Executive summary:

In spite of recent advances in describing the health outcomes of exposure to nanoparticles (NPs), it remains unclear how exactly NPs interact with their cellular targets. Size, surface, mass, geometry, and composition may all play a beneficial role as well as cause toxicity. Nanomedicine has great potential in the development of novel treatments for human diseases. However, recently voiced concerns of scientists, politicians and the public about potential health hazards associated with NPs, even in medical therapies, need to be answered. The main objective of the Seventh Framework Programme (FP7) NANOTEST project (see http://www.nanotest-fp7.eu online) was a better understanding of mechanisms of interactions of NPs with cells, tissues and organs. This will benefit medical sciences and provide key knowledge for the safe use of medical NPs and will contribute to the formulation of guidelines and recommendations for future risk assessment of NPs used in clinical practice.

In vitro experiments with cells representing different organ targets revealed that oxidative stress and cytotoxic effects induced by NPs depend on the NPs properties, the test used and the cell type. Polylactic-glycolic acid (PLGA-PEO) NPs induced little or no oxidative stress in any cell type compared with solid-core metallic NPs which generally produced ROS. NP transport is most affected by tightness of the cell barrier, increasing in the order: brain less than kidney less than gut less than placenta. NP coating is also important but improved model membranes and analytical techniques are required to further investigate a greater range of NPs. Genotoxicity induced by NPs depends on the cell type, the NPs, the dispersion protocol and the measured endpoint. It is crucially important to use non-cytotoxic concentrations when assessing genotoxicity. In vitro models are valuable for providing estimates of effects of exposure as well as information on mechanisms of action. Validation with in vivo tests is, however, necessary in the first instance.

In the in vivo study a single i.v. administration of TiO2 or Na-oleate-coated iron oxide (OC-Fe3O4) NPs (0.1, 1 and 10% of LD50) to young female rats did not elicit overt acute or subacute toxicity. Subtle differences were seen in some parameters between control and NP-administered groups shortly after NP exposure. A single intravenous exposure to NPs seems to have an immunomodulatory effect. The in vitro model of human peripheral blood cells generally reflected in vivo responses of peripheral blood immune cells to TiO2 and OC-Fe3O4 NPs in exposed rats. In vivo findings proved the reliability of our panel of immune assays proposed as biomarkers for assessment of immunotoxicity in vitro.

A NP testing strategy should contain at least 3 cytotoxicity tests and 2 representative cells per organ. Initially, non-cytotoxic concentrations of NPs are identified for in vitro studies. Positive and negative controls should be included in both in vitro and in vivo models. Oxidative stress endpoints are important to investigate the mode of action of NPs in biological systems. A testing strategy for assessment of immunotoxic effects of NPs should contain a panel of assays covering several aspects of immune response. For genotoxicity, the micronucleus and comet assays together can give a reliable picture of potential genetic instability as they measure different endpoints, while H2AX assay is an interesting end-point for automated procedures.

With proper refinement of computational models and methodologies, PBPK may serve as an alternative testing strategy, replacing experiments that are expensive both in time and resources.

Project Context and Objectives:

Nanoparticles (NPs) have unique, potentially beneficial properties, but their possible impact on human health is not known. The area of nanomedicine brings humans into direct contact with NPs and it is a pressing need to understand how engineered NPs can interact with the human body following exposure. The Seventh Framework Programme (FP7) project NANOTEST (see http://www.nanotest-fp7.eu online) addresses these requirements in relation to the toxicological profile of NPs used in medical diagnostics. A better understanding of how properties of NPs define their interactions with cells, tissues and organs in exposed humans is a considerable scientific challenge but one that must be addressed to achieve responsible use of biomedical NPs. NANOTEST has evaluated toxic effects and interactions of NPs used in nanomedicine. There are a number of different NP characteristics which will influence transport and toxicity including size, surface area, coating and charge. With the use of a suitable panel of NPs of the highest purity, we have determined how these characteristics relate to possible adverse health effects.

NANOTEST integrates the investigation of toxicological properties and effects of NPs in several target systems by developing a battery of in vitro assays using cell cultures derived from different biological systems; blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems. As the activity of NPs is likely to involve oxidative stress (reactive oxygen species –ROS production) we focused on the cross-cutting areas of transport through barriers; oxidative stress, inflammation, cellular toxicity, immunotoxicity, genotoxicity and related endpoints. Following development of Standard Operating Procedures and generation of a common database, and in parallel with in silico assays (QSAR, PBPK modelling), NANOTEST is evaluating toxic effects and interactions of NPs used in nanomedicine. Finally, we will propose recommendations for evaluating the potential risks associated with new medical NPs, which will be communicated to the scientific and industrial community.

The overall aim of NANOTEST was to develop alternative testing strategies and high-throughput toxicity-testing protocols using in vitro and in silico methods which are essential for hazard identification and risk assessment of these NPs. To be able to achieve this ambitious goal, the specific aims of NANOTEST were:
a) To carry out a detailed characterization of selected NPs in order to define their main physicochemical properties;
b) To study specific and nonspecific interactions of NPs with molecules, cells and organs and to develop in vitro methods which can identify the toxicological potential of NPs;
c) To validate in vitro findings in short-term in vivo models, to study manifestation of particle effects in animals and humans, and to assess individual susceptibility in the response to NPs;
d) To perform both Structure-Activity modelling and physiologically-based pharmacokinetic (PBPK) modelling of NPs;
e) To adapt the most advanced and promising assays for high-throughput automated systems and to prepare for validation by the European Centre for the Validation of Alternative Methods.

The specific objectives of WP1 were to provide a complete and advanced physico-chemical characterization of selected engineered NPs for the assessment of their toxicological profile and to support project partners

in their toxicological evaluation. The incorporation of the physicochemical data of NPs into a knowledge base is of crucial importance in order to investigate their potential biological effects, to show which materials are safe or possibly hazardous and to ensure that results are reproducible. Due to the influence of physico-chemical properties upon biological activity, these had to be fully characterized in any toxicology studies of NP both at the time of sample administration as well as at the conclusion, if possible. Adopted exposure conditions might influence the properties of NPs, thus the characterization of administered sample was considered essential. All pertinent physico-chemical properties (size distribution, shape, specific surface area, porosity, chemical composition, impurities of concern, surface chemistry, surface charge, crystal structure, dispersion and stability in aqueous and biological media, aggregation state as powdered and dispersed form) of the selected NPs have been determined.

WP2 was a comprehensive work package with several sub WPs. The specific objectives of WP2 were:
1. the investigation of general mechanisms of NPs toxicity with specific focus on cytotoxicity, oxidative stress, uptake into and transport across cells, inflammation /immunotoxicity, and genotoxicity as cross-cutting topics
2. the co-ordination and harmonisation of all in vitro studies
3. the development of automated assays to generate high quality data for input into modeling activities, and for future robust testing applications

Several tasks were defined to achieve these objectives:
1. to define representative organs which can be used as predictors to evaluate in cellular models the biological response of cell exposure to selected medical NPs (defined in WP1) and to define reference cell lines of these organs which can predict the biological and physiological response of exposure to medical NPs which can be used by other researchers and technologists to test the possible toxicity of medical NPs.
2. to define objective reference biological markers, using these in vitro relevant models which can be used by other researchers and technologists to test the possible toxicity of medical NPs.
3. to provide databases where all protocols and cells assays are described in details as Standard Operating Procedures (SOPs) and where the obtained information is statistically evaluated and collected.
4. to automate the most promising candidate assays being applied within the project consortium in order to systematically assess the performance of the assay and to generate high quality data for input to modelling activities (WP4).
5. to propose recommendations for evaluating the potential risks associated with new medical NPs, which can be proposed to the scientific and industrial community.

In WP2, to achieve these goals and define the most promising biological markers, we had proposed to utilize cell cultures (in vitro models), organotypic cell culture models and organs (ex vivo models), to expose them to the selected NPs (characterized in WP1), and to determine their reaction in order to define reference markers and reference cellular models to evaluate for potential health hazards, and provide recommendations. In order to perform these evaluations, different biological systems (WP2.1. the blood; WP2.2. the vascular system; WP2.3. the liver; WP2.4. the respiratory system; WP2.5. the placenta; WP2.6. the

digestive system; WP2.7. the central nervous system; and WP2.8. the kidney) have been selected and the interaction with the NPs have been be assessed for the different systems within the cross-cutting areas of transport across cellular barrier models cytotoxicity, oxidative stress, inflammation/immunotoxicity, and genotoxicity. The related endpoints were proposed to be collected in databases (WP2.0.) and to be used to develop common reference materials and techniques to be proposed to the scientific community, under the form of SOPs (WP2.0.) and procedures for automation (WP2.9.) of the evaluation of NPs and statistical tools (WP2.0.) to assess the interactions of NPs with biological tissues, and to devise a detailed implementation plan.

Then, in order to compare the information obtained, cross-cutting topics (CCT) were defined covering the information gained in analyzing the transport of NPs across relevant cell layers the oxidative stress (CCT-2: oxidative stress), (CCT-3: transport of NPs), inflammation / immune toxicity (CCT-4: inflammation / immune toxicity), and potential genotoxicity (CCT-5: genotoxicity). No CCT had been initially planned for cytotoxicity (CCT-1: cytotoxicity), but has been added, according to the necessity to evaluate for this endpoint early in the process of the testing strategy.

In WP3 in vivo study the objective was to validate the findings of the alternative in vitro assessment of the toxicological profile of NP used in diagnostics or therapeutics, as planned by WP2, by experiments on animals. First, LD50 was defined for two selected NPs by performing acute toxicity study (OECD TG 425). In the in vivo experiment, animals were divided into 5 groups: negative control, positive control and 3 exposed groups, and exposed intravenously under combined ketamine/xylazine anaesthetization. There was a single exposure at the beginning of the study with subsequent sacrificing after 1 day, 1 week, 2 and 4 weeks.

The following biomarkers were measured:
- cardiotoxicity (mitochondrial coenzyme Q, oxidative phosphorylation)
- hepatotoxicity (serum cholesterol and triacylglycerols, ALT, AST, bilirubin, serum proteins, mitochondrial CoQ,oxidative phosphorylation)
- nephrotoxicity (serum urea and creatinine, histological alterations of kidney structures).
- neurotoxicity (mitochondrial coenzyme Q)
- damage in lung tissue cells (acid phosphatase, lactic acid dehydrogenase, viability and phagocytic activity of alveolar macrophages, cathepsin D)
- genotoxicity (micronuclear test in lymphocytes and bone marrow, oxidative DNA damage in white blood cells (comet assay)
- oxidative stress (malondialdehyde, reduced glutathione, antioxidant enzymes and vitamins as non-enzymatic antioxidants – in liver, lung brain, heart tissues and in blood)
- immunotoxicity (immune function assays: proliferation activity of lymphocytes, phagocytic activity and respiratory burst; immunophenotypic analysis of leukocytes, expression of adhesion molecules on leukocytes, cytokines/chemokines in serum or spleen)
- routine basic haematological examinations undertaken at sacrifice

In WP4 the objective was to identify descriptors relevant for the experimentally determined toxicitity endpoints and suitable for modelling from the available data. Structure-activity modelling is based on the assumption that similar chemical structures exhibit similar biological activities thus enabling development of mathematical models relating

chemical structure to activity. Following a preliminary review of the literature, it was clear that traditional statistically-based QSAR modelling, which requires multiple chemical descriptors and a reasonable number of high quality biological datapoints, would not be feasible. Therefore, a more theoretical approach was adopted, using expert judgement for the selection of descriptors. In particular, the reactivity of the NPs was chosen as a predictor of adverse effects mediated by oxidative stress.

Regarding PBPK modelling the work was divided in two stages. In the first stage we explored the possibility of formulating a simple compartment-based model to describe the biodistribution of NPs. In the second stage we employed computational fluid-particle dynamics to analyze the behaviour of NPs in (i) the respiratory system and (ii) the cardiovascular system. Lung deposition was calculated by further developing/refining an available one-dimensional model, which permits to calculate the deposition in all generations of the lung.

Project Results:

WP1 Characterization

Aim of WP1
The aim was to provide a complete and advanced physico-chemical characterization of selected engineered NPs for the assessment of their toxicological profile used in medical diagnostics and to support project partners in their toxicological evaluation.

