumption that can be questioned. Indeed, Balashazy and co-workers (2003) computed the patterns of particle deposition in airway bifurcations and found that for micron-sized particles the local deposition in the tracheobronchial region can increase doses several hundredfold; while in the case of NPs the deposition at these bifurcational 'hot spots' was enhanced from about 5- to 60-fold (Balashazy et al, 2003, Oberdörster et al, 2009). Therefore, when estimating cellular doses, a factor of ×10 is sometimes applied to account for the uneven distribution of NPs (Paur et al, 2011).

Better correlation using air-liquid interface exposures?

Most *in vitro* studies use cells exposed to particles suspended in a liquid (i.e. mixed into the cell medium). This will lead to interactions between the cell culture medium and the NPs and to agglomeration of the NPs, which could affect the biological response. Another disadvantage of submerged cell exposure to NPs is that the motion of NPs in liquids is primarily driven by random diffusion. Consequently, under submerged conditions a substantial fraction of the NPs will either remain in the medium or be lost to the lateral walls of the cell culture vessel, thereby altering the cellular dose. Indeed, the behavior of NPs in cell culture media could be a major explanation for reported differences in NPs toxicity (Teeguarden et al, 2007). An alternative approach is to use direct exposure of the cells at the air-liquid interface (ALI). In this case, the cells are cultured on transwell membranes with no cell medium covering the cells, thus enabling cell exposure to an aerosol of particles. Moreover, such exposure is more comparable to inhalation of NPs. The cellular dose can be analyzed on-line using a Quartz-Crystal Microbalance (QCM); estimated by e.g. measuring input and output concentrations; or quantified by chemical analysis (e.g. ICP-MS or AAS).

A variety of ALI cell exposure systems have been described in the literature (e.g. Aufderheide and Mohr, 1999; Lenz et al., 2009). Most of them rely on diffusion and/or gravitational settling for deposition and since NPs tend to follow the air stream, obtaining appropriate deposition onto the cells can be challenging (Elihn et al, 2013). One option to increase the deposition onto cells is to use electrostatic deposition, where the particles are first charged and deposition enhanced by an electrostatic force generated beneath the exposure chamber. Most of the procedures described in the literature have been developed in-house, but systems such as the CULTEXs exposure technology (http://www.cultex-laboratories.com) and the VitroCell system (http://www.vitrocell.com) are also commercially available. The *in vitro* lung cell models exposed via ALI can be more or less advanced. The construction of 3D models and co-culture systems enable cell-matrix and cell-cell interactions, and are thereby more similar to the physiological situation.

2.3 Description of the work carried out

This is an inter WP deliverable and only relevant information is shown per study. The complete set of experimental results per study has been reported in their corresponding deliverables. To produce this deliverable a revision of all *in vivo* and *in vitro* studies carried out under NANoREG was initially perfomed. This scanning exercise concluded that the following studies could be introduced into this deliverable since both *in vivo* and *in vitro* data had been produced in parallel and the timeframe for data collection had allowed pairwise comparison of results. Unfortunately the long *in vivo* study with NM212 could not be included in this deliverable since full set of results will only become available after 2018. Studies included in this deliverable are reported below:

Each study is summarised below:

Three different sets of experiments, combining both *in vitro* and *in vivo* data related to pulmonary toxicity or uptake have been used for performing *in vitro-in vivo* correlations.

1. *In vivo* and *in vitro* pulmonary toxicity of TiO_2 (NM100 and NM101) and CeO_2 (NM212)

a. *In vivo*: WP4, Task 4.5.7. Acute and repeated nose-only inhalation toxicity study
b. *In vitro*: WP5, Task 5.4. Inhalation toxicity modelling/*in vitro*

2. *In vivo* and *in vitro* uptake of CeO_2
    a. *In vivo*: WP4, Task 4.4. Pattern of particle distribution in organs
    b. *In vitro*: WP5, Task 5.6. Develop a rapid high throughput screening methodology

3. *In vivo* and *in vitro* pulmonary toxicity of nanocellulose
    a. *In vivo*: WP4, Task 4.7. Acute immunotoxic and genotoxic effects of fibrous nanomaterials
    b. *In vitro*: WP5, Task 5.5. *In vitro* toxicity assays connected to regulatory questions

As an extra experimental set for this deliverable, the following study is also included

4. *In vivo and in vitro* immunotoxicity of SiO_2 (NM203).
    a. *In vivo:* WP4, Task 4.5.5. Repeated dose 90 day oral toxicity study.
    b. *In vitro*: WP5, Task 5.5. *In vitro* toxicity assays connected to regulatory questions.

2.4 Results

2.4.1 TiO_2 and CeO_2 studies (toxicity studies)

***In vivo* study**

NM to be tested were chosen among the 19 NANoREG core group and obtained from JRC or RIVM. NanoTiO_2 NM100 (JRCNM0100a) and NM101 (JRCNM0101a) were selected for their differences in surface properties (size and coating) and compared to TiO_2 NM105 which is not in the core group but which has been extensively studied for pulmonary toxicity in rats.

The initial aim of task 4.5.7 was to perform acute and sub-acute respiratory study in rats, using TiO_2 NM100 and NM101, in order, first, to compare the toxicological responses of these two TiO_2 (differing from their surface properties) and, second, to compare the results obtained *in vivo* to those obtained *in vitro*, in task 5.4 (inhalation toxicity modeling/*in vitro*).

Due to technical reasons, the inhalation toxicity study, however, was replaced by an instillation study, at doses (5, 50, 500 µg/lungs) similar to those expected for the acute and repeated inhalation study. To increase the scope of the the analysis, additional low soluble NM (CeO_2 NM212) was also included in the study as *in vitro* data were available from partners of task 5.4.

The experimental design is summarized in the table below.

Animals	Rats WISTAR RjHan:WI, male
Nanomaterials	TiO_2 NM100 and NM101, + TiO_2 NM105 as reference NM, CeO_2 NM212
Suspension protocol	MilliQ water and sonication with cup-horn
Exposure method	Unique instillation (100 µL) after hyperventilation
Theoretical dose	**500, 50, 5 µg/lung**
Exposure duration	3h, **24h**, 5d, + (35d and 90d for biodistribution and histology)
Endpoints	On bronchoalveolar Lavage fluids(BALF): **Cytotoxicity, Inflammation, Oxidative stress** (3h, **24h,** 5d) On blood smear**:** µ-nucleus assay Biodistribution: lungs, tracheobronchial nodes, spleen, liver, kidneys (35 and 90d) On animal tissue**:** Histology (35d and 90d only)
Characterization of suspensions	Dynamic Light Scattering (DLS)
Dosimetry	ICP-MS

In bold: parameters and time-points included in the *in vivo-in vitro* correlation analysis

The complete set of results obtained in the *in vivo* pulmonary toxicity study is reported in detail in Deliverable D4.15, including all the results at the different time points. Since this report is focussed on correlation between *in vivo* and *in vitro* studies, only results obtained 24h post-exposure have been selected for comparison purposes with the *in vitro* approach.

***In vitro* studies**

The cell models used were a monoculture composed only of A549 or a co-culture system composed of A549 + THP-1 (Fig. 2). Cells were exposed to 3 different TiO_2 (NM100, NM101, NM105) and CeO_2 (NM212). The opportunity to compare *in vitro* and *in vivo* data has been considered for the choice of NM in task 5.4. Details of *in vitro* experimental designs are reported in D5.4.

As no BSA was added in suspensions for aerosol generation (*in vitro* ALI exposures) and *in vivo* instillations, size distribution and aggregation state of the NM when dispersed in different vehicles using the Nanogenotox protocol (reference protocol) or INERIS protocol in milliQ water have been evaluated and compared. The resulting cellular toxicity was also studied. As reported in D5.4., size distributions and cell biological effects were similar for a given NM whatever the dispersion protocol used. So in the present deliverable, submerged *in vitro* results are reported for the dispersion protocol in milliQ water only.

At the air-liquid interface (ALI), cells were exposed to NM aerosols obtained by nebulisation of NM suspensions in milliQ water during 3 hours and then kept at the incubator for a remaining 21h (total exposure duration: 24h).

In submerged conditions, cells were exposed to NM suspensions for 24h. To assess the role of the timing of the dose delivery and of the support, an intermediate design between the ALI and submerged cultures was set-up: cells were exposed in submerged conditions in inserts for 3h and then kept in the incubator for 21h.

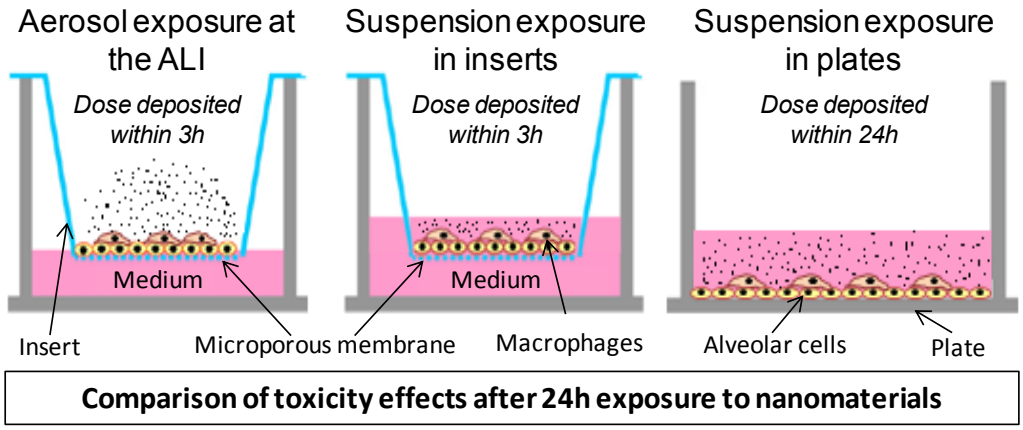


Fig 2. Different *in vitro* cell exposure protocols used to mimic *in vivo* pulmonary exposure.

At the ALI, the deposited doses on the cells were assessed by ICP-MS measurements. In submerged conditions, the deposited doses were estimated using the *In vitro* Sedimentation Diffusion and Dosimetry (**ISDD**) model, in order to take into account the sedimentation of the NM in suspension. To fill the model, the hydrodynamic diameters were measured by Dynamic Light Scattering (**DLS**) and the effective densities were measured by the Volumetric Centrifugation Method (**VCM**).

Biological parameters assessed 24h later were the cytotoxicity, the oxidative stress, by the measurement of intracellular ROS, and the pro-inflammatory properties by the measurement of the cytokine release.

***In vivo-in vitro* correlations**

In order to compare the *in vitro* to the *in vivo*, it was first necessary to select common dose metrics applicable for both methodologies. The doses are often expressed in concentrations, including mass/volume of suspension *in vitro* and mass/volume of air *in vivo* for inhalation of aerosols. However, using concentrations in mass per volume don't take into account the real contact between the NMs and the cells. This seems not really suitable for comparisons between *in vitro* and *in vivo*, more particularly if poorly soluble NMs, as their toxicity are mainly due to

their surface reactivity. Moreover, these dose metrics are not compatible between exposures to aerosols *in vivo* by inhalation or *in vitro* at the ALI and exposures to suspensions *in vitro* in submerged conditions or *in vivo* by intratracheal instillation. *In vivo*, the total mass of NMs administered by lungs, animal or per mass is often used as a dose metric. This dose metric takes into account the deposition in the overall organ, but it cannot be used *in vitro*. Nevertheless, both *in vivo* and *in vitro*, it is possible to normalize the final doses by the surface area of the deposition (fig.1), more particularly because poorly soluble NMs are toxic via surface reactivity. Doses were normalized *in vivo* by the total alveolar surface and by the surface of the cell layer *in vitro*. Moreover, as the NM used were not considered to be soluble, the release of free ions was not measured.

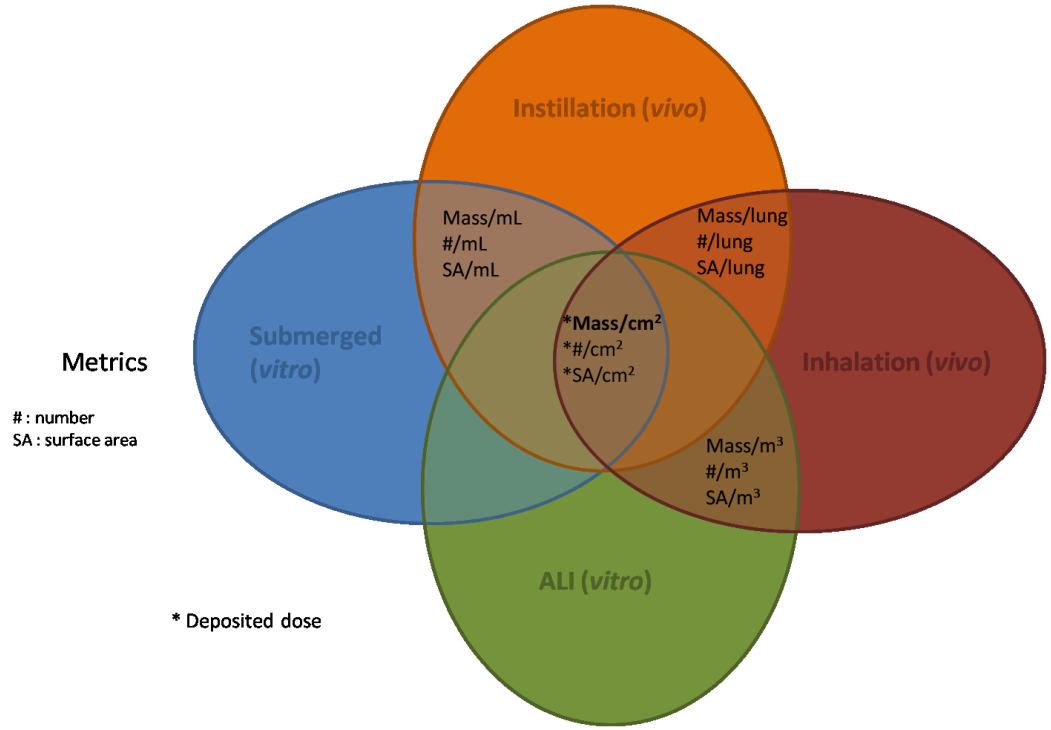


Fig1. Compatibility of the metrics used *in vivo* and *in vitro*

Both *in vivo* and *in vitro* the biological responses after 24h of exposure were thus expressed in function of the deposited doses on the cells or into the lungs in µg/cm^2. *In vivo,* doses were roughly estimated by dividing the initial lung charge (obtained by ICP-MS dosage) by the surface of rat alveolar region which has been estimated to 4000 cm^2 [1]. *In vitro*, doses were normalized by the surface of the cell layer (4,67 cm² in inserts and 2 cm² in plates).

As a reminder, all the material and methods for the *in vitro* and the *in vivo* experiments and detailed results are available respectively in D5.4 and D4.15.

According to the dose metric selected (mass/surface), LOAELs (Low Observed Adverse Effects Levels) were determined for several biological endpoints (cytotoxicity, inflammation, oxidative stress), for each methodology of exposure and NM used. These LOAELs were used to perform *vivo-vitro* comparisons. Nevertheless, as we did not observed any significant effects with the A549 monoculture exposed at the ALI (reported in D. 5.4.), *in vitro-in vivo* correlations were performed only using the *in vitro* results obtained with the co-culture of A549 and THP-1 cells.

The LOAELs (in µg/cm² for 24h exposure) determined using the co-culture model for inflammation, cytotoxity and oxidative stress are presented in the Tables 1 and 2.

[1] Stone *et al.* 1992, Allometric relationships of cell numbers and size in the mammalian lung. *Am.J.Rspir.Cell.Mol.Biol.* **6**:235-243.

The inflammatory effects were mainly observed at lower doses than cytotoxic effects and oxidative stress, both *in vivo* and *in vitro* for a given methodology (tables 1 and 2). This is less obvious for submerged exposure in plates but, as reported in D5.4., significant interactions were observed between NM and cytokines (signal decreases leading to underestimation of cytokine secretion). This may induce an underestimation of LOAELs for inflammatory effects in submerged conditions in plates. Interactions were minimized in submerged conditions using inserts due to the method used as described in D5.4.

Because inflammation was our most sensitive endpoint, *both in vitro* and *in vivo*, we focused on the pro-inflammatory responses to perform *in vivo–in vitro* correlations. According to the LOAELs determined for the pro-inflammatory effects, we ranked the different methodologies in term of sensitivity: *In vivo* (IT) > *in vitro* ALI > *in vitro* submerged in inserts > *in vitro* submerged in plates

Table 1. Lowest observed adverse effect levels (LOAEL in µg/cm² for 24h exposure) determined for inflammation. To assess inflammation, levels of IL-1b, IL-6, IL-8 or Kc-Gro, TNF-a (*in vitro* in culture medium and *in vivo* in BALF) and cytology (*in vivo* in BALF) were assessed. *In vitro* **A549 + THP-1 co-culture** were exposed at the ALI to aerosols of NM or in submerged conditions (on inserts or in plates) to suspensions of NM using INERIS dispersion protocol. *In vivo*, rats were exposed by intratracheal instillation (IT) to suspensions of NM.

LOAEL in µg/cm²		**Inflammation**				
		In vitro (cytokines IL-1β, IL-6, IL-8, TNF-α)			*In vivo* (cytokines)	*In vivo* (Neutrophils)
		ALI (3h+21h)	Submerged (3h+21h)	Submerged (24h)	IT	IT
TiO_2	NM105	**1**	**3**	**10 - 20**	**0.1**	**0.1**
	NM101	**1**	**3**	**10**	**0.1**	**0.1**
	NM100	**1-3**	> 10	> 20	> 0.1	> 0.1
CeO_2	NM212	**1-3**	**10**	> 20	**0.1**	> 0.1

significant effects allowing the determination of a LOAEL

no significant observed effects

Table 2. Lowest observed adverse effect levels (LOAEL in µg/cm² for 24h exposure) determined for cytotoxicity and oxidative stress. To assess cytotoxicity, LDH levels (*in vitro* in culture medium and *in vivo* in BALF), cell metabolism (*in vitro* in culture medium), and proteins levels (*in vivo* in BALF) were assessed. To assess oxidative stress, levels of intracellular ROS were assessed both *in vitro* and *in vivo*. *In vitro* **A549 + THP-1 co-culture** were exposed at the ALI to aerosols of NM or in submerged conditions (on inserts or in plates) to suspensions of NM using INERIS dispersion protocol. *In vivo*, rats were exposed by intratracheal instillation (IT) to suspensions of NM.

		Cytotoxicity*				Oxidative stress			
		In vitro			*In vivo*	*In vitro*			*In vivo*
		ALI	Subm Inserts	Subm plates	IT	ALI	Subm Inserts	Subm plates	IT
TiO_2	NM105	**1**	**10**	**10**	>0.125	> 3	**10**	**10**	>0.125
	NM101	> 3	**10**	**10**	>0.125	**1**	> 10	>20	>0.125
	NM100	> 3	**10**	> 20	>0.125	> 3	**10**	>20	>0.125
CeO_2	NM212	> 3	**10**	> 20	>0.125	> 3	> 10	>20	ND

*LOAEL indicated represent significant cytotoxicity > 5%.

significant effects allowing the determination of a LOAEL

no significant observed effects

Considering the LOAELs for pro-inflammatory effects, we provided a ranking of the four NMs used in our study and the NM were ranked as following:

- When comparing ALI versus submerged exposures (Table 1):

NM were ranked similarly whatever the *in vitro* exposure method used: NM105~NM101 > NM212 > NM100

- When considering *in vivo* data only (Table 1):

NM105~NM101~NM212 > NM100

Thus, TiO_2 NM were ranked similarly *in vivo* and *in vitro but :*

- CeO_2 NM appeared more toxic than expected from *in vitro* data, when considering *in vivo* results.

To conclude,we showed that:

- *In vivo* approach remains the reference method to assess pulmonary effects of poorly soluble NM.
- ALI seems to better simulate *in vivo* adverse effects, regarding biological activation levels
- *In vitro* methods seem to provide reliable results regarding the ranking of TiO_2 NM.