Results
After a detailed survey of potential candidates, and after evaluating prons and cons of each material (availability, relevance, etc.), the following panel of commercial ENPs has been selected.

All pertinent physico-chemical properties (size distribution, shape, specific surface area, porosity, chemical composition, impurities of concern, surface chemistry, surface charge, crystal structure, dispersion and stability in aqueous and biological media, aggregation state as powdered and dispersed form) of the selected ENPs have been determined prior to toxicological testing, as well as their potential interactions with culture media components, their size distributions in stock solution/dispersion and their behaviour after addition to culture media (see Deliverable 1.3). A wide set of techniques/procedures/methods were employed in order to get necessary set of information, such as SEM, TEM and AFM for size distribution determination, isotherm N2 absorption/desorption measurements (BET) of specific surface area and pore size distribution, ESR for surface reactivity, NMR for coating characterization, ICP-MS for elemental analysis and determination of inorganic impurities, HPLC-MS and GC-MS analysis for determination of organic impurities. In addition, experiments to evaluate agglomeration in selected biological media for in vitro experiments have been performed. Specific protocols for optimal dispersion of selected ENPs were also developed according to specific partner specifications. Analytical protocols for the determination of selected ENPs uptake and distribution in biological tissues have been also developed.

Conclusion
The comprehensive detailed physical and chemical characterization results obtained in WP1, especially the investigation on NPs behavior in culture media, greatly supported the project in deeply understanding the toxicological behavior of selected ENPs in cell and animal systems for ENPs. Not only the basic physical and chemical properties of pristine nanomaterials (“primary” characterization) have to be considered, but also their behavior under real conditions in biological systems (“secondary” characterization) must be investigated in detail in order to get the correct set of information required for an effective testing strategy for NPs.

WP2 In vitro screening tests
Aim of sub WP2.0: Databases: The aim of this subWP was to coordinate in vitro studies; to develop database and create SOPs for set of in vitro tests for automation and validation; to perform statistical evaluation of NPs toxicity in different systems.

Strategy: A common approach for handling experiments, analyzing and comparing data from different systems and for statistical analysis, a crucial aspect of the assessment of health risks of therapeutic NPs, has

to be performed from the results of laboratory tests. To obtain experimental results which can be compared between them, the experimental conditions must be carefully described and be similar in all participating partners of the Consortium. To collect the data arising from all investigated systems, a common NANOTEST database must be developed and be available to all participants.

Summary of Results: To establish and validate methods and take into consideration standardization of assays, the factors affecting inter- and intra-experimental variability, including for example, the number of replicate treatments, the numbers of cells per samples, or the amount of NPs added to the cells, have been considered in the experimental design so that statistical analysis can be reliably applied. The experimental procedures have been described in a common database of SOPs developed for the most relevant methods, and the most relevant selected for validation for automation. The data from the experiments have been collected, organized in a database and appropriate statistical analyses carried out to determine the nature and extent of cell uptake, transport and cytotoxicity of the NPs over time, using the various outcome measurements. Statistical significance has been assessed by ANOVA, followed by post hoc tests to investigate whether any difference is a statistically significant result.

The statistical analyses focused on results from the cytotoxicity and genotoxicity assays with the principal objective of examining whether there were significant differences between assay types (cytotoxicity) and whether these differences varied by dose, cell-line or nanomaterial (cytotoxicity and genotoxicity). Relevant data files were identified from the database file inventory and the data for analysis extracted from each individual datafile.

Results from the cytotoxicity analysis showed that the WST-1 assay showed the strongest negative dose-response association (with higher doses associated with lower %Viability) and this was apparent for all cell-lines witin this assay. Less significant negative dose-response associations were seen for the RGA and MTT assays. The PI assay provided very different outcomes to the other assays. Among the nanomaterials, OC-Fe3O4 consistently showed the strongest association between dose and outcome, followed by the two SiO2 nanomaterials. Results from the genotoxicity assay also showed differences in dose-response associations between nanomaterials but not between cell-lines, For both Strand Breaks and Net FPG strong dose-response associations were seen for OC-Fe3O4, consistent with the findings of the analysis of the cytotoxicity data. No other nanomaterials showed a consistent association for either Strand Breaks or Net FPG.

All NANOTEST procedures and data have been collected and organized in a consortium specific database installation and integrated in the web-based IT platform NANOhub which is hosted by European Commission's JRC and provides an inventory of information on NPs from various projects. From the optimization of the test protocols, the assessment of within laboratory variability, transferability, between-laboratory variability, cell models and end-point parameters have been identified and selected, and integrated in a platform to be used for routine testing of the potential effects of NPs in representative cells of representative organs. From these databases, experimental procedures, which are suitable for automation, have been extracted and validated using the platforms available.

Conclusions: In summary, all the necessary data have been provided, database was developed and validated by the different partners of the Consortium, and is ready to be uploaded to the European Commission JRC NANOhub. The information has been made accessible to the partners of NANOTEST for approval, and will be made accessible to the scientific community when approved by the European Commission. The information obtained using cell models from the different organs is summarized below, and a testing strategy is proposed to evaluate the interactions of NPs with cells.

Aim of sub WP2.1: Blood: After intravenous administration of NPs, blood is primary target organ and secondary target after access of NPs to systemic circulation during other route of administration. Direct toxic effect of NPs on blood components can be the result of cytotoxicity, effect on growth and maturation of immune cells and genotoxicity. Consequence of such interactions can be, inflammation or modulation of immune response resulting in allergy or genotoxic effect. On the other hand, immunotoxic effect can lead to increased susceptibility to infection and cancer as well.

Strategy:
Cytotoxicity methods used were trypan blue exclusion, cell proliferation assay, clonogenic assay, mitotic index.

Genotoxicity endpoints and methods: the cytokinesis block micronucleus (CBMN) assay to detect mutagenicity, clastogenicity and aneugenicity; the comet assay (CA) to detect strand breaks, and formamydopyrimidine (FPG) modified comet assay (FPG CA) for oxidised DNA lesion especially 8-oxoGuanine.

Immunotoxicity of NPs: proliferative activity of lymphocytes in vitro stimulated with mitogens and CD3 antigen to assess functionality of the lymphocytes, phagocytic activity and respiratory burst of leukocytes to evaluate function of neutrophil granulocytes and monocytes (macrophages), natural killer cell activity to determine function of natural killer cells, immunophenotypic analysis to monitor proportion of lymphocyte subsets in peripheral blood, expression of adhesion molecules to evaluate adhesive capabilities of blood cells, expression of interleukins to assess inflammatory and immune response.

Cell components used to evaluate the genotoxicity and immunotoxicity induced by NPs: human peripheral blood from volunteers and TK6 cell line. The cells were exposed in complete culture medium to increasing concentrations of dispersed NPs from 0.12 µg/cm2 to 75 µg/cm2 and analyzed after up to 72h of exposure.

Summary of results and conclusions
a) Assessment of genotoxic effect: Both CBMN and the CA data on TK6 and human peripheral blood cells show consistent results. TiO2 and OA-Fe3O4 show positive effect with the CA. Micronucleus assay data show no genotoxic or clastogenic effect. For details see deliverable report and CCT report.

b) Assessment of immunotoxic effect: For details see deliverable report and CCT report.
Immunotoxicity induced by NPs depends on cell type, NPs, dispersion protocol and measured endpoint. Several immune biomarkers have been used

to monitor the possible effects of NPs on humoral and cellular immune response. From functional assays, proliferative activity of lymphocytes demonstrated to be sensitive biomarker of specific cellular immune response. Similarly, cytotoxic activity of natural killer cells and phagocytic activity and respiratory burst of leukocytes proved to be reliable indicator of function of natural immune response. On the other hand, non-functional assays were less (but still) sensitive biomarkers of NPs exposure. Immunophenotypic analysis proved to be simple but less sensitive indicator of possible alterations in leukocytes subsets after NPs. In our set up of experiments, only changes in expression of CD62L adhesion molecule were found, expression of CD54 did not show any effect.

Conclusions: These findings contributed to the development of testing strategy for monitoring of potential genotoxic and immunotoxic effects of NPs on blood cells.

Aim of sub WP2.2: The vascular system: The vascular system provides the interface between blood and organs, maintaining tissue homeostasis. Thus, insults to the vascular system, in particular oxidative stress, may perturb the correct functioning of a whole organism. Therefore it is of particular interest and relevance to evaluate if and which NPs can interfere with the functions of cells of the vascular walls, and the consequences of such interactions, in particular on the survival, oxidative stress, and adhesive properties for circulating cells of endothelial cells.

Strategy: The murine Ecp23 pulmonary, the rat EC219 rat and human HCEC brain endothelial cells were selected. The cells were exposed in complete culture medium to increasing concentrations from 0.4 µg/ml (0.12 µg/cm2) to 235 µg/ml (75 µg/cm2) of dispersed NPs and analyzed after up to 72h of exposure. The MTT and thymidine incorporation assays were selected for cytotoxicity; the 5/6carboxy-2,7-dichloro-dihydro-fluorescein (DCFH-DA assay), the dihydroethidium (DHE), the bromobimane and the nitric oxide assays for oxidative stress; the CA for genotoxicity studies, and their adhesive properties for human TK6 lymphoblastic cells and human myelomonocytic THP-1 cells by endothelial cells exposed to NPs.

Summary of results

a) Evaluate uptake and transport of NPs across endothelium: The uptake of the NPs by the three endothelial cells was determined. All the solid-core NPs were well taken up by endothelial cells, with the exception of OC-Fe3O4 NPs, which are taken up only at very low levels, however, no subsequent release by or transport across the cells could be demonstrated.

b) Identify effects of NPs on endothelial cells: neither U-Fe3O4 NPs nor PLGA-PEO NPs were cytotoxic, Fl-25 SiO2 NPs and TiO2 NPs were slightly cytotoxic whereas OC-Fe3O4 NPs were highly cytotoxic for all endothelial cells. The three solid-core metallic NPs, but not PLGA-PEO or Fl-SiO2 NPs, induced an oxidative stress in endothelial cells. A decrease of cellular thiol levels was observed in all three endothelial cell lines after exposure to Fe3O4 NPs, but not to TiO2 NPs. Only uncoated Fe3O4 NPs stimulated NO production by rodent endothelial cells. TiO2 NPs induced DNA damage, but only slight DNA damage was induced by U-Fe3O4 and OC-Fe3O4 NPs and by both-sized Fl-SiO2 NPs whereas PLGA-PEO NPs did not induce DNA damage. Exposure of human endothelial cells to NPs did not modify their adhesive properties for cells of the human immune lineage.

Conclusions: In summary, only OC-Fe3O4 NPs were cytotoxic, while U-Fe3O4 NPs and OC-Fe3O4 NPs and TiO2 NPs induced an oxidative stress in endothelial cells. Only TiO2 NPs induced damage to DNA. Even if endothelial cells were able to internalize NPs, no direct transport across an endothelial cell layer and no modification for the adhesion of cells of the immune lineage were demonstrated. A testing strategy is proposed to evaluate the interactions of NPs with endothelial cells.

Aim of sub WP 2.3: the liver: The liver is a heterogeneous organ and the major site for biotransformation and defense against foreign materials and xenobiotics. Thus, in assessing the risk of hepatotoxicity of NPs, in particular cytotoxicity, oxidative stress and genotoxicity, the in vitro interactions between the selected NPs and the most representative liver cells were studied.

Strategy: Hepatocytes, Kupffer cells and liver sinusoidal endothelial cells (LSEC) were the cell types chosen for hepatocytoxicity studies. Hepatocytes represent more than 70% of liver cells while Kupffer cells and LSEC are also relevant due to their phagocytic phenotype and fenestrated structure, respectively. Basal cell viability (MTT test), oxidative stress (DCFH-DA assay) and genotoxicity (CA) were the endpoints analyzed in these cells exposed to increasing concentrations (0.384 µg/ml (0.12 µg/cm2) to 240 µg/ml (75 µg/cm2) of the selected NPs.