We also highlighted several key points which should be considered to improve the *in vitro* predictivity after acute exposure to poorly soluble NMs:

- Assessing the real mass of NM deposited on the cell surface *in vitro* is fundamental
- Using compatible and relevant dose metrics between the *in vivo* and the *in vitro* is critical
- It appears important to use more realistic cell models (macrophages ++) and exposure methods

- It seems very important to use similar timing of the dose delivery and exposure duration *in vitro* and *in vivo*

Nevertheless, some clarifications are still needed and we thought that further investigations may be necessary in the future to strengthen these conclusions. Thus, we recommend:

- To perform acute exposure of rats to NMs aerosol by inhalation using the TiO_2 NM100 and NM101 and the CeO_2 NM212
- To assess more accurately the doses *in vivo* considering the following points: which region of the lung is concerned? How to manage the non homogeneous deposition within the lungs? What are the doses at the hot spots?
- To find, if possible, other accurate dose metrics compatible between the *in vivo* and the *in vitro* (e.g. mass per cell)

2.4.2 CeO_2 inhalation study (uptake study)

The comparison of *in vivo* and *in vitro* uptake based on the knowledge on intracellular effective dose in culture cells and tissues was performed by means of ion beam microscopy (IBM) techniques. IBM as label-free imaging technique represents a unique possibility performing the spatial resolved element analysis at single cell level. IBM techniques as micro-proton induced X-ray emission (μPIXE) and micro-Rutherford backscattering (μRBS) analysis were applied to quantify the intracellular concentration of NPs in cells under *in vitro* and *in vivo* condition. Knowledge of cellular uptake under *in vivo* conditions is the basis for addressing the question of the *vitro-vivo* relevance on a quantitative basis.

***In vivo* study**

Lung tissues of control and exposed to 25 mg/m^3 CeO_2 rats from the 28 days study on inhalative toxicity and carcinogenicity (BASF) were analyzed by means of IBM. IBM was demonstrated to be a powerful imaging tool for visualization of nanomaterials and their environment in culture cells and in tissues (3rd and 5th technical reports from ULEI). One specific advantage of IBM is that it allows for quantification of NPs (NP) uptake at the level of single cells.

The NP element content and its distribution were studied in macrophages, alveolar and bronchiolar walls as well as in the interior of alveoli in lung tissues of Wistar rats (Fig.1).

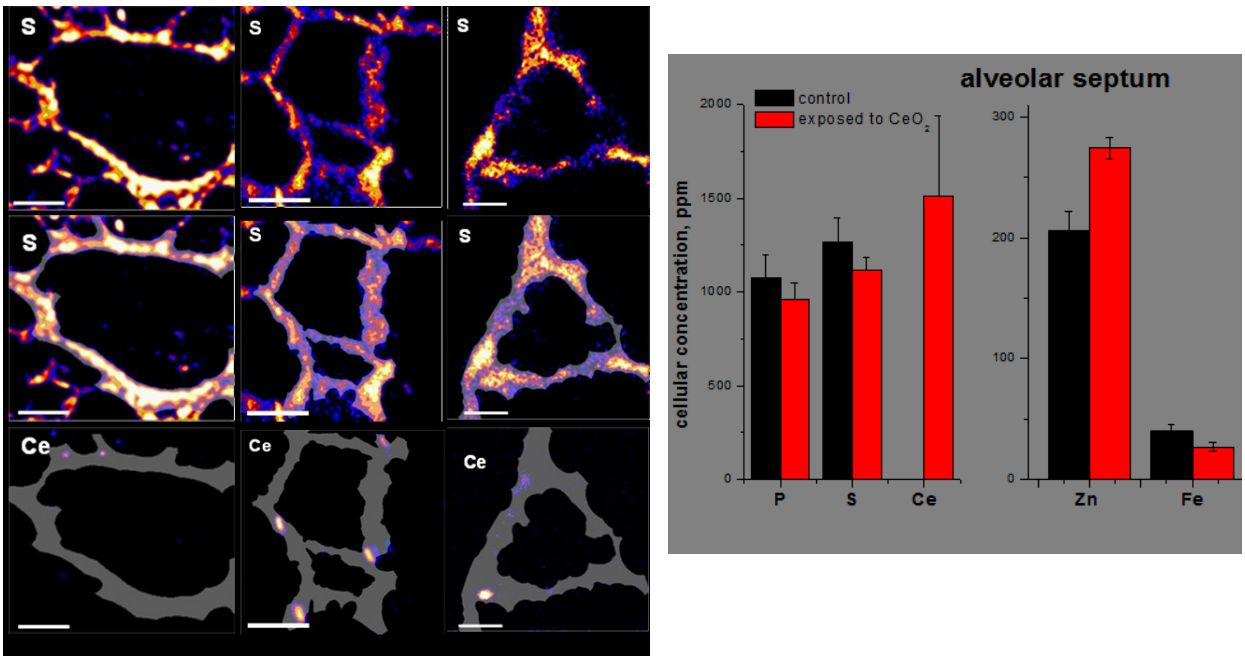


Fig 1. *Left*: µPIXE images of alveolar regions of lung tissue of exposed rat. The grey area indicates the region-of-interest, in which the cellular element content and NPs element concentration were determined.

Right: Quantification of cellular element concentrations and cerium content in alveolar septum for the control and 25 mg/m^3 groups. The elemental concentrations are given in ppm

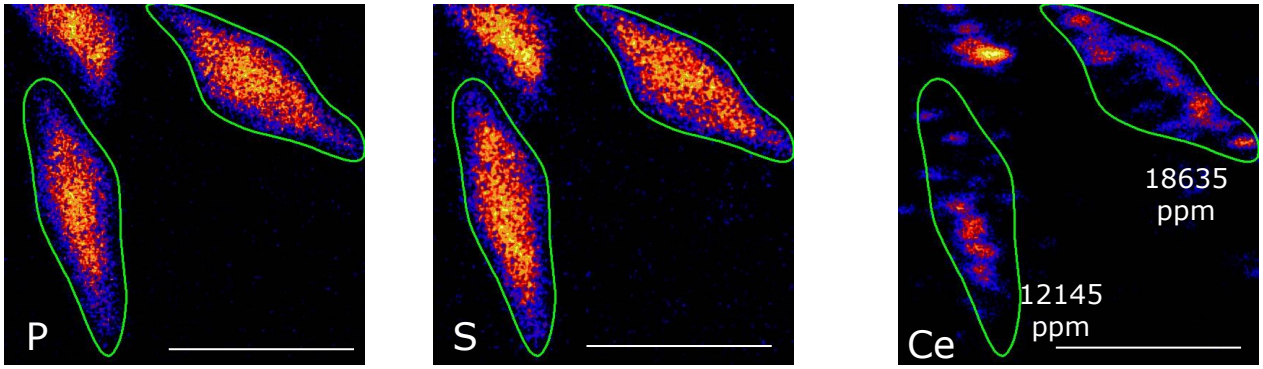
The histogram of the NP content revealed a rather inhomogeneous CeO_2 NP distribution in alveolar walls and/or alveolar capillaries. Hot spots of NPs with concentrations above 5000 ppm were detected. The average Ce concentration was about 1700 ppm in alveolar septum and in pneumocytes. The intracellular concentration of Ce in alveolar walls was comparable in magnitude with that of phosphor and sulphur. Furthermore, the applied NPs had a significant impact on the concentration of metabolically relevant intrinsic trace elements. The Zn concentration increased by 20-25%, whereas the Fe concentration decreased by about 30% in alveolar septum of exposed animals.

The NPs were mainly found in macrophages as well as in residues of macrophages located inside alveoli. The mean Ce loading of intact macrophages reached very high levels of about 26000 ppm. These findings may lead to the establishment of local threshold concentrations of accumulated nanomaterials, at which the onset of pathological processes cannot be excluded.

***In vitro* studies**

A549 cells were cultivated on superfibronectin coated polypropylene (PP) foil. After methanol fixation, the culture cells exposed to CeO_2 NPs over 24 h were analyzed by means µPIXE and µRBS. The element content and intracellular distribution of NPs at a single cell level were studied and compared to the cellular uptake from the 28 day CeO_2 inhalation study of Wistar rats.

A)



B)

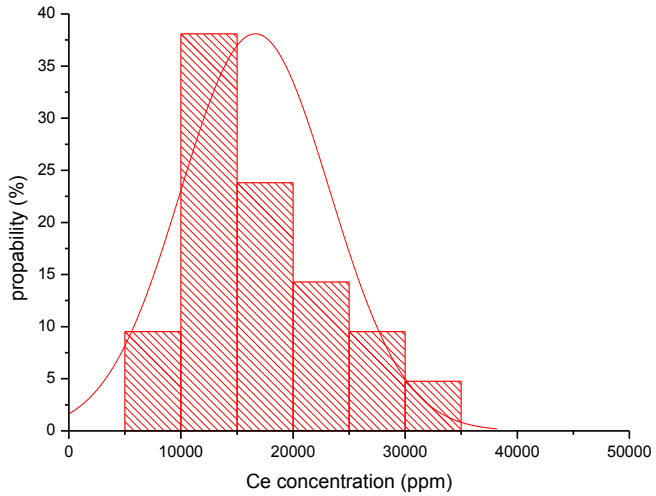


Fig 2. A: µPIXE analysis of A549 cells exposed to 10µg/ml CeO_2 over 24 hours. The bar indicates 25 µm.
B: Histogram of NP content in 21 A549 cells. The single cell concentration is expressed in part per million, ppm.

Phosphor (as main component of lipid membranes, DNA & RNA, energy carrier) and sulfur (as component of amino acids, coenzymes and proteins) elements were mapped to visualize single cells. Fig. 2 shows the P , S and Ce element distributions of cells exposed to 10µg/ml CeO_2 over 24h The Ce element concentration in 21 treated cells was calculated by using RBS und PIXE spectra extracted from individual cells.

Histogram of NP cellular content in Fig. 2B shows that the NPs were homogeneously distributed over cell assemble. 38% of the A549 cells contained about 15000 ppm and half of them about 20000 ppm of Cerium. The average and median concentrations amount to 16246 ppm and to 17140 ppm, respectively.

***In vivo-in vitro* correlations**

The comparison of *in vivo* and *in vitro* uptake results is summarized in Fig. 3. The mean NP concentration in septum of single alveola was by a factor of 10 lower then as in *in vitro* experiments in culture A549 cell at relative low applied concentration of CeO_2 NPs of 10µg/ml. Even the concentration in "hot spots" in alveolar septum was by a factor 3 lower.

The comparison of *in vitro* and *in vivo* intracellular concentrations has a fundamental meaning for addressing the question of the *vitro-vivo* relevance on quantitative basis. There could be two way for assessing *vitro-vivo* relevance question: i) intracellular concentration under *in vitro* condition can be matched to *in vivo* concentrations by means of adjusting the applied dose; i) comparison of toxic effects *in vitro* and *in vivo* on the basis of knowledge on intracellular effective dose in culture cells and tissues.