Summary of results:
a) Liver cell isolation: the preparation of hepatocytes and Kupffer cells were set up with an acceptable purity as revealed by immunofluorescence staining. However the culture of Kupffer cells was limited to 48 h. LSEC were not efficiently isolated, even after improving the cell isolation procedures of published protocols.

b) Basal cytotoxicity: Comparison of basal cytotoxicity of PLGA-PEO and Fl-SiO2 NPs in liver macrophages and hepatocytes showed that Kupffer cells were more susceptible to NPs toxicity than hepatocytes. The differences may be due to a different internalization pattern of the NPs. On the contrary, cell viability could not be assessed for TiO2 and Fe3O4 NPs due to their interference with the colorimetric MTT assay.

c) Measurement of NP-induced oxidative stress with the DCFH-DA probe resulted in interference of the particles with the dye. Despite that free radical formation could be observed for both Fe3O4 NPs in the two cell types while TiO2 NPs only induced oxidative stress in Kupffer cells.

d) Genotoxicity: DNA damage was also determined in these cells. TiO2 NPs were the only NPs inducing genotoxicity in a dose-dependent manner in hepatocytes. In contrast, all tested NPs but PLGA-PEO NPs caused a genotoxic effect in Kupffer cells even after a short time exposure (4h).

Conclusions: In summary, Kupffer cells are the liver cells initially exposed to NPs and the most susceptible to NP toxicity. Oxidative stress was shown for Fe3O4 and TiO2 NPs in hepatocytes and Kupffer cells, respectively. DNA damage was more relevant in Kupffer cells than in hepatocytes even after exposure to low concentrations of NPs and for a short period of time. A testing strategy is proposed to evaluate the interactions of NPs with liver cells.

Aim of sub WP2.4: the respiratory system: Lung epithelial cells are the first target cells after inhalation but also secondary targets after

injection of NPs especially at the alveolar level due to the small distance between the epithelial cells and the capillary. The objective of this sub-WP was to compare the responses of lung epithelial cells to the panel of NPs in terms of endocytosis, cytotoxicity, oxidative stress induction and inflammatory response.

Strategy: The effects of NPs (up to 75 µg/cm2) were determined after 24 and 48 h of exposure o the bronchial (16HBE) and alveolar (A549) cells. Endocytosis was evaluated by measuring the right angle scattering of the flow cytometer laser, oxidative stress by DHE oxidation in flow cytometry, anti-oxidant enzyme mRNA expression by RT-qPCR, metabolic activity and membrane integrity, by WST-1 and propidium iodide uptake, respectively, genotoxicity by the CA, and the inflammatory response by measuring cytokine mRNA expression by RT-qPCR. All these methods were firstly studied for their suitability for NP testing. The molecular and cellular mechanisms of toxicity, uptake and inflammatory response were determined by the use of inhibitors and by immunocytochemistry.

Summary of results: First the suitability of the assays to test the toxicity of NPs was controlled. ELISA and neutral red uptake are prone to interferences with NPs, thus the WST-1 and flow cytometric analysis methods were used to avoid interferences. The comparison of the dose-response analysis (WST-1, PI uptake, DHE oxidation) revealed that OC-Fe3O4 NPs induced the greatest responses whereas U-Fe3O4 NPs are only slightly toxic, suggesting a coating effect. The effects of silica NPs weare size dependant as Fl-25 SiO2 NPs are cytotoxic at lower concentrations than Fl-50 SiO2 NPs. TiO2 NPs induced responses comparable to Fl-50 SiO2 NPs whereas PLGA-PEO NPs have almost no effects in both cell lines used. The greatest pro-inflammatory response was induced by SiO2 NPs, showing that the inductions of cytokine and anti-oxidant enzyme genes are greater in 16HBE compared to A549 cells. These NPs are taken up and induce DNA strand breaks at non-cytotoxic concentrations in contrast to Fe3O4 and TiO2 NPs. The MAPK p38 but not NF-B signalling pathways were involved in the inflammatory response induced by TiO2 NPs and the lysosomal pathway in apoptosis induction.

Conclusions: In summary, based on the results of the thorough dose response studies a testing strategy could be proposed for the lung. PLGA-PEO NPs can be used as negative control NPs. TiO2 NPs are relevant positive controls but SiO2 NPs revealed even better candidates as they induce greater responses and interfere less with the assays. Both cell lines and different cellular endpoints and assays should be assessed to accurately evaluate the potential toxicity of NPs as they differ in their sensitivity, user friendliness and possible interferences.

Aim of sub WP2.5: the placenta: The objective of this sub-WP was to determine whether NPs can cross the placenta to reach the fetus and to evaluate the interaction of NPs with placental cells in terms of cytotoxicity, oxidative stress, and DNA damage, in order to consider potential impact of exposure to engineered NPs on the fetus during pregnancy.

Results: The human BeWo b30 placental cell line was selected for in vitro study utilizing the Transwell model (polyester membrane, 3 µm pore diameter) for transport studies (up to 24h) and compared results using the ex vivo isolated dually perfused human placenta model (up to 6h). For toxicity studies, cells were exposed in complete culture medium to dispersed NPs from 0.12 - 75 µg/cm2 and analyzed after 0.5h to 48h of

exposure. From several possible protocols, lactate dehydrogenase (LDH), WST-1 and Annexin V/PI assays were selected for cytotoxicity; IL-6 CK release for inflammation; ROS detection by DHE oxidation by flow cytometry and DCFH-DA assay for oxidative stress; the CA for genotoxicity.

Summary of results
a) Placental uptake and transport of NPs: OC-Fe3O4 NPs were rapidly transported across BeWo cells in a dose-dependent manner whereas there was negligible transport of U-Fe3O4 NPs. Both NPs were internalized by cells and increased concentration led to increased cell uptake of U-Fe3O4 but no effect on OC-Fe3O4 uptake. Both sizes of SiO2 NPs were rapidly transported but particle size and concentration did not affect extent of transport or NP uptake. In the perfused placenta system, the OC-Fe3O4 NPs showed no transport and for SiO2 NPs there was only very limited transport, with fetal levels very close to the limit of detection.

b) NP-induced cytotoxicity, inflammation, oxidative stress and genotoxicity in placental cells: All NPs tested caused significant LDH release but only at 75 µg/cm2 for SiO2 NPs. With WST-1, Fe3O4 were cytotoxic at 3-75 µg/cm2, SiO2 at 75 µg/cm2 and TiO2 and PLGA-PEO NPs were not cytotoxic. IL-6 release was increased with SiO2 (75 ug/cm2) and U-Fe3O4 (15-75 µg/cm2) NPs, decreased with OC-Fe3O4 (75 µg/cm2) and not affected by TiO2 or PLGA-PEO NPs. Exposure to the panel of NPs did not affect apoptosis or necrosis. BeWo cells were not suitable for oxidative stress or MN assays. TiO2 NPs caused DNA damage at 0.6–75 ug/cm2, increasing with time and dose. There was no significant damage by PLGA-PEO, SiO2 or U-Fe3O4 NPs. OC-Fe3O4 NPs caused damage at 75 µg/cm2 but this was invalidated by decreased cell viability.

Conclusion: In summary NPs can cross the placental barrier in vitro but transport is reduced ex-vivo. Overall, OC-Fe3O4 NPs were most cytotoxic and TiO2 most genotoxic. Given the importance of oxidative stress pathways, BeWo cells may not be suitable for toxicity testing.

Aim of sub WP 2.6: the gastrointestinal tract: The gastrointestinal tract is important to assess the oral absorption of NPs. Therefore, the in vitro absorption and cytotoxicity of the selected NPs were determined in several in vitro intestinal models that mimic the physiological conditions of the human intestine.

Strategy: Caco-2 cells, CacoReady and Caco-Goblet cell systems were selected for this evaluation. When grown in Transwells® devices the cells form polarized monolayers after 21-day of culture. In addition, CacoGoblet are able to secrete mucus mimicking the terminal part of the intestine. Under those culture conditions, two independent compartments are formed, the apical (upper compartment) and the basal (lower compartment) compartments. NPs were applied to the apical compartment and samples from the basal compartment were analyzed at different time-points. The samples were analyzed by ICP-MS/MS to detect transport. NPs were also applied with the Lucifer Yellow reagent to study the integrity of the monolayers. The disruption of the monolayers (disruption of tight junctions) is an indirect measurement of cytotoxicity in these systems. Comparison of the experiments on CacoReady and CacoGoblet models indicate how the presence of mucus could affect the cytotoxicity and cell permeability for the NPs.

Summary of results:

a) Uptake studies: The transport of the metallic NPs was analyzed by ICP-MS/MS. Preliminary results showed that only very low amount of Fl-SiO2 NPs were detectable in both cell models. However, neither TiO2, nor U-Fe3O4, nor OC-Fe3O4 NPs could be detected in the basal compartment, probably due to the technical characteristics of the Transwell membranes rather than the incapacity of the NPs to cross the cellular barriers.

b) Basal cytotoxicity: Cell viability studies performed with cells seeded in plastic dishes showed that OC-Fe3O4 NPs were cytotoxic for CacoReady cells after 4h and 24h of exposure and that only Fl-25 SiO2 NPs and synthetic amorphous silica NPs were cytotoxic after 24h of exposure. Similar information was obtained for CacoGoblet cells, including cytotoxicity of Fl-50 SiO2 NPs after 24h of exposure. Cell viability studies for cells grown on Transwells were technically not possible. From the tested NPs only U-Fe3O4 NPs were cytotoxic for the CacoReady cells at 100 µg/ml after 1h and 4h of exposure, whereas at 25 µg/ml they were only cytotoxic after 4h of exposure. No cytotoxicity was found for any tested NP in CacoGoblet cells at any concentration or time-point used in this assay.

Conclusions: In summary, despite the low cytotoxicity of this set of NPs in these model cell systems, the presence of mucus could protect NP-induced cytotoxicity but does not play any role in NP transport. However, Transwell devices impair transport studies for some types of NPs. A testing strategy is proposed to evaluate the interactions of NPs with intestine cells.

Aim of sub-WP2.7: the central nervous system: The central nervous system (CNS) is very sensitive to insults, in particular to oxidative stress, but xenobiotics must be transported across the blood-brain barrier (BBB) to reach the brain parenchyma. Therefore it is of particular interest and relevance to evaluate if and which NPs can get access to this organ, and what might be the consequences of the interactions of NPs with cells forming the BBB, the immune defensive cells of the CNS, the microglial cells, or with cells of the astrocytic lineage.

Strategy: For the experiments human brain-derived endothelial cells, murine N11 microglial cells and human LN229 glioblastoma cells were selected as representative cells of the brain. The cells were exposed in complete culture medium to increasing concentrations from 0.4 µg/ml (0.12 µg/cm2) to 235 µg/ml (75 µg/cm2) of dispersed NPs and analyzed after up to 72h of exposure. Several protocols were selected, the MTT and thymidine incorporation assays for cytotoxicity; the DCFH-DA assay, the DHE, the bromobimane and the nitric oxide assays for oxidative stress; the CA and oxidative DNA damage (FPG comet) assays for genotoxicity and the Transwell model (polyester membrane, pore diameter 3 µm) for uptake and transport studies by brain endothelial cells.

Summary of results

a) Interaction of NPs with cerebral endothelial cells: Human brain endothelial cells dose-dependently and time-dependently internalized all NPs with the exception of OC-Fe3O4 NPs, but none were released or transported by the cells following uptake. TiO2 and Fe3O4 NPs were cytotoxic, induced an oxidative stress and/or DNA damage in endothelial cells.

b) Evaluation of the effects of NPs on microglial and astrocytic cells: Only U-Fe3O4 NPs, but neither OC-Fe3O4 nor Fl-SiO2 NPs were taken up by

N11 microglial cells and LN229 glioblastoma cells, but only OC-Fe3O4 NPs displayed cytotoxicity for N11 and LN229 cells. No oxidative stress reaction could be demonstrated with these cells.

Conclusion: In summary, NPs can gain access to the CNS if the BBB is disrupted, since brain endothelial cells can internalize NPs but cannot transport them. The internalization of the NPs by brain endothelial cells, in particular for metallic-core NPs, results in cytotoxicity, the induction of an oxidative stress and potential genotoxicity for the cells. The uptake of NPs by brain parenchyma cells, the murine N11 microglial cells and the human LN229 glioblastoma cells was demonstrated only for U-Fe3O4 NPs, but only OC-Fe3O4 NPs were cytotoxic for these cells. No oxidative stress was detected following uptake of NPs by these cells. A testing strategy is proposed to evaluate the interactions of NPs with brain-derived cells.

Aim of sub WP2.8: the kidney: The accumulation and elimination of engineered NPs may be via the kidney. The objective of this sub-WP was to evaluate the interactions of the selected NPs with representative cells of the kidney addresing cytotoxicity, oxidative stress, DNA damage, cell uptake and subsequent release and transport of the NPs across epithelial kidney cell layers.

Strategy: From several possible cell lines available, the MDCK (distal tubule) and LLC-PK (proximal tubule) porcine kidney epithelial cells, human embryonic kidney HEK293 cells and the Cos-1 monkey kidney fibroblasts were selected. The cells were exposed in complete culture medium to concentrations from 0.4 µg/ml (0.12 µg/cm2) to 235 µg/ml (75 µg/cm2) dispersed NPs and analyzed after 0.5h to 72h of exposure. From several possible possible protocols, the MTT, proliferation (relative growth activity) and plating efficiency (PE) assays were selected for cytotoxicity; the DCFH-DA assay and the bromobimane assays for oxidative stress; the CA and oxidative DNA damage (FPG comet) assays for genotoxicity and the Transwell model (polyester membrane, pore diameter 3 µm) for transport studies.

Summary of results

a) Uptake, subsequent release and transport of NPs by kidney cells: The uptake by the MDCK and LLC-PK cells of U-Fe3O4 NPs, but not OC-Fe3O4 NPs was time dependent, cell line dependent and proportional to the amount of NPs added. Significant release of U-Fe3O4 NPs by MDCK cells but not by LLC-PK cells was observed, following uptake. The uptake, but not the release by the cells of Fl-25 SiO2 NPs, but not Fl-50 SiO2 NPs, was observed only at the highest concentration tested. Following uptake the release of TiO2 NPs by both cell types could be observed. Neither Fl-SiO2 NPs nor OC-Fe3O4 NPs were transported across the renal MDCK and LLC-PK cell layers

b) NP-induced cytotoxicity, oxidative stress and genotoxicity in kidney cells: OC-Fe3O4 NPs were the most cytotoxic NPs among all tested NPs, already cytotoxic at the highest tested concentration after 24 h. TiO2 NPs were the only NPs tested inducing the production of high amounts of ROS in exposed MDCK and LLC-PK cells, whereas U-Fe3O4 NPs induced a significant decrease of cellular thiol content in MDCK cells and in LLC-PK cells. In Cos-1 and HEK293 cells, only OC-Fe3O4 NPs and TiO2 NPs increased DNA damage. For TiO2 NPs, the effect was related to the agglomeration state of the NPs.

Conclusion: In summary epithelial renal cells can take up and release, but not transport, NPs following their uptake. OC-Fe3O4 NPs and TiO2 NPs were the most damaging NPs for renal cells, considering cytotoxicity, oxidative stress and DNA damage. A testing strategy is proposed to evaluate the interactions of NPs with representative cells of the kidney.

Aim of sub WP2.9: automation. In vitro assays proposed by the NANOTEST partners had to be evaluated for automation feasibility and implemented on the robotic platform for high throughput screening (HTS) with data generation on all NPs identified by NANOTEST.

Strategy: The robotic platform was tested for automation and simultaneous screening of several NPs, including the development of a generic dispersion protocol for the different NPs, an accurate serial dilution of the NPs, treatment of cells and measurement of relevant endpoints.

From 35 assays compiled by the partners, few were retained as suitable for automation on our platform and 2 endpoints were successfully implemented (with generation of SOPs and data): oxidative stress (ROS production) and genotoxicity (double strand breaks). A multi-parametric analysis was performed (High Content Imaging-HCI), measuring 4 parameters simultaneously (cell count, nuclear intensity, nuclear size and either ROS or double strand breaks) what allowed the prediction of possible mechanisms of action leading to cytotoxicity. For both assays, Balb/c 3T3 cells were exposed to 11 concentrations ranging from 0.31 µg/cm2 to 316 µg/cm2 for each NP dispersed in complete culture medium and analyzed after 24h exposure (ROS) or 4h exposure (double strand breaks).

Summary of results

a) Oxidative stress (ROS production): PLGA-PEO NPs and Endorem showed no cytotoxicity and did not induce oxidative stress while with TiO2 NPs a trend towards cell toxicity was observed possibly due to the significant increase in ROS. SiO2 NPs had a clear cytotoxicity effect but not ROS mediated. Fl-25 SiO2 and Fl-50 SiO2 NPs were both cytotoxic with the 25 nm NP having a bigger effect. ROS activity could not be measured as these NPs had the same excitation wavelength as the ethidium dye used to measure ROS levels. Both OC- Fe3O4 NPs and U-Fe3O4 NPs were found to decrease cell viability. The OC-Fe3O4 NPs were clearly more toxic though apparently not through ROS production.

b) Genotoxicity (double strand breaks): PLGA-PEO NPs and Endorem revealed no cytotoxicity or genotoxicity at the time points and concentrations tested. TiO2 NPs had no effect on cell viability but induced DNA breaks to different extents. SiO2 NPs led to variable results between experiments but induced a clear DNA damaging effect at high exposure. Fl-25 SiO2 and Fl-50 SiO2 NPs led to significant DNA damage though cytotoxicity was not obvious. OC-Fe3O4 affected cell viability but the mechanism does not seem to involve double strand DNA damage.

Conclusion: The data generated via HTS and HCI for the endpoints oxidative stress and genotoxicity are in concordance with the observations of the NANOTEST partners: OC-Fe3O4, Fl-25 SiO2 and TiO2 NPs are damaging for cells in various degrees and the cause may be attributed in some cases to oxidative stress or DNA damage. In summary, HTS for nanotoxicity was shown to be feasible and reliable.

Aim of CCT-1: Cytotoxicity: The objective of this CCT was to determine whether NPs induce cytotoxicity and which assay is the most sensitive and appropriate for in vitro toxicity testing of NPs. Cytotoxicity is basal toxicity where basic functions of cells are affected and thus result in cellular damage. Therefore it is of particular interest to develop assays to be used for fast screening of potential cytotoxicity of NPs and to know whether selected NPs can be cytotoxic.

Strategy: The cytotoxicity and determination of LC50 can be evaluated by measuring different endpoints. We used three types of assays to measure: a) cellular metabolic activity; b) membrane integrity; and c) cell number and cell proliferation. Membrane integrity was determined by LDH assay, Trypan blue exclusion, Neutral red uptake, and Propidium Iodide uptake. The mitochondrial activity of cells was assessed by MTT and WST-1 assays, metabolically active cells by Alarm Blue assay using fluorescence. Cell apoptosis was measured by Annexin V-FITC/PI Staining and by flow cytometry. Cell proliferation was measured by DNA synthesis using thymidine incorporation, the proliferation or the relative growth activity (RGA), and the colony forming (PE) assays. Additionally several cytotoxicity tests were used to detect cytotoxicity in human peripheral blood cells. The following organs were evaluated for sensitivity to NPs-induced cytotoxicity using representative cell lines: blood (TK6, human peripheral blood cells), lung (16HBE, A549); CNS (HCEC, LN229, N11); liver (primary hepatocytes and Kupffer cells); kidney (MDCK and LLC-PK, COS-1, HEK293); vascular system (endothelial cell line Ecp23 and EC219), placenta (BeWo b30) cells.

Summary of results

a) Development of sets of cytotoxic tests for NPs cytotoxicity testing. Comparison of assays: All cytotoxicity assays were able to detect dose response. However, results showed that colorimetric assays such as MTT, WST-1, LDH, Neutral red uptake are likely to interfere with NPs and need protocol adaptation and additional control to avoid false positive results. On the other hand cell number - proliferation assays (DNA synthesis, RGA and PE assays) do not show any interference, but are more tedious. Cellular metabolic activity assays were the most sensitive assays recognising early cytotoxic events. Cell number methods reflected the overall cell response to NPs cytotoxicity and are suitable for acute and chronic toxicity assessment.

b) Evaluation of the cytotoxic effects of NPs and cell sensitivity: All NPs except PLGA-PEO NPs exhibited some level of cytotoxicity. OC-Fe3O4 NPs were the most cytotoxic NPs followed by TiO2, U-Fe3O4 Fl-SiO2 25, Fl-SiO2 50, PLGA-PO NPs and Endorem. But the sensitivity of cells to NPs-induced cytotoxicity was cell-dependent.

Conclusion: In summary, the cytotoxicity induced by NPs depends on the NPs, the cytotoxicity test and the cell type. Testing strategy of NPs should contain at least three different cytotoxicity tests and 2 representative cells per organ.

Aim of CCT-2: Oxidative stress: The objective of this CCT was to determine whether NPs can generate an oxidative stress and which assays permit the best evaluation of ROS production, thiol depletion and antioxidant enzymes expression. Oxidative stress is a common mediator of toxicity which may lead to damage to the cell membranes, mitochondria and nuclei.

Strategy: The intracellular production of ROS was evaluated using two methods: DHE oxidation in fluorescence multiwell plate reader or flow cytometry and DCFH-DA assay in fluorescence plate reader. Thiol depletion was measured using the monobromobimane assay. The induction of anti-oxidant enzymes (SOD-2 and HO-1) mRNA expression was measured by RT-qPCR. The following organs were evaluated for the oxidative stress induced by NPs using representative cell lines: lung (16HBE, A549); CNS (microglial N11 cells and LN229 glioblastoma, HCEC human brain endothelial cells); kidney (MDCK and LLC-PK); liver: (primary hepatocytes and Kupffer cells); vascular system (endothelial cell lines Ecp23 and EC219).

Summary of results

- Lung: The flow cytometric protocol for measuring DHE oxidation was used successfully unlike the fluorescent plate reader to evaluate the intracellular production of ROS induced by the panel of NPs. TiO2 NPs induce an oxidative stress in both cell lines only at cytotoxic concentrations. In comparison PLGA-PEO NPs, despite their non-cytotoxicity, induced a weak production of intracellular ROS. For the other NPs, all of them were able to induce a dose-dependent increase of ROS. The induction of anti-oxidant enzymes (SOD-2 and HO-1) mRNA expression could be observed at lower concentrations than the ROS production.
- Kidney: DCFH-DA and monobromobimane assays were used. TiO2 NPs were the only NPs tested inducing the production of high amounts of ROS in exposed MDCK and LLC-PK cells whereas U-Fe3O4 NPs induced a significant decrease of cellular thiol content in MDCK cells and in LLC-PK cells.
- CNS: TiO2 and Fe3O4 NPs induced an oxidative stress in endothelial cells but not in microglial and astrocytic cells.
- Vascular system: The three solid-core metallic NPs, but not PLGA-PEO or SiO2 NPs, induced an oxidative stress in endothelial cells. A decrease of cellular thiol levels was observed in all three endothelial cell lines after exposure to Fe3O4 NPs, but not to TiO2 NPs.
- Liver: Measurement of NP-induced oxidative stress with the DCFH-DA probe resulted in interference of the particles with the dye. Despite that, free radical formation could be observed for Fe3O4 NPs in both cell types while TiO2 NPs only induced oxidative stress in Kupffer cells.

Conclusions: In summary,

1. The oxidative stress induced by NPs depends on the cell type and on the NPs. PLGA-PEO NPs induced no or very weak oxidative stress in the entire cell types instead of solid-core metallic NPs which generally induced ROS production.

2. The detection of fluorescent probes by flow cytometry avoids interferences of free NPs but takes more time than detection by fluorescence plate readers and could not be used for high-throughput screening. DHE assay is well adapted to flow cytometry measurement and DCFH-DA and bromobimane assays to microfluorimetry. RT-qPCR revealed to be a suitable and more sensitive method to study antioxidant enzyme mRNA but is time consuming and induced delays in testing.

Aim of CCT-3: Transport: The objective of this CCT was to determine whether NPs can cross specific barriers to be further disseminated throughout the body (e.g. via the gut) or to reach particularly sensitive areas e.g. brain or fetus. If NPs can reach such specific targets then the potential impact of any toxicity from engineered NPs may need more careful consideration.

Strategy: The following organs were evaluated for NP barrier function using representative cell lines: lung (16HBE, NCl-H292, Calu 3); placenta (BeWo b30); gut (CacoReady and CacoGoblet); blood brain barrier (BBB) (HCEC); kidney (MDCK and LLC-PK).