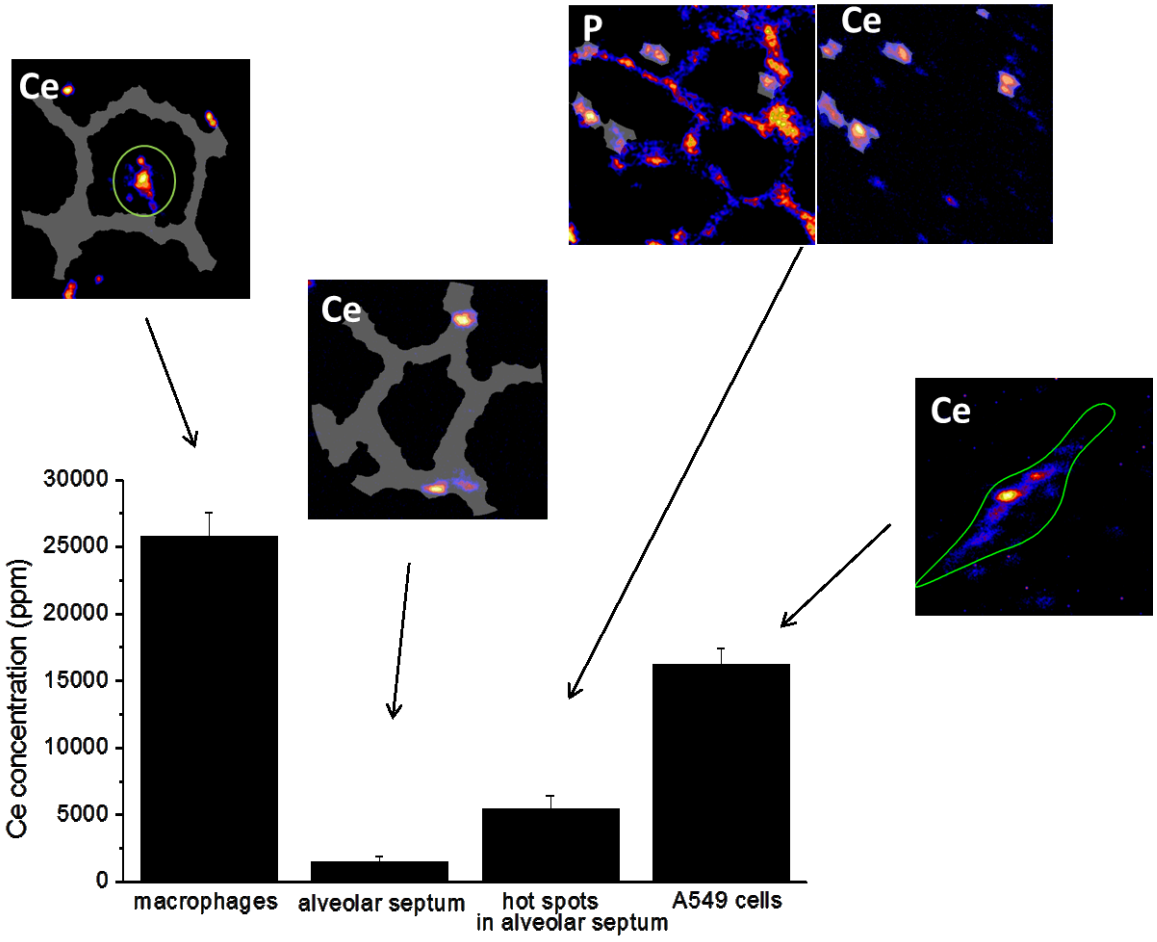


Fig 3. Uptake, cellular localization and distribution of CeO_2 NPs in lung tissue slices from 28 day CeO_2 inhalation study of rats as well as in culture A549 cell at applied concentration of CeO_2 NPs of 10µg/ml . The cellular concentrations were measured in selected region of interest.

2.4.3 Nanocellulose studies (toxicity studies)

***In vivo* study**

The present study focused on assessing the genotoxic and immunotoxic effects of nanofibrillar cellulose (NFC) materials provided by Stora Enso and UPM-Kymmene, as part of NFC hazard identification. The *in vivo* model was used to investigate responses 24 h and 28 days after a single administration to the lungs of C57BL/6 female mice (doses 10, 40, 80 and 200 µg/mouse for genotoxicity and doses 10 and 40 µg/mouse for inflammation). Epithelium alveolar surface in mouse lungs has been reported to be around 500 cm^2 [2] so doses can be roughly estimated to be from 0,02 to 0,4 µg/cm^2.
Genotoxicity was assessed *in vivo* in BAL fluid cells and in the lung cells by the comet assay and in bone marrow erythrocytes by the micronucleus assay. BAL was also used to assess the recruitment of inflammatory cells in airways. Histolopathological examination of the lung tissue was performed by analyzing Hematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS) and Picro Sirius Red (PSR, 28 d samples only) -stained tissue sections. mRNA expression levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, anti-inflammatory cytokine IL-10, T helper (Th) 2 type cytokine IL-13 and Th1 type cytokine IFN-γ were assessed after 24 h while IL-1β, TNF-α, IL13 and fibrosis related cytokine TGF-β were assessed after 28 days by RT-qPCR from the lung tissue.

Results

In vivo studies revealed genotoxicity that was not observed in the *in vitro* assays. Most of the tested materials induced DNA damage, as seen in the comet assay (Table 1**.**). This effect was clearly dose-dependent for NFC material 2 in lung cells. No systemic genotoxic response in the bone marrow was seen in the micronucleus assay.

Test material	**Genotoxicity**					
	Comet assay				MN assay	
	24 h		28 d		24 h	28 d
	Lung	BAL	Lung	BAL		
1	-	-	+	+	-	(+)
2	++	+	++	+	-	-
3	-	-	-	-	-	-
4	-	-	+	+	-	-
5	-	-	+	+	-	-

Table 1. Summary of the *in vivo* genotoxicity results. Legend -, negative; (+), equivocal; +, positive; ++, highly positive

All tested materials were able to trigger recruitment of neutrophils after 24 h. NFC materials 2 and 4 induced a clearly stronger effect compared with other NFCs. In addition, materials 2 and 4 induced a slight influx of eosinophils, and also a minor lymphocyte infiltration was observed in response to material 2 after 24 h (Table 2.) 28 days after the exposure, only the presence of macrophages and no significant increases in the numbers of other cell types was seen in BAL, indicating that the inflammation had resolved.
Data obtained from mRNA expression analysis supported the results of the cytological and histological assessments (Table 2**.**). NFC material 2 induced the strongest response, as it triggered the expression of a wide range of cytokines including IL-1β, TNF-α, IL-6, IL-10 and IL-13 after 24 h. Material 1 triggered a significant elevation of IL-1β and IL-6 expression. Material 3 was able to trigger an increased expression of IL-6 and IL-13, while material 4 induced the expression of IL-1β, TNF-α, IL-6 and IL-13 cytokines. The bulk-sized reference material (5) was

2 Stone *et al.* 1992, Allometric relationships of cell numbers and size in the mammalian lung. *Am.J.Rspir.Cell.Mol.Biol.* **6**:235-243.

able to trigger mRNA expression of IL-1β and IL-6, suggesting that also bulk-sized cellulose is able to cause some pro-inflammatory responses.
mRNA expression levels of IL-1β, TNF-α, IL-13 and TGF-β were assessed 28 days after the exposure. None of the cellulose materials induced the expression of the measured cytokines, except for a minor IL-13 expression induced by NFC materials 1 and 4. The mRNA expression results support the cytological and histological findings, suggesting that the inflammation observed after 24 h resolved by day 28.

Test material	**BAL –cells**								**mRNA expression**													
	Macr.		Neutr.		Eos.		Lymph.		IL-1β		TNF-α		IL-6		IL-13		IL-10		IFN-γ		TGF-β	
	24 h	28 d	24 h	28 d	24 h	28 d	24 h	28 d	24 h	28 d	24 h	28 d	24 h	28d	24 h	28 d	24 h	28 d	24 h	28d	24 h	28 d
1	-	-	+	-	(+)	-	-	-	+	-	-	-	+	NA	-	-	(+)	NA	-	NA	NA	-
2	-	-	++	-	+	-	(+)	-	+	-	++	-	+	NA	+	-	+	NA	-	NA	NA	-
3	-	-	+	-	-	-	-	-	-	-	-	-	+	NA	(+)	-	-	NA	-	NA	NA	-
4	-	-	++	-	(+)	-	-	-	+	-	+	-	+	NA	+	-	-	NA	-	NA	NA	-
5	-	-	+	-	+	-	-	-	+	-	-	-	+	NA	-	-	-	NA	-	NA	NA	-

Table 2. Summary of the *in vivo* immunotoxicity results performed with NFC. Legend (-), negative; (+), equivocal; +, positive; ++, highly positive; NA=not assessed

***In vitro* studies**

Summary of work

In vitro genotoxicity of four nanofibrillar cellulose materials (1-4) and bulk-sized reference material (5) was assessed in human bronchial epithelial (BEAS 2B) cells by the comet assay (24-h exposure, doses 0-250 µg/cm^2) and by the cytokinesis-block micronucleus assay (48-h exposure, doses 0-250 µg/cm2). These assays were preceded by cytotoxicity studies using the 24-h and 48-h time points. Immunotoxic effects *in vitro* were investigated in human THP-1 derived macrophages at 1, 10, 100 and 1000 µg/ml. Cytotoxicity, mRNA expression and protein secretion of pro-inflammatory cytokines IL-1β, and TNF-α were assessed after 3-h, 6-h and 24-h exposure

Results

The NFC materials tested did not induce cytotoxic effects, DNA strand breakage or chromosomal damage (micronuclei) *in vitro* in BEAS 2B cells. NFC material 2 induced a dose- and time-dependent decrease in THP-1 cell viability and up-regulated mRNA expression (Table 3) and protein secretion (Table 4) of pro-inflammatory cytokines *in vitro*. Further studies are ongoing to clarify the reason behind the observed effect associated with exposure to material 2.

Test material	**Cytotoxicity**					**Genotoxicity**	
	Trypan Blue (BEAS-2B)		LDH (THP1)			Comet assay	MN assay
	24 h	48 h	3 h	6 h	24 h	24 h	48 h
1	-	-	-	-	-	-	-
2	-	-	+	+	+	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	+	-	-	-	(+)	(+)

Table 3. Summary of the *in vitro* cytotoxicity and genotoxicity results performed with NFC. Legend (-), negative; (+), equivocal; +, positive; ++, highly positive

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 310584

Test material	Immunotoxicity											
	IL-1β mRNA			TNF-α mRNA			IL-1β protein			TNF-α protein		
	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h
1	-	-	-	-	-	-	-	-	-	-	-	-
2	+	++	++	+	+	+	++	++	++	++	++	++
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. Summary of the *in vitro* immunotoxicity results performed with NFC. (-), negative; (+), equivocal; +, positive; ++, highly positive

***In vivo-in vitro* correlations**

From a global evaluation of the genotoxicity and immunotoxicity results obtained in the *in vivo* instillation study in mice with NFC, the following conclusions can be gathered. Conclusive inflammatory and genotoxic effects were observed with strongest response for NFC material 2: NFC materials, except one, caused DNA damage in lung or BAL cells, as determined by the comet assay. For one NCF, the effect was dose-dependent in lung cells both 24 h and 28 days after the exposure. None of the NFCs was shown to possess systemic genotoxic properties as measured by the micronucleus assay in bone marrow. Two NFCs showed activation of inflammatory response. One of them induced a decrease in cell viability and up-regulated mRNA expression and protein secretion of pro-inflammatory cytokines *in vitro*. Furthermore, the recruitment of neutrophils and eosinophils in BAL and lung tissue, and mRNA expression of several cytokines was found in response to this NFC after 24 h *in vivo*. The other material did not activate macrophages *in vitro*, but showed similar effects as the first material *in vivo*. The remaining two NFCs caused a mild acute inflammatory response in the mice. None of the NFCs caused prolonged inflammation.

Regarding the *in vitro* approach, the results highlighted the capacity of one NFC (material 2) to induce cytotoxicity and modulate inflammatory response in the monocyte/macrophage THP-1 cell line. No clear effects were observed with the bronchial epithelial cell line BEAS-2B.

Undoubtedly, the instillation *in vivo* study is able to yield much more sensitive and refined information on the effects of pulmonary exposure to NFC. *In vitro* approach underlines the importance of the cellular model used and shows less sensitivity than *in vivo* approach. However, both approaches highlighted the particular toxicity of NFC material 2.

As a conclusion, the combined analysis of the two methodologies supports the fact that *in vivo* approach remains at that time the reference strategy to characterise NMs immunotoxicity and genotoxicity. *In vitro* approach may provide valuable information regarding the relative ranking of NM if the cell model used is sensitive enough.

2.4.4 Nanosilica study

***In vivo* study**

NM used

NM to be tested was chosen among the 19 NMs core group and obtained from JRC. Pyrogenic SAS NM203 (JRCNM02003a) was selected for oral testing due to its relevance in food safety (it corresponds to the food additive E551) and successfully tested in short-term oral studies in

rodents. The opportunity to compare *in vitro* and *in vivo* immunotoxicity (Task 5.5 in WP5) has been also considered.

Size distribution and aggregation state of the NM when dispersed in different vehicles using the Nanogenotox protocol (reference protocol) have been evaluated. The characterization work has been done using FFF-UV-MALS-ICP-MS as the analytical technique.

Route of exposure

A repeated-dose 90-day oral toxicity study in rat has been carried out on the basis of the OECD TG 408. This protocol has been considered by EFSA (EFSA, 2011) as the minimum requirement to identify hazards and obtain dose-response data to characterize the hazards of nanomaterials relevant for food safety.

Animal model: young adult Sprague Dawley rats, 10 sex/group; oral administration by gavage 5 days/week for 90 days.

Dose levels of NM203:

- Control, vehicle only
- *2 mg/kg bw*
- *5 mg/kg bw*
- *10 mg/kg bw*
- *20 mg/kg bw*
- *50 mg/kg bw*

Endpoints addressed

A comprehensive panel of general and specific toxicity endpoints have been addressed. Among these, immunotoxicity and immunological function have been studied by ex vivo assays on treated and control rats. Organs and tissues for the isolation and analysis of immune cell populations and/or soluble factors (blood, spleen, lymph nodes) have been obtained from groups of 5/6 rats of both sexes at the end of the exposure period (day 90). The following parameters have been evaluated, taking into account the suggestions of the ICH S8 Guideline on Immunotoxicity evaluation as well as the data already available in literature on immunomodulatory/immunotoxic effects of nanomaterials:

- alterations in immune system organ weights and histopathology (e.g. changes in thymus, spleen, lymph nodes) have been recorded;
- function of resident peritoneal macrophages has been assessed by *in vitro* LPS-induced NO and cytokine production;
- beside central immune organ (spleen), the lymph node draining the exposure site (mesenteric lymph node, MLN) has been selected to evaluate local effects, according to the suggestions of the ICH S8 Guideline;
- mitogen (PHA)-induced lymphocyte proliferation and lymphoid population (monocytes, T cells, B cells, NK cells) analysis by FACS analysis have been assessed in spleen and MLN;
- blood counts have been performed soon after blood collection and sacrifice, with particular attention to hematologic changes such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis or lymphopenia/lymphocytosis;
- individual serum samples have been collected and stored to evaluate levels of serum total immunoglobulins (IgM, IgG, IgA isotypes), and inflammatory cytokines (leptin, IL-6).

As far as possible, the immunological and immunotoxicity studies have been performed in compliance with Good Laboratory Practice (GLP).

Results

The complete set of results obtained in the *in vivo* oral toxicity study is reported in details in the Deliverable D4.11, including all the results of immunotoxicity evaluation. Since this report is

focussed on correlation between *in vivo* and *in vitro* studies, only results regarding the function of rat peritoneal resident macrophages have been selected for comparison purposes with murine macrophage cell line studied *in vitro* approach.

LPS-induced NO production by rat macrophage

The effect of NM203 oral exposure on the ex vivo LPS-induced NO production has been assessed on peritoneal resident macrophages obtained by post-mortem peritoneal washing procedures (Fig. 1). No significant effects have been observed in female rats, whereas male rats showed a significant increase at 5mg/kg dose. Basal NO production has been recorded at different doses in both sexes.

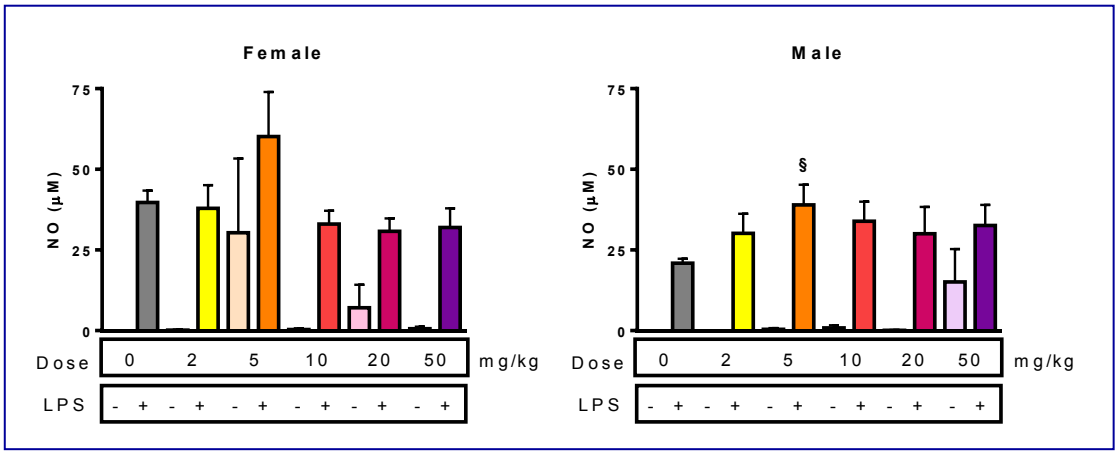


Fig 1. See above text. § $p < 0.05$

LPS-induced cytokine production by rat macrophage

The production of IL-6, TNFα and IL-10 cytokine has been studied in the same experimental setting (Fig. 2). Also in this case no significant effects have been observed in female rats. At least for selected doses, female experimental groups showed higher intra-group variability, probably affecting the significance of potential differences with control group. At the dose of 5 mg/kg, marked basal production (i.e. in the absence of LPS stimulation) of the three cytokines has been recorded. On the other hand, male rats showed significantly higher production of IL-6, associated with a significant reduction of IL-10 (10 mg/kg group), and a significant reduction of TNFα (20 mg/kg group). The highest treatment dose (50 mg/kg) was associated with basal production of all the three cytokines in male rats.

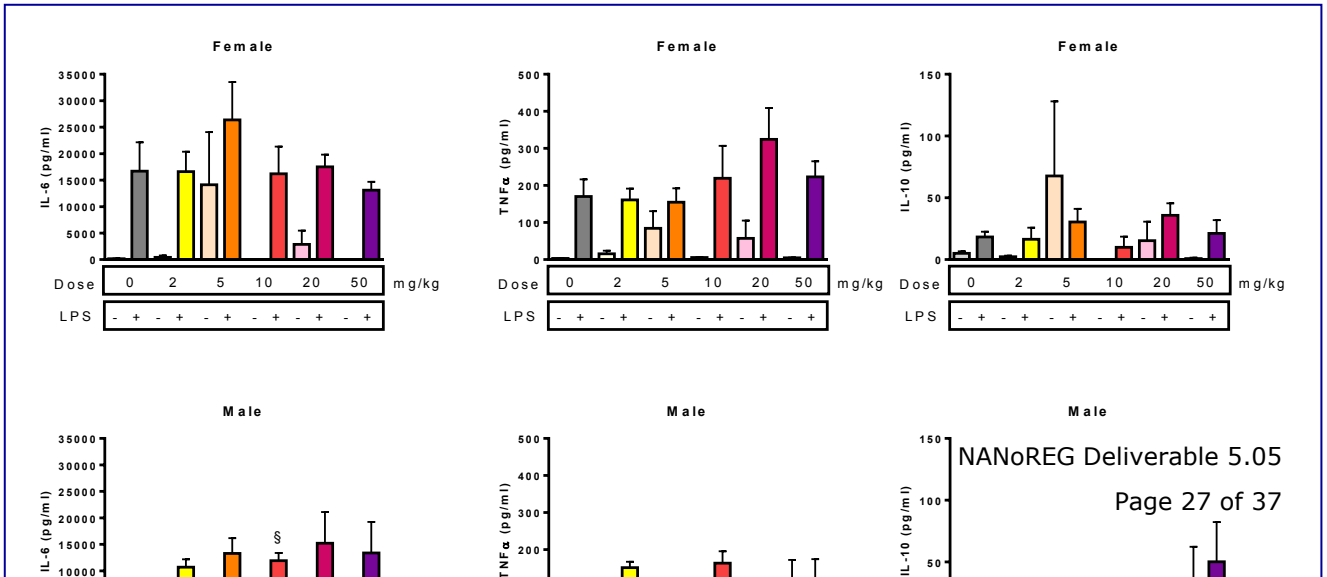


Fig. 2 See above text. § $p < 0.05$

***In vitro* studies**

NM used

Pyrogenic SAS NM203 (JRCNM02003a) was selected due to the opportunity to compare *in vitro* and *in vivo* immunotoxicity (Task 4.5.5 in WP4).