The Transwell model (polyester/polycarbonate membrane, 3 µm pore diameter) was utilised for in vitro transport studies (up to 24h) and additionally for the placenta, the ex vivo isolated dually perfused human placenta model (up to 6h). Studies were restricted to TiO2, Fe3O4 and Fl-SiO2 NPs. Cells were grown to confluence and barrier integrity was determined by visual assessment, measurement of trans-epithelial electrical resistance (TEER) or transport of permeability dyes. NP transport was determined by analysis of samples removed from the apical and basal chambers at appropriate time intervals after exposure. Cellular uptake of NPs and effect of NPs upon barrier function were also determined.

Summary of results
a) Lung: Only Calu 3 cultures were suitable for transport showing a network of tight junctions (TJ) and elevated TEER values. With fl-TiO2 NPs greater than50% were trapped in the membrane and only 1% detected in basal compartment demonstrating that Transwell inserts are not suitable for TiO2 NP study. b) Placenta: rapid dose-dependent transport in vitro of OC-Fe3O4 but not U-Fe3O4 NPs. Both NPs were internalized by cells and increased dose led to increased cell uptake of U-Fe3O4 but no effect on OC-Fe3O4 uptake. Both sizes of SiO2 NPs were rapidly transported but particle size and concentration did not affect extent of transport or NP uptake. NP exposure did not affect barrier permeability. In the perfused placenta, there was no transport of OC-Fe3O4 NPs and very limited transport of SiO2 NPs. c) BBB: Human brain endothelial cells dose-dependently and time-dependently internalized all NPs with the exception of OC-Fe3O4 NPs, but none were released or transported by the cells following uptake. d) Gut: both Caco models transported SiO2 NPs but transport was not evident for Fe3O4 NPs e) Kidney: epithelial renal cells could take up and release, but not transport, NPs following their uptake.

Conclusion: In summary NP transport is most affected by tightness of the cell barrier: transport across BBB less than kidney less than gut less than placenta. NP coating is also of importance but improved model membranes and analytical techniques are required to further investigate a greater range of NPs. In vivo correlation of in vitro findings is essential but in vitro models may provide worst case exposure estimate.

Aim of CCT-4: Immunotoxicity: The objective of this CCT was to determine whether NPs may induce immunotoxic effects and which assays are sensitive and appropriate for in vitro immunotoxicity testing of NPs. Immune dysregulation may occur in various forms as immunosuppression, immunostimulation, hypersensitivity and autoimmunity. It is of particular interest to develop panel of assays to be used for screening of potential immunotoxicity of NPs.

Strategy: Immune system is complex network of cooperating cells, therefore panel of assays have been proposed for monitoring: Natural cellular immune response - phagocytic activity and respiratory burst of leukocytes, natural killer cell activity. Acquired cellular immune response: proliferative activity of lymphocytes in vitro stimulated with mitogens and CD3 antigen. Non-functional assays: immunophenotypic

analysis of leukocytes, expression of adhesion molecules on leukocytes. Humoral immune response: interleukins.

Cell components used to evaluate the immunotoxicity induced by NPs: 1) human peripheral blood cells from volunteers were exposed in complete culture medium to increasing concentrations of dispersed NPs from 0.12 µg/cm2 to 75 µg/cm2 and analyzed after up to 72h of exposure. 2) Rat peripheral blood and spleen cells from exposed animals. Female Wistar rats have been intravenously exposed to single dose of TiO2 NPs: 0.59 mg/kg or 3 doses of OC-Fe3O4 NPs: 0.03642 mg/kg, 0.3642 mg/kg, 3.642 mg/kg and killed 1 day, 1 week, 2 weeks and 1 month after exposure. For TiO2 and OC-Fe3O4 NPs, results of in vitro assays have been compared with in vivo findings using the same immune assays.
Summary of results: Comparison of in vitro and in vivo findings

Phagocytic activity of granulocytes: In vitro, both tested NPs stimulated phagocytic activity of granulocytes. In vivo - significant stimulatory effect of TiO2 and no significant alterations in animals exposed to OC-Fe3O4. Stimulatory effect of in vivo exposure to TiO2 NPs is in agreement with in vitro studies. Phagocytic activity of monocytes: in vitro, high dose of OC-Fe3O4 NPs suppressed the cell function. Exposure of rats to high dose of OC-Fe3O4 also significantly depressed phagocytic activity. Respiratory burst of phagocytes: TiO2 NPs - stimulated respiratory burst of phagocytes after in vitro and in vivo exposure.

Proliferative response of lymphocytes: Basal response: in vitro as well as in vivo exposure to TiO2 NPs significantly stimulated basal proliferative activity of peripheral blood cells. T-lymphocyte response: stimulatory effect of TiO2 NPs on proliferative activity of T-lymphocytes (stimulated with Con A) is in agreement with in vitro study. Expression of adhesion molecule CD54 on lymphocytes: no effect of in vitro as well as in vivo NP exposure was found.

Conclusion: In vitro model of human peripheral blood cells reflected to the certain extent in vivo response of animal peripheral blood immune cells to TiO2 and OC-Fe3O4 NPs exposure seen in exposed rats. In vivo findings approved reliability of our proposed panel of immune assays used as biomarkers for assessment of in vitro immunotoxicity.

Human peripheral blood cells can be used as in vitro model for assessment of immunotoxicity. In first tier, cytotoxicity assays should be used to identify non-cytotoxic concentrations of the NPs for in vitro studies. Moreover, including positive and negative controls in both in vitro and in vivo models is strongly recommended. Testing strategy for assessment of immunotoxic effect of NPs should contain panel of immune assays to cover several aspects of immune response.

Aim of CCT-5: Genotoxicity: The objective of this CCT was to investigate the potential genotoxic effects of NPs and to evaluate suitable models for genotoxicity. Genotoxic compounds can be mutagenic and thus potentially carcinogenic. It is therefore of particular interest to develop suitable genotoxicity assay for NPs as essential part of testing strategy.

Strategy: the following assays and endpoints were used to evaluate genotoxicity of NPs - the CBMN assay to detect mutagenicity, clastogenicity and aneugenicity; the CA to detect strand breaks, FPG modified comet (FPG CA) for oxidised DNA lesions (oxoGuanine and Fapy

derivates); phosphorylation of Histone2A.X (pH2AX) assay for double strand breaks. The following organ-representative cells were evaluated for NPs-induced genotoxicity: blood (TK6, human peripheral blood lymphocytes); kidney (MDCK and LLC-PK, Cos-1 and HEK293); vascular system (endothelial cell line Ecp23 and EC219); lung (16HBE); placenta (BeWo); liver (hepatocytes and Kupffer cells); CNS (HCEC); 3T3 mouse fibroblasts for automation procedures.

Summary of results

Blood: TK6 cells and peripheral blood lymphocytes were used to evaluate genotoxicity (using CBMN and CA/ FPG CA assays). The CA: a) Exposure of TK6 cells to U-Fe3O4, PLGA-PEO, Fl-25 SiO2 and Fl-50 SiO2 and Endorem NPs did not cause genotoxic effect. TiO2 NPs induced DNA damage in TK6 cells dependent on the dispersion protocol used. OC-Fe3O4 NPs increased SBs and oxidized bases. b) CA on peripheral blood lymphocytes confirmed the results o TK6 cells. Additionally, individual susceptibility to NPs exposure was demonstrated. CBMN assay: the CBMN assay was adapted for NPs due to potential interferences of NPs with cytochalasin B. Results on TK6 and on human peripheral blood lymphocytes suggest no clastogenic effect.

Vascular system: TiO2 NPs induced DNA damage, but U-Fe3O4, OC-Fe3O4 and both-sized Fl-SiO2 NPs induced only slight DNA damage whereas PLGA-PEO NPs were negative.

Liver: TiO2 NPs were the only NPs dose-dependently inducing genotoxicity in hepatocytes. All tested NPs but PLGA-PEO NPs caused a genotoxic effect in Kupffer cells even after a short time exposure (4h). DNA damage was also more relevant in Kupffer cells than in hepatocytes.

Lung: DNA damage was significantly induced only after Fl-25 SiO2 NPs exposure.

Placenta: CA results showed a dose related response to TiO2 NPs in BeWo cells. U-Fe3O4 (75 ug/cm2) and Fl-25 SiO2 NPs induced only mild DNA damage. OC-Fe3O4 NPs did not induce genotoxicity.

Central Nervous system: TiO2 and Fe3O4 NPs induced DNA damage in endothelial cells.

Kidney: PLGA-PEO and U-Fe3O4 NPs did not cause genotoxic effects using the CA in Cos-1 cells. In contrary OC-Fe3O4 NPs induced a mild but significant increase of DNA damage and oxidative DNA lesions. Fl-25 SiO2 and Fl-50 SiO2 NPs caused slight increase in SBs and oxidized bases in Cos-1 cells. TiO2 NPs caused DNA damage (HEK293, Cos-1) but the effect was dependent on the NPs dispersion protocol used.

Conclusion: Genotoxicity induced by NPs depends on the cell type, the NPs, the dispersion protocol and the measured endpoint. It is crucially important to use non-cytotoxic concentrations when assessing genotoxicity. Both CBMN and CA, especially with lesion specific enzymes, can give a reliable picture of potential genetic instability as they measure different endpoints, while pH2AX assay is an interesting end-point for automated procedures.

WP3 In vivo studies to validate the in vitro findings

Aim of WP3: The objective of the in vivo study was to validate the findings of the alternative in vitro assessment of the toxicological profile of NPs used in diagnostics or therapeutics, as planned by WP2, by experiments on animals.

Strategy: First the acute toxicity study was performed in order to define the LD50 for TiO2 NPs and OC-Fe3O4 NPs after intravenous (i.v.)

administration to adult female rats. The study was performed according to OECD guidelines 425. LD50 for TiO2 NPs was established to be 59.22 mg/kg with confidence interval from 55 to 70 mg/kg. For OC-Fe3O4 NPs, the LD50 was 36.42 mg/kg with confidence interval (0 - 20 000 mg/kg). In the in vivo experiment, animals were divided into five groups: negative control (vehicle); reference control - i.v. administered TiO2 NPs in a dose equal to 1% of LD50/ kg body weight; and three exposed groups receiving OC-Fe3O4 NPs in doses: 0.1, 1 and 10% of LD50/kg body weight (established in the acute toxicity study). Female outbred Wistar rats (age 8 weeks, weight 205.5 ± 8.5 g) from Velaz, Prague were used for the experiment. Intravenous injection was performed under combined ketamine/xylazine anaesthetization. Each group consisted of 8 animals/battery of tests. There was a single exposure at the beginning of the study with subsequent sacrificing after 1 day, 1 week, 2 and 4 weeks.

The following biomarkers were measured:
- cardiotoxicity and hepatotoxicity (oxidative phosphorylation parameters in isolated heart and liver mitochondria: oxygen consumption after stimulation by ADP, basal oxygen consumption, rate of oxidative phosphorylation, mitochondrial membrane integrity, coupling of oxidation with phosphorylation, measurement of complex I activity by the use of NAD substrate glutamate or malate, concentrations of oxidised forms of coenzymes Q- CoQ9ox and CoQ10ox, and alpha-tocopherol levels in isolated myocardial and liver mitochondria, contents of cholesterol and triacylglycerols in the liver tissue);
- damage in lung tissue cells (inflammatory bronchoalveolar lavage (BAL) biomarkers: count of alveolar macrophages (AM), differential count of cells (AM, granulocytes and lymphocytes), immature forms of AM, multinucleated lung cells and total amount of protein; cytotoxic BAL parameters: phagocytic activity of AM, viability of AM, lactate dehydrogenase activity (in cell - free lavage fluid), acid phosphatase activity (in cell - free lavage fluid and in BAL suspension), cathepsin D activity (in cell - free lavage fluid and in BAL suspension);
- nephrotoxicity (plasma concentration of glucose, albumin, creatinine, urea, bilirubin, total cholesterol, triacylglycerols, sodium, potassium, calcium, magnesium, phosphate, iron; enzyme activities of AST, ALT, GTT, lipase, and creatinine kinase), and urine chemistry (concentration of urea, creatinine, sodium, potassium, calcium, magnesium, phosphate), creatinine and urea clearance was calculated, renal excretion of urea, creatinine, minerals, and ions and protein excretion per 24 h was calculated, fractional excretion of ions and minerals was calculated, plasma advanced oxidation protein products (AOPPs), advanced glycation end-products, plasma immunoreactive insulin, rat-specific hsCRP, carbonyl-proteins and rat-specific asymmetric dimethylarginine and kidney injury molecule-1 (KIM-1) and calbindin concentrations in urine were measured, TGF- 1, collagen IV and TNF-a gene expression in kidney cortex homogenates were measured);
- basic haematological examination was undertaken at sacrifice (erythrocytes, leukocytes, platelets, hemoglobin, hematocrit)
- organ weight (kidneys, liver, spleen, brain, heart, lungs), organ-to-body weight ratio was determined;
- genotoxicity (CBMN test in bone marrow, CA detecting strand breaks (SBs), oxidative DNA damage and sensitivity of DNA to hydrogene peroxide in white blood cells by the CA;
- oxidative stress (concentrations of malondialdehyde (MDA) and activities of antioxidant enzymes (glutathione peroxidase (GPx), catalase, glutathione S-transferase, superoxide dismutase (SOD) and ceruloplasmin oxidase) – in liver, lung, brain, heart tissues and in

blood, levels of reduced glutathione (GSH) and vitamins tocopherol, tocopherol, carotene, retinol, xanthophyll and lycopene) as non-enzymatic antioxidants in blood);
- immunotoxicity - natural cellular immunity: phagocytic activity of granulocytes and monocytes and respiratory burst of phagocytes, specific cellular immunity: lymphoproliferative assay (LTT), lymphocyte subsets in peripheral blood using flow cytometry, expression of adhesion molecules CD11b and CD54 on peripheral blood leukocytes, humoral immunity: levels of tumour necrosis factor alpha, interleukin-10 and interleukin-4 in blood and spleen cell supernatants);