A batch dispersion of 2.56 mg/mL NM203 NPs (NPs) was prepared following the NANOGENOTOX dispersion protocol (NANOGENOTOX Joint Action). NM203 was suspended by probe sonicator calibrated according to the "SOP for probe sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *in vivo* toxicological testing". Time of sonication determined by calorimetric method for our sonicator (Vibracell, 750W, 20 kHZ, amplitude 20 %, probe sonicator: 13 mm) is 8 minutes and 53 sec.

Characterisation of batch dispersions and of batch dispersions diluted at the culture concentrations in complete DMEM and in DMEM w/o PR was performed, at the beginning and the end of treatment, by DLS analysis at 25 °C using a Malvern Zetasizer Nano according to the NANoREG refined NANOGENOTOX dispersion protocol and the "SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)".

In one out of three experimental replicates, TEM analysis has been performed as recommended in the "NANoREG Technical Guidance document for characterization in toxicological testing". Samples of batch dispersions and of batch dispersions diluted at the culture concentrations in complete DMEM and in DMEM w/o PR, at the beginning and the end of treatment, were prepared according to "Proposed method for preparation of TEM specimens suitable for qualitative analysis of nanomaterials dispersed in biological medium" provided by Partners 31 (CNR) and 41 (ECAMRICERT). Samples have been analysed by Dr. Alessandro Ponti (Institute of Molecular Science and Technologies, CNR, Milan, Italy, Partner 31).

Cell type

Mouse macrophage cell line RAW 264.7 (ECACC 91062702, derived from blood monocyte/macrophage cells) has been obtained by Sigma Italia. After initial thawing, cells were expanded in semi-adherence in DMEM complete medium containing 10% FBS. Several identical aliquots were frozen at the fourth passage to be used in each of the functional experimental replicate. Cell aliquots were thawed five days before exposure to NMs, expanded in semi-adherent culture, and plated in 24-well dishes the day before exposure at the conditions described for each endpoint. Two different media were used: DMEM complete medium for apoptosis/necrosis and cytokine production assays, or DMEM complete medium without Phenol Red (DMEM w/o PR) for NO production evaluation to avoid interference with Greiss Reagent assay.

Doses and exposure times

On the basis of the results obtained in the MTS cytotoxicity assays, performed according to the SOP developed by NanoValid FP7 Project for THP-1 cells with some modifications, the 10 and 1 ug/ml concentrations of SiO_2 NPs to be used in the functional assays have been defined:

The following culture conditions were selected for each endpoint:

Endpoint	**Time (h)**	**Medium**
Apoptosis/Necrosis	24	DMEM
IL-6 and TNFα secretion	24	DMEM
NO production	48	DMEM w/o PR

Endpoints addressed

To assess the impact of SiO_2 NM203 NPs on the functional response of the innate immune system, three major endpoints have been selected: induction of apoptosis/necrosis evaluated by cytofluorimetric analysis (Annexin V/PI staining), pro-inflammatory cytokine secretion (IL-6 and TNFα) evaluated by commercially available ELISA systems on culture supernatants, Nitric Oxide (NO) production evaluated by Greiss Reagent Assay on culture supernatants. Preliminary cytotoxicity evaluation by MTS assay has been carried out to select subtoxic concentrations of NM203 to be used in functional assays (see below). Three experimental replicates of functional assays, as established in the work program, have been performed. The relevant SOPs for the functional assays listed above have been circulated by other Partners involved in the Task 5.5 (for apoptosis/necrosis and pro-inflammatory cytokine evaluation by Gaiker, for MTS cytotoxicity by UNamur), or obtained from Nanotechnology Characterization Laboratory (NO production). They have been adapted to the cell line used and to the applied experimental conditions.

Results

DLS characterisation

Results of the characterisation of batch dispersions and of batch dispersions diluted in culture media are reported in the following tables as mean ± SD of three experimental replicates. Separate tables have been prepared according to the different experimental conditions applied to different endpoints.

The silica NPs was used at the concentrations of 10 and 1 μg/ml. Only the higher concentration could be analysed by DLS. At this concentration of NM203 (Tables 1-3) a strong decrease in Z average values and a strong increase in PDI values were observed after dilution in culture media independently of the time, suggesting that the dispersion of this NM in such media is not effective.

Sample	**Concentration (μg/ml)**	**Time (h)**	**Z_{Ave} (nm) ± SD**	**PDI ± SD**
Batch dispersion	2560		165.8 ± 4.84	0.249 ± 0.018
DMEM	10	0	23.5 ± 5.36	0.726 ± 0.160
DMEM	10	24	21.2 ± 4.84	0.544 ± 0.147

Table 1 NM203 dispersions for Apoptosis/Necrosis assay

Sample	Concentration (µg/ml)	Time (h)	Z_{Ave} (nm) ± SD	PDI ± SD
Batch dispersion	2560		163.07 ± 1.00	0.240 ± 0.001
DMEM	10	0	27.48 ± 6.43	0.836 ± 0.187
DMEM	10	24	24.94 ± 5.83	0.696 ± 0.226

Table 2 NM203 dispersions for cytokine assays

Sample	Concentration (µg/ml)	Time (h)	Z_{Ave} (nm) ± SD	PDI ± SD
Batch dispersion	2560		163.07 ± 1.00	0.240 ± 0.001
DMEM w/o RP	10	0	32.87 ± 0.70	1.000 ± 0.00
DMEM w/o RP	10	48	28.05 ± 3.67	0.759 ± 0.211

Table 3. NM203 dispersions for NO production assay

TEM analysis

Batch dispersions at 2.56 mg/ml and batch dispersions diluted at the two concentrations used in culture (10 and 1 μg/ml) have been analysed at the beginning and the end of treatment. Preliminary results of TEM analysis are reported in Fig. 1. Particles of amorphous silica have been observed in the batch preparation (Fig. 3, panel A), as well as in both the dilutions in culture medium at time 0 at lower concentrations proportional to the sample dilution (Fig. 3, panels B 10 μg/ml and C 1 μg/ml).

At the end of the treatments (time 24 and time 48), NM203 particles have been observed only in the higher concentration sample (10 ug/ml) at time 48 (Fig. 3, panel D).

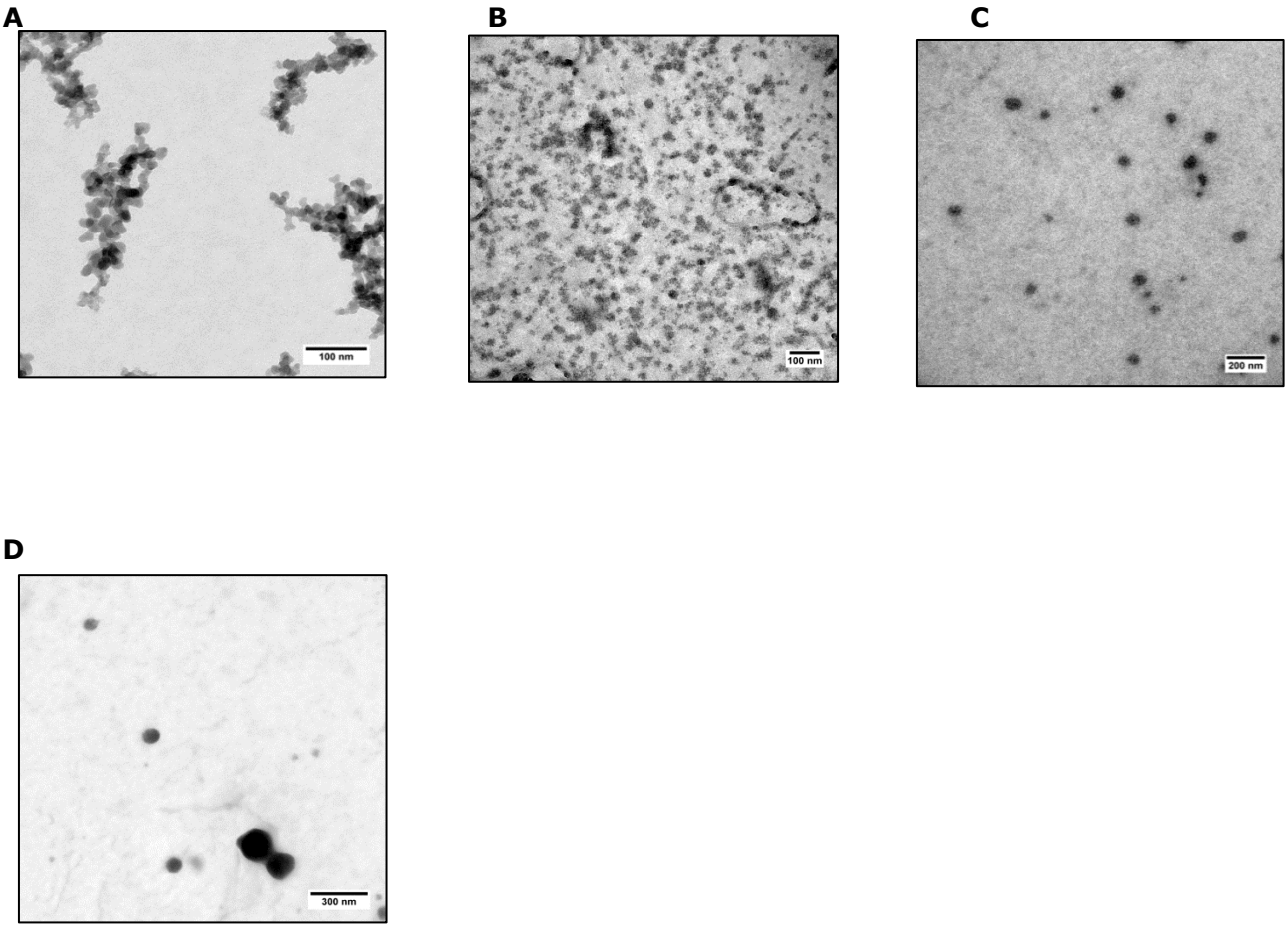


Fig. 3 See abovementioned text.

Immunotoxicity endpoints

Apoptosis/necrosis induction by 24 h *in vitro* exposure to NM203 has been evaluated by Annexin V and Propidium Iodide staining using the Annexin V-APC apoptosis detection kit. A total of 50,000 events was analysed by multiparameter flow cytometry immediately after staining. Camptothecin (4 μM) and Staurosporine (2 μM) were used as positive controls for induction of apoptosis and necrosis, respectively. Results are reported as frequency (%) of Annexin V-positive and PI-negative cells (early apoptotic cells), and Annexin V-positive and PI-positive (necrotic cells or late apoptotic cells).

As reported in Fig. 4, treatment of RAW cell with 1 or 10 μg/ml of SiO_2 NM203 did not induce significant levels of apoptosis, whereas frequencies of necrotic cells were significantly increased by exposure to both doses of NPs (Fig. 4).

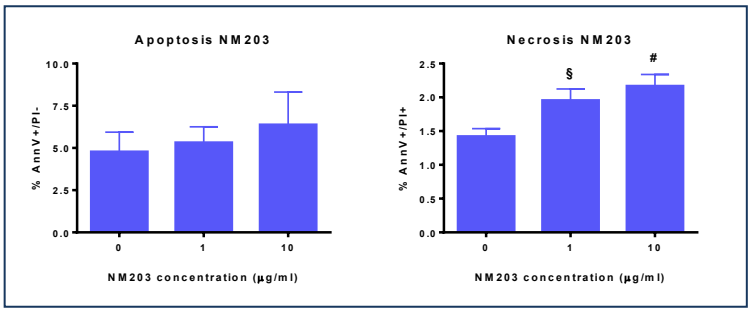


Fig 4. See abovementioned text. § $p < 0.05$, # $p < 0.005$

Levels of the proinflammatory cytokines IL-6 and TNFα have been measured in RAW cell line culture supernatants after 24 h co-culture with NM203 in the presence or absence of LPS as a positive control, by commercially available ELISA kits. This experimental system allows to evaluate both the capacity of NMs to induce inflammation and to modulate the inflammation induced by a known stimulus. No production of IL-12 cytokine has been observed in cell co-cultures with any NMs and with LPS control.

Significant production of TNFα was induced by exposure of RAW cells to the higher dose of NM203 (Fig. 5), whereas IL-6 production did not reach statistical significance. Levels were in any case much lower than those induced by positive control (LPS). No modulation of the LPS-induced production of TNFα was observed, whereas level of IL-6 was significantly increased by co-incubation with the higher concentration of silica NPs (10 μg/ml, Fig. 5, panel B).

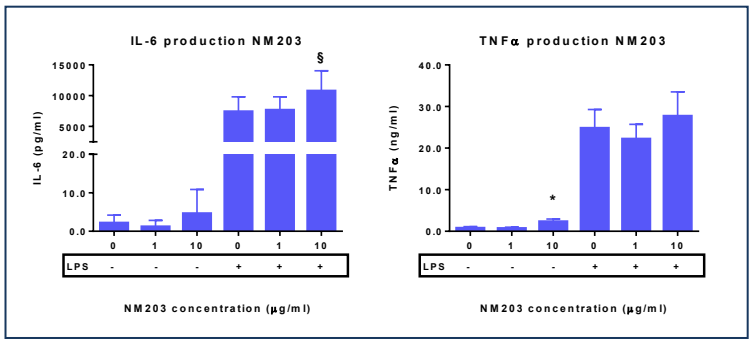


Fig. 5 See abovementioned text. § $p < 0.05$ * $p < 0.0001$

A protocol for quantitative determination of nitrite (NO_2-), a stable oxidative end-product of the antimicrobial effector molecule nitric oxide in cell culture medium, was used according to the NCL Method ITA-7_Detection of Nitric Oxide Production by the Macrophage Cell Line RAW264.7. The protocol is used to evaluate the capability of nanomaterials to induce nitric oxide (NO) production by macrophages. NO secreted by macrophages has a half-life of seconds; it interacts with a number of different molecular targets, resulting in cytotoxicity. In the presence of oxygen and water, NO interacts with itself to generate other reactive nitrogen oxide intermediates and ultimately decomposes to form nitrite (NO_2-) and nitrate (NO_3-). Evaluation of nitrite provides a surrogate marker and quantitative indicator of NO production. Nitrite has been measured in RAW cell line culture supernatants using the Greiss Reagent after 48 h co-culture with NMs in the presence or absence of LPS as a positive control. This experimental system allows to evaluate both the capacity of NMs to induce inflammation and to modulate the inflammation induced by a known stimulus.

No production of NO was observed after exposure of RAW cells to the two doses of NM203 (Fig. 6). Only the higher concentration of NM203 was able to significantly reduce NO production induced by the classical inflammatory stimulus LPS.

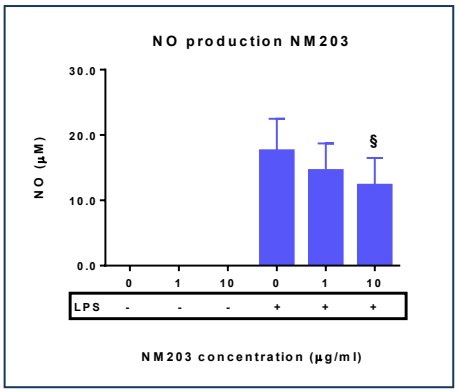


Fig. 6 See abovementioned text § $p < 0.05$

***In vivo in vitro* correlations**

From a global evaluation of the immunotoxicity results obtained in the *in vivo* oral study with NM203, the following conclusions can be gathered. Gender differences have been highlighted, since macrophages from male rats showed in general enhanced inflammatory responses (increased NO and IL-6 production, reduction of IL-10), whereas no significant differences have

been appreciated in female rats. This finding is probably associated in female rats with higher production of inflammatory cytokines in the control group and with higher intra-group variability. Moreover, difficulties in individuating a critical dose (effective in different parameters) and a dose-response relationship in the majority of immunological endpoints analysed have been found.

Regarding the *in vitro* approach, the results highlighted the capacity of NM203 to modulate inflammatory response induced by LPS in murine RAW cell line, in terms of reduction of NO production and increase of IL-6 secretion.

Undoubtedly, the oral *in vivo* study is able to yield much more refined information on the effects of chronic exposure to NMs, whereas *in vitro* approach allows simpler and reproducible evaluation of multiple parameters.

As a conclusion, the combined analysis of the two approaches supports the need for different experimental strategies (*in vivo* studies and *in vitro* models) to thoroughly characterise NMs immunotoxicity.

2.5 Evaluation and conclusions

The overall goal of NANoREG is to support industry and regulators in dealing with environment, health and safety issues of MNs. One aim of the project is, therefore, to propose new testing strategies for NMs adapted to innovation requirements. Within this context, WP4 and WP5 have used validated and non-validated but generally accepted test methods to assess the safety of NMs. The aim of this deliverable is to compare standard *in vivo* validated protocols to easily implemented *in vitro* strategies. The benefits of *in vitro* over *in vivo* experimentation are well recognised. *In vitro* experimentation is easier to implement, more economically viable, free from ethical implications and could be easily adapted into high-throughput methodologies and implemented into intelligent testing strategies. *In vivo* experiments remain, however, the gold standard in the field of toxicology. The aim of this deliverable is to address the potential of *in vitro* methodologies to be used as a tool to minimize *in vivo* experimentation in future testing strategies, speeding, in this way, NMs safety assessment. In line with WP5 task 5.7 two main end points have been selected as modules within the NANoREG testing strategy, namely genotoxicity and immunotoxicity. Both end points have been addressed *in vitro* and *in vivo* following standardised protocols. Unfortunately, and due to time constraints within the project, there was no access to the long term *in vivo* inhalation study carried out by BAuA, and only full access to data derived from 4 short term studies was available at the time of writing. The NANoREG published approach for risk assessment (RA) includes the following elements: exposure potential, dissolution, nanomaterial transformation, accumulation, genotoxicity and immunotoxicity (Fig. 1 of this section). Two such end points, namely gentoxicity and immunotoxicity have been compared *in vitro* and *in vivo* as key elements in a nanospecific RA scheme (Dekkers et al., 2016) and conclusions from the NANoREG experiences are summarised below.