Summary of results:
Cardiotoxicity and hepatotoxicity: Changes in functional parameters of heart mitochondria, CoQ and a-tocopherol content in the groups of 0.1% and 10% OC-Fe3O4 NPs could be caused by adaptation of the organism to short time exposure (1 day, 1 week). After longer exposure, there were no significant changes in these parameters. Our results did not indicate severe damage of heart mitochondria after all.

Oxygen consumption after stimulation by ADP, basal oxygen consumption and rate of oxidative phosphorylation (rate of ATP production) were significantly increased after 2 weeks of exposure in liver mitochondria in the group of 10 % OC-Fe3O4 NPs. However, according to cholesterol and triacylglycerol concentrations in the liver tissue, the administered NPs did not cause any serious toxic liver damage.

Damage in lung tissue cells: The most expressive response after exposure to NPs compared with the corresponding control was found in acute phase (1 day after exposure). 7 and 14 days after exposure, less pronounced response of bronchoalveolar lavage (BAL) parameters was found, but still the response was comparable with that, found one day after exposure. 28 days after exposure, BAL parameters were moderately changed in comparison with control; significant differences between rats exposed to 0.1%, 1% and 10% LD50 OC-Fe3O4 NPs and control animals disappeared. The highest biological activity was recorded in rats administered with high dose – 10% LD50 OC-Fe3O4 NPs. TiO2 NPs influenced BAL parameters in less extent - but its biological effect is not negligible. Significance of differences in inflammatory BAL parameters after longer period of time after exposure (28 days) was decreasing. The cytotoxic parameters showed increasing trend with time after exposure. The lack of significance could be affected by large interindividual differences in animals.

Basic haematological examinations and renal toxicity: Administration of NPs did not affect significantly the metabolic parameters studied (glycaemia, insulin-to-glucose ratio, lipid profile, liver enzyme activities and creatine kinase activity). No clinically significant differences among the groups were observed in parameters characterizing renal function (plasma urea and creatinine concentration, creatinine and urea clearance regardless of the way of their expression, renal excretion of urea, creatinine, proteins, minerals and ions, as well as in fractional excretion of ions and minerals). Our findings suggest some disturbance in ions and minerals handling one week after exposure to NPs, but these changes were not systematic, and appeared to be within the normal ranges. Two weeks after administration of NPs urinary pH of the control animals was significantly higher in comparison with all NPs administered groups. In any of analyzed time intervals after the administration of NPs we observed no significant signs of uremic toxins accumulation in circulation in experimental animal groups when compared

to controls. No changes in white blood cell counts, and hsCRP concentrations were revealed between the controls and NPs administered animals in either time interval. No significant differences between the groups in the expression of pro-fibrotic (TGF-1 and collagen IV) or pro-inflammatory (TNF-a) genes in renal cortex were detected in any of time intervals studied.

Genotoxicity: Neither mutagenic (incidence of micronucleated immature erythrocytes) nor cytotoxic (decrease in the proportion of immature erythrocytes) effects were found when testing the selected NPs in mammalian erythrocyte micronucleus test in bone marrow of animals. The selected NPs in tested doses were non mutagenic or the negative result was a consequence of the fact that the NPs did not reach the target tissue (bone marrow) and this test is not appropriate to use in this case.
Most pronounced increase in strand breaks and oxidative damage of DNA was detected 2 weeks after exposure to both TiO2 and OC-Fe3O4NPs in all concentrations. In the case of TiO2 NPs a moderate genotoxic effect was recorded.
The strongest genotoxic effect was induced by OC-Fe3O4 NPs of the lowest concentration (0.1 % of LD50), where 3 times increase in SBs and 2.9 times increase in FPG sensitive sites was observed after 2 weeks of exposure.

Oxidative stress: Most significant differences after OC-Fe3O4 NPs exposure were observed after 1 day in brain and after 1 week in liver and lung. Most sensitive biomarkers were the activities of GPx and SOD. Regarding the results of measurements in blood the decreases in concentrations of non- enzymatic antioxidants were most notable after 4 weeks of exposure.
According to the concentrations of both MDA and GSH (which have not been changed significantly), we can assume, that exposure to NPs could evoke certain changes in the production of ROS, but moderate changes in the activities of AOEs. Changes in concentrations of non –enzymatic components of antioxidant system were able to prevent them in exerting significantly deleterious effect in the cell membranes of corresponding tissues.

Immunotoxicity: In exposed animals, immunomodulatory effect of TiO2 and OC-Fe3O4 NPs manifested very early, from one day to one week after exposure. In first phase, phagocytic activity and respiratory burst of leukocytes was significantly altered by the exposure to NPs. While stimulatory effect of TiO2 NPs on phagocytic function of granulocytes and monocytes and respiratory burst of phagocytes was found, high dose of OC-Fe3O4 NPs suppressed phagocytic activity of monocytes. Moreover, one week after exposure, treatment of animals with OC-Fe3O4 NPs increased percentage of (CD8+) cytotoxic T-cells in peripheral blood of exposed rats. Enhanced levels of interleukin-10 (produced primarily by monocytes) found in spleen cell cultures in rats exposed to low dose of OC-Fe3O4 NPs might indicate the effort to regulate immune response. Later, 4 weeks after exposure, functionality of the lymphocytes was changed measured by proliferative activity after stimulation of the cells with panel of mitogens and CD3 antigen. Although significant stimulatory effect of both NPs on proliferative activity of T-lymphocytes and T-dependent B-cell response was found 4 weeks after exposure, most changes in TiO2 exposed groups were detected. In OC-Fe3O4 group, weak dose-dependency was observed. Moreover, significantly increased levels of tumour necrosis

factor-alpha (4 weeks) in animals exposed to low dose of OC-Fe3O4 NPs have been found.

Conclusion: Our in vivo study documented that single i.v. administration of TiO2 NPs and OC-Fe3O4 NPs in above mentioned doses to young female rats did not elicit overt acute or subacute toxicity. Subtle differences in some parameters between the control and NPs administered groups were revealed mainly short time after exposure to NPs. These findings indicate possible immunomodulatory effect of single intravenous exposure to OC-Fe3O4 less than TiO2 NPs in exposed animals.

WP4 Structure Activity and PBPK modeling

Aim of sub WP4.1: Structure-Activity Modelling: The objective of this task was to explore the feasibility of developing structure-activity models for NPs. This was interpreted in a broad sense to include any relationship between the structure of NPs (using suitable descriptors) and their (toxicological) effects at any level of biological organisation.

Results: Development and evaluation of a theoretical model for predicting the oxidative stress potential of metal oxide NPs. Following a preliminary review of the literature, it was clear that traditional statistically-based QSAR modelling, which requires multiple chemical descriptors and a reasonable number of high quality biological datapoints, would not be feasible. Therefore, a more theoretical approach was adopted. Building on previous findings (Meng et al, 2009) linking the oxidative stress potential of NPs with adverse outcomes, including inflammation, cytotoxicity, and in vivo toxicities, a theoretical model was developed (Burello & Worth, 2011) for predicting the reactivity of metal oxide NPs as well as their ability to cause oxidative stress through the generation of ROS. The model, which is based on reactivity descriptors (electronegativity/Fermi energy and the band gap of the bulk oxide), calculates and compares the energy band structure of metal oxide NPs with the range of redox potentials exhibited by cellular reactions. On this basis, the model predicts whether electron transfer, and consequently ROS generation/oxidative stress, is likely to occur between the nanomaterial and adsorbates from the cellular environment. The predictions for 70 metal oxide NPs were validated against in vitro data taken from the scientific literature as well as in vitro data generated by NANOTEST partners. Overall, it was found that the model can qualitatively predict the oxidative stress potential of metal oxide particles through ROS generation. In addition, in the case of titania, the model could predict the photocatalytic activity of this material and the isoelectric point of the planes exposed by rutile crystals. An independent and experimental verification of the oxidative stress model has recently been published by Zhang et al. (2012)

Conclusion: The theoretical model for predicting oxidative stress potential could be used in the assessment of NPs by prioritising metal oxides for further evaluation, and by forming part of a more extensive battery of models and in vitro tests for characterising metal oxide toxicity.

Aim of sub WP4.2: PBPK Modelling: The objectives of the Physiologically Based Pharmacokinetic modelling were a) the formulation of simple compartment-based model to describe biodistribution of NPs and b) the use

of computational fluid-particle dynamics (CFPD) to analyze the behavior of NPs in the respiratory and the cardiovascular system.

Results

a) Integration of a lung transport & deposition model and a lung clearance/retention model to predict internal doses by inhalation exposure data: The combination of the deposition model with the clearance model allows for the connection of the external exposure to the internal dose, though further research is required on the extension of this model to account for the distribution of the inhaled particles to other targets organs in the human body. This will be feasible in time, as the particle transport mechanisms into the body become clearer and the experimental data increase. Overall, the presented study demonstrates that the combination of representative measurements of the full size spectrum of the inhaled aerosol, mechanistic mathematical modelling of deposition and of clearance/retention and in vitro toxicological assays can lead to derivation of exposure limits and risk assessment.

b1) Employment of CFPD to estimate particle behavior in the respiratory system: Transport and deposition of particles in a physiologically based bifurcation created by the 3rd and 4th lung generations, was calculated for different flow conditions and particles sizes using an in-house computational model. The study showed that total deposition fraction increases with increasing particle size, but does not change significantly for the different flow conditions. These results are in agreement with earlier experimental findings. Moreover, it was shown that there is significant deposition both at the bifurcation and the walls of the daughter tubes. In the latter case, the deposition sites and the particle concentration profiles change significantly between the different flow cases. In the next step, particle transport and deposition will be studied in real lung geometries obtained by imaging techniques (CT-scans, MRI).

b2) Employment of CFPD to estimate particle behavior in the cardiovascular system: The computational model was used to predict the transport and deposition of superparamagnetic particles suspended in a liquid under the influence of an external magnetic field. Particle deposition fraction was calculated for different liquid velocities and viscosities, as well as for different magnetic fields and the results were compared to previous experimental findings. In all examined cases the fully Eulerian model describes successfully the qualitative characteristics of the experimental deposition fraction curves, although in some cases underestimates deposition. This is an indication of missing deposition mechanism and further studies are in order.

Conclusion: In summary, all the above studies showed that with proper refinement the developed computational models and methodologies, may serve as an alternative testing strategy, replacing experiments that are expensive both in time and resources.

Strategy and suggested battery of tests

Overall aim

The overall aim of NANOTEST was to provide a testing strategy for NPs used in medical diagnostics.The specific objective of the project was to develop a set of Master SOPs for at least 2 assays for each type of toxicity. The most advanced and standardised techniques will be adapted for automation and prepared for validation. NANOTEST aimed to provide

testing strategies for hazard identification and risk assessment of NPs, and to propose recommendations for evaluating potential risks associated with new medical NPs. A battery of assays that can be directly applied to fulfill regulatory requirements (REACH) will also help to decide whether new regulations are needed for risk assessment of NPs. In vitro and in silico methods will have an impact on the use of animals for toxicity testing. The development of these methods and strategies can also be utilised for the assessment of health effects of NPs used and applied in other areas (cosmetics, etc.) and thus can have wider impact on all 3 Rs (Replacement, Reduction and Refinement).