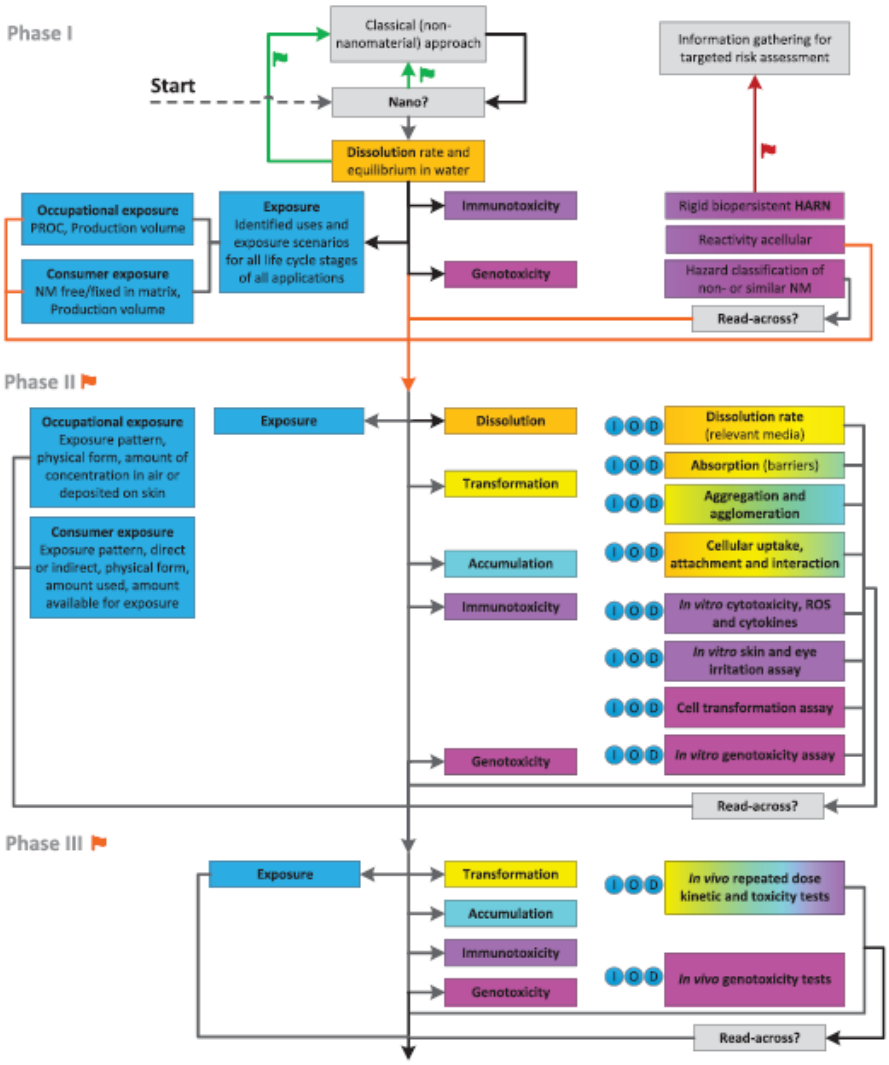


Fig 1 Overview of the different phases of the flow chart. Green arrows: the material is no nanomaterial or has such a high dissolution rate in water that it falls apart into its molecular or ionic form before it reaches its target => the classical (non-nanomaterial) risk assessment approach can be performed. Red arrow: the material is a "rigid and biopersistent High Aspect Ratio Nanomaterial (HARN)" => substitution or information gathering for targeted risk assessment to evaluated the potential to cause mesothelioma is needed. Orange arrows: the material does not meet the criteria for classical (non-nanomaterial) risk assessment or targeted risk assessment to evaluate the potential to cause mesothelioma ¼> use information of phase I for prioritisation and/or further evaluation following the proposed elements related to the kinetics, toxicity and exposure in phase II, III and further. Black arrows: evaluation of the nanomaterial following the proposed elements related to the kinetics, toxicity and exposure in phase I, II, III and further. PROC = process and operational conditions. I: inhalation route of exposure. O: oral route of exposure. D: dermal route of exposure.

2.5.1 *Lessons learnt from the in vivo in vitro correlation exercise. Input for the NANoREG Framework*

The main focus of the abovementioned studies was the inhalation route though an oral toxicity study has been included since the oral route is also a potential exposure route of NM into the body.

From sets of experiments described above, several conclusions can be drawn regarding *in vivo-in vitro* correlations.

- *In vivo* approach appears to be the most sensitive and exhaustive one to assess absolute pulmonary toxicity of poorly soluble NM.
- *In vitro* approach may provide valuable information regarding relative ranking of NM, provided that the cell model is sensitive enough. From both sets of experiments, it seems that monocultures of lung epithelial cells (A549 or BEAS-2B) are poorly sensitive models. THP-1 cells were able to provide significant signals in response to NM exposure, both in monoculture or when co-cultivated with epithelial lung cells.
- The mode of exposure is also to be considered. When cultivated and exposed at the ALI to aerosols of poorly soluble NM, cells show responses at lower doses compared to submerged exposure.
- *In vitro* experiments may contribute to prediction of toxic effects once knowledge on the intracellular effective dose is available for both cultures and tissues.
- The development of more complex *in vitro* models, mimicking closer lung physiology and the reality of environmental exposure to assess pulmonary toxicity of low soluble NM should be a priority for the upcoming years.

Although some investigations seem still needed to strengthen these conclusions, we highlighted several key points which should be considered before implementing *in vitro* experiments, to improve the *in vitro* predictivity after acute exposure to poorly soluble NMs:

- Assessing the real mass of NM deposited on the cell surface *in vitro* is fundamental
- Using compatible and relevant dose metrics between the *in vivo* and the *in vitro* is critical
- It appears important to use more realistic cell models (macrophages ++) and exposure methods
- It seems important to use similar timing of the dose delivery and exposure duration *in vitro* and *in vivo* to assess the acute toxicity of NMs using *in vitro* methods

As a general conclusion from all abovementioned points *in vitro* experimentation stands as the main serious alternative for NM safety assessment in the coming years due to its potential to be implemented in an Intelligent Testing Strategy. At present, *in vitro* technologies were able to rank NM by toxicity potential though the following issues must be taking into account: cell lines should be chosen with care as they have different sensitivities towards NM exposure (the reader is also referred to D5.06), more complex *in vitro* systems showed a better toxicity trend when compared to *in vivo* results (co-cultures ALI exposure) and further efforts should be taken into this direction, even though *in vivo* studies were more accurate at predicting toxicity outcomes, *in vitro* methodologies were able to rank NM by toxicological outcomes.

2.6 Data management

Data has been uploaded either into ISA-TAB templates or into the NANoREG Database entry tool. At the time of writing some ISA-TAB templates did not have a corresponding entry into the NANoREG data entry tool. This issue is being dealt with TNO and interested partners.

3 Deviations from the work plan

No deviations from the work plan reported. Final deliverable submission scheduled 31 August 2016.

4 References / Selected sources of information (optional)

Asgharian, B., W. Hofman, R. Bergmann (2001). Particle deposition in a multiple-path model of the human lung. Aerosol Science and Technology 34, 332-339.

Asgharian B., O. T. Price, M. Oldham, L.C. Chen, E.L. Saunders, T. Gordon, V.B. Mikhee, K.R. Minard, J.G. Teeguarden (2014). Computational modeling of nanoscale and microscale particle deposition, retention and dosimetry in the mouse respiratory tract. Inhal Toxicol 26(14):829-42.

Anjilvel, S., B. Asgharian (1995). A multiple-path model of particle deposition in the rat lung. Fundamental and applied toxicology: official journal of the Society of Toxicology, 28, 41-50.

Aufderheide, M., U. Mohr (2004). A modified CULTEX system for the direct exposure of bacteria to inhalable substances. Exp Toxicol Pathol 55(6):451-454.

Bachler, G., S. Losert, Y. Umehara, N. Von Goetz, L. Rodriguez-Lorenzo, A. Petri-Fink, B. Rothen-Rutishauser, K. Hungerbuehler (2015). Translocation of gold nanoparticles across the lung epithelial tissue barrier: Combining *in vitro* and in silico methods to substitute *in vivo* experiments. Particle and fibre toxicology 12,18.

Balashazy, I., H. Hofmann, T. Heistracher (2003). Local particle deposition patterns may play a key role in the development of lung cancer. J Appl Physiol May;94(5):1719-25.

Cho, W.S. R. Duffin, M. Bradley, I.L. Megson, W. MacNee, J.K. Lee, J. Jeong, K. Donaldson (2013). Predictive value of *in vitro* assays depends on the mechanism of toxicity of metal oxide nanoparticles. Part Fibre Toxicol. Oct 25;10(1):55.

Cohen J.M., DeLoid G.M, and Demokritou P (2015). A critical review of *in vitro* dosimetry for engineered nanomaterials. Nanomedicine 10(19): 3015-3032.

Dekkers S., Oomen A.G., Bleeker E.A.J., Vandebriel R.J., Micheletti C., Cabellos J., Janer J., Fuentes N., Vazquez-Campos S., Borges T., Silva M.J., Prina-Mello A., Movia D., Nesslany F., Ribeiro A.R., Leite E.P., Groenewold M., Cassee F.R., Sips A:J.A.M. , Dijkzeul A, van Teunenbroek T., Wijnhoven S.W. (2016). Towards a nanospecific approach for risk assessment. Regulatory Toxicology and Pharmacology 80:46-59.

Donaldson, K., P.J. Borm, G. Oberdorster, K.E. Pinkerton, V. Stone, C.L. Tran (2008). Concordance between *in vitro* and *in vivo* dosimetry in the proinflammatory effects of low-toxicity, low-solubility particles: the key role of the proximal alveolar region. Inhal Toxicol Jan;**20**(1):53-62.

Elihn, K., P. Cronholm, H.L. Karlsson, K. Midander, I. Odnevall Wallinder, L. Möller (2013) Cellular dose of partly soluble Cu particle aerosols at the air-liquid interface using an *in vitro* lung cell exposure system. J Aerosol Med Pulm Drug Deliv Apr;26(2):84-93.

Han X, Corson N, Wade-Mercer P, Gelein R, Jiang J, Sahu M, Biswas P, Finkelstein JN, Elder A, Oberdörster G (2012). Assessing the relevance of *in vitro* studies in nanotoxicology by examining correlations between *in vitro* and *in vivo* data. Toxicology. 297(1-3):1-9.

Hinderliter, P.M., K.R. Minard, G. Orr, W.B. Chrisler, B.D. Thrall, J.G. Pounds, J.G. Teeguarden (2010). ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for *in vitro* toxicity studies. Part Fibre Toxicol **7**:36.

ICRP (1994). Human Respiratory Tract Model for Radiological Protection. ICRP Publication 66. Ann. ICRP 24 (1-3).

Lenz, A.G., T. Stoeger T, D. Cei, M. Schmidmeir, N. Semren, G. Burgstaller, et al (2014). Efficient bioactive delivery of aerosolized drugs to human pulmonary epithelial cells cultured in air-liquid interface conditions. American journal of respiratory cell and molecular biology **51**(4):526-35.

Sayes, C.M., K.L. Reed, D.B. Warheit (2007). "Assessing toxicity of fine and nanoparticles: comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles. Toxicol Sci 97:163–180.

Paur, H.F., F.R. Cassee, J. Teeguarden, H. Fissan, S. Diabate, M. Aufderheide, W. Kreyling et al. (2011). In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung - A dialog between aerosol science and biology. Journal of Aerosol Science 42: 668–692.

Rushton EK, Jiang J, Leonard SS, Eberly S, Castranova V, Biswas P, Elder A, Han X, Gelein R, Finkelstein J, Oberdörster G (2010). Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J Toxicol Environ Health A. (5):445-61.

Teeguarden, J.G., P.M. Hinderliter, G. Orr, B.D. Thrall, J.G. Pounds (2007). Particokinetics *in vitro*: dosimetry considerations for *in vitro* nanoparticle toxicity assessments. Toxicol Sci 95(2):300-12.

Teeguarden, J.G., V.B. Mikheev, K.R. Minard, W.C. Forsythe, W. Wang, G. Sharma, N. Karin, S.C. Tilton, K.M. Waters, B. Asgharian, O.R. Price, J.G. Pounds, B.D. Thrall (2014). Comparative iron oxide nanoparticle cellular dosimetry and response in mice by the inhalation and liquid cell culture exposure routes. Part Fibre Toxicol **11**:46.

Wiemann M, Vennemann A, Sauer UG, Wiench K, Ma-Hock L, Landsiedel R (2016). An *in vitro* alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials. J Nanobiotechnology. **14**:16