Testing strategy

One of the main obstacles for assessing the toxicity of nanomaterials is the lack of knowledge of how physicochemical properties relate to the interaction of NPs with biological system and the mechanism of toxicity. It is clear that physical and chemical properties can influence NP behaviour and may have an impact on toxicity; they must therefore be an integral part of toxicity testing. This is one of the key aspects of toxicity screening strategies (Dusinska et al. 2009, 2011, 2012, 2013). Both primary and secondary characterisation of tested NPs are crucial, including in situ characterisation during exposure. The physico-chemical properties that should be considered for assessing toxic effects of nanomaterials include as a minimum chemical composition, particle size, shape, surface properties, size distribution, agglomeration state and crystal structure. Regarding the likelihood of biomolecular corona formation, it is also important to set up experimental conditions that can mimic exposure in humans. As NPs change their properties depending on surrounding milieu we recommend at least two different exposure conditions for testing the NPs effects (Magdolenova et al., 2012a).

The question also was to determine if the commonly used assays for chemicals could be applied to NPs. Our results show that it is not possible to use these assays without careful adaptation because interference is always possible, especially between NPs, the dye and the optical detection or with the assay components during the experiment. It is therefore of crucial importance to test possible interference of all studied NPs with the foreseen methods prior to evaluating cellular responses to NPs. Furthermore, all the assays do not have the same sensitivity and it is important to choose the most sensitive appropriate assay. From our results, the oxidative stress markers: thiol depletion and induction of antioxidant enzymes seem to be more sensitive that the measure of ROS. Our proposal for further evaluation of testing strategy is to perform first a battery of assays for validation of the effects of a representative set of unknown NPs on the target cells (detailed below), then if appropriate and available, to screen larger banks of unknown NPs using automated procedures on a predefined set of representative cells. Appropriate statistical analyses must always be implemented.

From the evaluation of the different cell models, depending on route of exposure and use of NPs there should be several organ models used for testing. Blood is an important model both as a direct target as well as surrogate target tissue and gives an indication of toxicity. Peripheral blood lymphocytes are suitable cells but unfortunately not always accessible, thus the TK6 (lymphoblastic) cell line is an alternative. We additionally propose that commercially available human cell lines for each representative organ be included in the testing strategy e.g. for lung cells available cell lines (A549 cells is one alternative), CaCo2

cells (colon), LN229 cells (glioblastoma), and HEK293 or MDCK (porcine kidney). The strategy proposed is:

1) to determine possible cytotoxicity and induction of oxidative stress
- For cytotoxicity studies: basal cellular toxicity tests such as RGA and PE and the MTT and WST-1 assays and a time course of 24 h and 72 h, using OC-Fe3O4 NPs as positive control NPs and PLGA-PEO NPs as negative control NPs. However, the cytotoxicity of Fe3O4NPs seems to depend on the coating rather than the iron oxide core itself.
- For oxidative stress: the thiol depletion measured by monobromobimane assay (and possibly DCFDA) and the induction of antioxidant enzyme mRNA expression measured by RT-qPCR (Guadagini et al., 2013b), 4 h and 24 h time-course, using uncoated TiO2 NPs as positive control NPs and PLGA-PEO NPs as negative control NPs
2) then to determine the uptake and possible release, following uptake, of the NPs by relevant cells of the different organs, at non cytotoxic concentrations of the NPs,
- For uptake and release studies: 24 h uptake followed by 24 h release, using U-Fe3O4 NPs as positive control NPs.
- For transport studies: 24 h time-course, using OC-Fe3O4 NPs as positive control NPs, limiting these experiments to NPs which do not agglomerate in the culture conditions.
3) then, to determine possible genotoxic effect (see review Magdolenova et al., 2012b,2013)
- For DNA damage: cells exposed for 24h to NPs, using the Comet assay for DNA SBs and oxidized DNA lesions (TiO2 or OC-Fe3O4 as positive control NPs and PLGA-PEO NPs as negative control NPs, at non-cytotoxic concentrations of NPs).
- For mutagenicity and clastogenicity: CBMN modified protocol for NPs genotoxicity testing. However, positive and negative controls should be further specified.
4) The H2AX assay is an interesting end-point for automated procedures, but little information so far exists about predicting NP-induced genotoxicity using this test. The following experimental testing strategy is proposed for assessment of immunosafety of newly developed NPs. Human peripheral whole blood or isolated peripheral blood mononuclear cells (PBMC) as representatives of human blood cell model are proposed for in vitro screening of the immunotoxic potential of nanoproducts. The main strength is the complexity of the model containing several cell components in a relatively intact environment. Testing strategy for assessment of immunotoxic effect of NPs should contain a panel of immune assays to cover several aspects of immune response. Cellular immune response: phagocytic activity and respiratory burst of leukocytes, natural killer cell activity, proliferative activity of lymphocytes in vitro stimulated with mitogens and antigen (LTT). Humoral immune response: production/expression of cytokines. In first tier, cytotoxicity assays should be used to identify non-cytotoxic concentrations of the nanoparticles for in vitro studies. Moreover, inclusion of positive and negative controls in both in vitro and in vivo models is strongly recommended:
- For LTT test: Fl-25 SiO2 (75µg/cm2) as immunosuppressive control and TiO2 NPs as possible candidate for immunostimulatory control (75µg/cm2).
- For phagocytic activity and respiratory burst assay: U-Fe3O4 as stimulatory control (0.12-3µg/cm2), and OC-Fe3O4 (75µg/cm2) as suppressive nano-control.
- For natural killer cell activity: OC-Fe3O4 NPs as suppressive control (75µg/cm2) and Fl-25 SiO2 (75µg/cm2) as stimulatory control.

- For cytokine gene expression:OC-Fe3O4 NPs as suppressive control (75 µg/cm2) for IL-6
5) finally, to perform the more selective evaluations, as required by the particular characteristics of the organ-representative cells, such as cytokine production, cellular localization of NPs inside cells using techniques such as confocal or electronic microscopy techniques, etc

For high-throughput and automated procedures: Once the target cell lines and the representative assays have been defined (see above), high throughput screening and automation can be implemented for the testing of a large number of NPs. Increased throughput of the CA for detection of SBs and specific DNA lesions is suggested for robust testing together with automation and high throughput of assays for cytotoxicity (measuring cell count, nuclear intensity, nuclear size) and alternatively also for genotoxicity (H2AX assay). It is recommended to use a multi-parametric analysis (High Content Imaging-HCI) which provides more information and can allow us to determine the cause of the cytotoxic effect. HTS assays provide several benefits, including an upscaling of number of NPs to be tested; for optimization and precision of the assays; as a support to validations; and if applicable also for industrial use.

Technical limitations of the assays:
a) interference with specific assays was observed for metallic oxide solid core NPs. Thus, the evaluation of possible interference is required to ensure reliable results. This is mainly relevant for cytotoxicity assays, oxidative stress responses of cells, and the production by the cells of bio-molecules such as peptides, proteins, or others (Guadagnini et al., 2013a).
b) the release and transport studies are limited to NPs that can be detected at low concentrations in buffers, and are also limited by the physical properties of the membranes used to develop 2-chamber models and to NPs which do not agglomerate under cell culture conditions.

The structure-activity models (Q)SARs seems to be promising tool in testing strategy for future. One approach used in NANOTEST is the theoretical model predicting oxidative stress potential that could be used in the assessment of NPs by prioritising metal oxides for further evaluation. The proposed model could be used to guide the development of more rational and efficient screening strategies; in addition, it can create a more coherent conceptual framework when additional toxicity related physicochemical properties (e.g. agglomeration and solubility in water) are included (Burelo and Worth, 2013).

Conclusions and remarks
For NP toxicity testing the primary and secondary characteristics of NPs should be included as an integral part of the testing strategy. We proposed that at least 3 cytotoxicity tests (the MTT, WST-1 and plating efficiency assays or RGA), a set of (at least 3-5) representative cells and 5 NPs concentrations are used for each NPs. Initially, cytotoxicity response to the NPs must be determined, then oxidative stress response using at least 2 assays. A testing strategy for assessment of immunotoxic effects of NPs should contain assays covering several aspects of immune response (inducible proliferative response, phagocytic and respiratory burst). For genotoxicity, the modified comet assay for DNA damage (strand breaks as well as oxidised DNA lesions) should be included in the testing strategy together with the micronucelus assay, optionally the H2AX for automated procedures. The evaluation of internalization of NPs by cells is not always easily accessible, and the devices designed to evaluate NPs transport across cell layers need the technology improving before being safely proposed.

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Potential Impact:

The rapid growth in the use of nanomaterials in medicine has led to a concern about possible health risks. There is a serious lack of information available to predict health effects due to nanomaterial exposure. In a regulatory context, nanoscale materials are still mostly treated in the same way as conventional chemicals and there is no consensus or clear nano-specific guideline for their regulation (Dusinska et al. 2009; 2011; 2012; 2013). The NANOTEST project (see http://www.nanotest-fp7.eu online) was one of the initiatives set up by European Commission to fill the knowledge gaps in this area, by studying the interactions of representative therapeutic nanomaterials with living cells, and developing and validating appropriate high-throughput toxicity testing protocols using in vitro models. Keeping in mind the 3 Rs (refine, reduce, replace), the testing strategy should reduce in vivo experiments. To achieve this, a comparison of in vitro and in silico with in vivo results is an important and critical aspect of the validation of in vitro and in silico methods. One of the main problems encountered in testing NPs for possible human hazard include the lack of appropriate standard protocols.

The NANOTEST project started in April 2008 and lasted four years. A range of medically relevant NPs were screened using a broad range of toxicity assays, across cell lines representing up to eight organs, with a view to development of alternative high throughput testing strategies to assess the toxicological profile of NPs used in medical diagnostics. During the subsequent four years, twelve partners were involved in the project, bringing together top scientists from the whole of Europe. Harmonisation and quality assurance have been the key elements in every step, and we have used standard protocols and the same data templates in order to be able to compare results, as well as using NPs from the same batch.

Appropriate risk assessment in relation to health and safety of NPs used in medicine is essential, in order to ensure ethical, societal and regulatory acceptance, and maintain public confidence. Many knowledge gaps concerning the health impact assessment of therapeutic NPs must be filled. There is presently no consensus on the most suitable toxicity tests, models for exposure assessment, and standardized testing strategies to evaluate possible hazards of therapeutic NPs for human health. Long-term effects of chronic exposure in humans of these NPs must be determined and tools for detecting NPs in situ must be developed for therapeutic NPs. Bio-transformation of therapeutic NPs in the human body and their interaction with biological systems should be studied in conjunction with a characterisation of NPs in terms of size distribution, surface properties, biopersistance, and stability of original and modified NPs in different biological media. The accurate and precise analytical (for characterisation of therapeutic NPs) and biological methods (for efficacy and/or safety assessment), and appropriate in vitro and in vivo models must be developed and validated in order to analyze solubility, biodegradability and dissolution of NPs in biological media, their interactions with living organisms, their reactivity and uptake by cells and tissues. The NANOTEST project contributed substantially to this complex task.

In order to develop suitable alternative testing strategies, it was essential to bring together a panel of experts from different disciplines in order to maximise the benefit that can be obtained from different and novel approaches to this problem. This was an opportunity for the

cosortium of European researchers to push this important research area forward. This was possible only through the collaboration of highly competent experts in an interdisciplinary network combining the highest levels of European expertise. For NANOTEST to be successful it was essential that we had the necessary critical mass in terms of resources, knowledge and facilities and this could not be achieved at a national level. NANOTEST outcomes are remarkable and their potential impacts are listed below:

Impact on toxicity testing of NPs by developing tests and testing strategy

The main goal of the NANOTEST was to develop alternative testing strategies and high-throughput toxicity-testing protocols using in vitro and in silico methods for the assessment of the toxicological profile of NP used in medical diagnostics. The NANOTEST approach focused on eight different target tissues and organs (blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems) and crucial toxicology pathways – oxidative stress, inflammation, immunotoxicity and genotoxicity. Additionally detailed characterization of NPs was performed. Toxicity and uptake studies, methods development and high-throughput and assays automation allowed us to deliver a battery of assays relevant to targeted organ for the development and validation of suitable biomarkers. Additionally, NANOTEST research highlighted problems connected with interaction of NPs with toxicity tests, proteins and other components in biological fluids or with detection analysis which may impede their detection for the assessment of the biological effects of NP exposure. We observed important differences in NP interference with cytotoxicity assays depending on the type of NPs, their characteristics, and the test method. Thus standard toxicity testing strategies for chemicals need to be adapted by integrating NP-specific considerations. Interference can be assay specific as well as NP specific. It is therefore of crucial importance to test possible interference of all studied NPs with the foreseen methods prior to evaluating cellular responses to NPs. The physico-chemical analysis of NPs may thus allow the pre-selection of suitable assays. The testing strategies should thus take into consideration possible interference for test reliability but also the feasibility of the replacement techniques. To summarise, one of the major impacts of NANOTEST is the establishment of a framework of toxicity tests that can be applied to a broad range of common cell lines to evaluate any NP. Additionally, publications from the project highlight the potential pitfalls posed by the interference of NPs with established toxicity assays. In combination, these have the benefit of providing guidance to the community and enabling comparison against a well characterised bank of nanoparticles, reducing wastage of time, money and other resources through the pursuit of inappropriately designed experimental strategies. With ever increasing interest in the area of nanoscience, the outcomes from the project provide an invaluable guide to both new and experienced researchers who are drawn into this area from other disciplines. Such investigators may be unfamiliar with the specific requirements and considerations that apply when working with NPs.

The research results also provide a framework for the study of in vivo toxicity and clarifies which parameters are of importance when switching from in vitro to in vivo or vice versa. The outcomes highlight the importance of working with a range of doses and time points. The study demonstrates the influence that different NP characteristics may have and highlights the assays most suitable for use in high throughput testing. NANOTEST has provided a robust body of evidence that will enable us to move forward with the implementation of suitable and appropriate safety guidelines.

Contribution to 3Rs

The information on toxicity of NPs can be obtained using animals and in vivo experimentation but it can be difficult to isolate the exact mechanisms and toxicological pathways involved in relation to specific NP characteristics in addition to ethical concerns regarding animal use. NANOTEST focused on development of in vitro and ex vivo models in a more

efficient manner to define the markers which can determine the toxicological potential of NP, before pre-clinical evaluation of new biomedical nanomaterials. Such an approach clearly decreases the numbers of animals necessary for pre-clinical validation and spares a lot of suffering for animals, and results in a drastic decrease of the number of animals which would be necessary to obtain this information.
Extrapolation from in vivo studies in nanotoxicity testing is even more challenging than with chemical toxicology, and due to the enormous variety of NPs being produced, alternative in vitro toxicity tests will have to be considered further. Within NANOTEST the key toxicology endpoints, uptake and transport studies have been addressed together with detailed in situ characterization in tested media using a broad range of human and mammalian cells in vitro. Knowledge gained by the NANOTEST consortium on the suitability of cell models and sensitivity of cells to NPs exposure will have an impact on further development and use of alterative toxicity testing beyond the nanotoxicology field and will further contribute to 3Rs. Additionally, several methods showed potential for robust high throughput testing (comet assay, micronucleus assay) and some of them had been developed for automation and High Content Imaging-HCI analysis. This will have further impact on use of animals in nanotoxicology and regulatory toxicology.

Contribution to European regulation and legislation
The ambition of the proposal was to develop suitable methods and testing strategies which could be further developed as robust automated assays and after validation, integrated into any new regulatory to complement existing European legislation. An approach to the safe, integrated and responsible introduction of nanotechnology into medical practice should thus be included at a fundamental scientific level, to assess all aspects of risk and to contribute to appropriate regulations for this new technology. This approach is fully in line with the Commission's European strategy for nanotechnology set out in the Communication "Towards a European Strategy for Nanotechnology" and its associated Action Plan.
NANOTEST investigated 6 selected NPs and collected new knowledge addressing the key toxicity endpoints. A better understanding of NP kinetics, molecular and cellular mechanisms, pathways of action, and their associations with health effects in exposed cells, organs, animals, and human blood achieved in NANOTEST will benefit medical sciences, provides knowledge required for risk assessments, and will support the definition of guidelines for the safe production, use, and disposal of NP.
Many standard assays have been modified for NP toxicity testing to address unique features of NPs and to avoid interference of NPs with the testing procedure or detection method and altogether 29 SOPs for test methods were developed. Additionally, primary and secondary characterisation of NPs was performed in tested media and suggested as an integral part of testing strategy.
Generally, chemicals are regulated under REACH (registration, evaluation, authorization and restriction of chemicals) and nanopharmaceuticals and nanomedical products and devices under the regulatory directives of EMEA in Europe (EMEA 2006), or FDA in the US. It is acknowledged (HSE 2005, SCENIHR 2005, 2007) that the current regulatory framework does not deal well with the specific challenges set by the novel properties of nanomaterials. However, it is not expected that new regulatory frameworks will be introduced to address these deficiencies. A more likely approach is that the detail and the interpretation of the regulations will evolve to take account of new information as it becomes available. A possible change would be to introduce a different limit based on particle size or

on surface area, reflecting the different toxicity (on a mass based concentration basis) of the NPs. There is a potential for changes to occur in the EU legislation, particularly given the entry into force on 1 June 2007 of the new REACH Regulation, whose two most important aims are to improve the protection of human health and the environment from the risks of chemicals while enhancing the competitiveness of the EU chemicals industry. The decision to change regulation could potentially have major implications for the health of the community and for the future prosperity of the European economy. It is critical that any decision is evidence based and that the evidence used is of the highest quality and most comprehensive. The outcomes of NANOTEST will contribute to the development of future regulatory activity in this area.

Contribution to standards and quality assurance
NANOTEST has developed a common approach and best practices of how to handle nanomaterial, how to use them in toxicity tests and developed 29 standard operating procedures (SOPs) for toxicity test methods as well as pioneering several new approaches, which are now being developed as best practices for broader dissemination to the community.

The SOP protocols were formatted into the harmonised (based on JRC and ENPRA) template and are placed on the NANOTEST website to be used by the Consortium and in future by a larger community of scientists and toxicologists. Several of these protocols are being made available to the community via the FP7 QualityNANO (formerly QNANO) and NanoImpactNet protocols database, which has been taken over by the NanoSafety Cluster.

Additionally new approaches have been proposed for avoiding interference of test methods with NPs. |The Consortium developed candidates for negative and positive control NPs for the same end-points. Since the validation of such positive and negative control NPs was outside the scope of NANOTEST they will be further validated in other projects such as the large infrastructure FP7 QualityNano project (formerly QNano), with the final plan being that they might be developed as certified reference materials.

Several of the approaches developed or optimised in NANOTEST are suitable for standardization and validation by JRC (formerly ECVAM). Several core scientists are members of ISO/CEN committees on nanomaterials. Some of the NANOTEST SOPs are good candidates also for ISO/CEN standardization.

Contribution to community
Nanotechnology is a promising tool for the development of innovative treatment strategies allowing us to overcome obstacles encountered by classical drug delivery. This has led to the development of nanomedicine. Nanomaterials may allow the controlled release of therapeutics, protection of drugs against degradation, targeted drug delivery and facilitated transport across barriers. While a lot of attention has been paid to the development of new engineered nanomaterials and to new applications of nanotechnologies, comparatively less research has been performed to assess the potential hazard of these new materials. There is public concern about the potential health hazards of these new materials, especially as a variety of nanoparticles have been shown to induce toxicity related to their nanometer size leading to the new field of nanotoxicology.
One of the objectives of the NanoNOTEST proposal was to increase the knowledge about health effects of medical NP for health risks and to develop in vitro and in silico methods for toxicity assessment of NP. NP

toxicity studies are mostly conducted with materials that are not designed for medical use and which are more relevant for environmental and occupational exposures. NANOTEST addressed nanomaterials potentially used in nanomedicine. The impact of the results obtained from NANOTEST is important as it brings new knowledge and methodology needed to ascertain the potential health risk of NP, legislation, best practice and standards relating the impact of NP on human health and the environment. Our study, we believe, helps to address societal concerns and uncertainties relating to the use of NP and to avoid the adverse economic consequences and restricted technological advances that may arise from a failure to identify any potential health risks.

Impact on the state of the art and future research

The NANOTEST project has already had, and will continue to have, impact far beyond its size and budget, in many areas of nanotoxicology research as it has facilitated the development of several insights that will have a durable impact on the toxicology and other research fields. Among the key scientific developments resulting from the project are the understanding of the interaction of NPs with biological systems, interference of NPs with methods detection systems, uptake and transport studies, the different effects of aggregation and agglomeration on toxicity outcomes, development and modifications of methods for NP toxicity testing, etc.

Database development and its impact

A large database was developed with all results reported on the same template. Data were stored in the project database which comprised a list of the 303 datafiles, along with their contextual information. This database will be included into NANOhub hosted by European Commission's JRC as a source of information for the scientific community and regulators and for later metaanalysis. The NANOhub database is based on the OECD endpoint-related templates. It provides an inventory of information on NPs from various projects.

Training and dissemination activities

During the NANOTEST project, several training courses (harmonisation of procedures, handling of nanomaterials), practical courses (genotoxicity course), thematic workshops (as part of annual meetings), scientific workshops (with ENPRA, NanoImpactNet and other projects) and dissemination activities (workshop with stakeholders, conferences, publications) have been provided and stimulated beyond the project itself. This comprises practical laboratory science between disciplines and poles of excellence, e.g. the NPs preparation dispersion protocols, the transfer of suitable methods for toxicological in vitro assessment, database (NANOhub) training.

Publication and dissemination activities

Dissemination activities planned in Annex 1 have been fully achieved. Key stakeholders relevant to the NANOTEST project have been identified and appointed to a panel whose role was to promote our findings among interested bodies in industry, health authorities and other bodies. To promote NANOTEST in the early phase of the project, we had two publications. This was important to reach stakeholders and other who might benefit from the outcome of our project.

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toxicological profile of nanoparticles used in medical diagnostics. The Parliament Magazine's RESEARCH European Research and Innovation Review, Issue 9, May 2009, p. 38.
- Dusinska M and NANOTEST consortium.(2009) Safety of nanoparticles used in medical application. Development of alternative testing strategies for toxicity testing. Science and Technology,Public service review, issue 4, 126-127.

Several Open workshop meetings have been hold as another main route for dissemination. A website was established and is available in the public domain and acts as a portal for information to interested individuals. Many of the results obtained during the development of the tests for effects of NP, as well as the NP characterisation and prevalidation studies, have been of interest to the scientific community and large network of nanosafety experts and had been disseminated via large European NanoCluster network, and FP7 NanoImpactNet, Quality NANO (formerly QNANO), NanoTOES and other projects. Significant findings have been published in the scientific literature. Considerable amount of presentations (talks and posters) have been given at international conferences, specialist workshop meetings and training courses. There had been also opportunities to present our findings and ideas at seminars and invited lectures.

During the four years of project course, the consortium managed to gain an excellent record of publications from research acknowledged to the NANOTEST project. Additionally to papers already published, the major dissemination achievement of NANOTEST even beyond the project course, is the special issue dedicated to the project. The consortium managed to prepare 16 manuscripts summarizing all main outcomes and most important results to be published for special issue in journal Nanotoxicology. Of the 16 publications, 12 are already submitted to the journal and are under review. The remaining four papers will be submitted in February/March 2013. The special issue (supplement of Nanotoxicology) is planned to be published in spring 2013. The list of papers is provided below.

Recommendations for further research

Our research has highlighted further needs to improve in vitro and ex vivo models for NPs safety assessment to correlate outcomes preferably with human but also with in vivo effects.

Research should be directed towards the real exposure conditions and the use of in vitro models that can mimic in the best way the in vivo situation, e.g. co-culture and 3D cell culture approaches, and towards comparative in vitro and in vivo studies under similar conditions.

Further research is needed to investigate uptake mechanisms; to improve existing and create new barrier models and develop clear in vitro-in vivo correlations, including appropriate culture conditions (such as serum free).

Interaction of NPs with biological macromolecules such as proteins could potentially be an indicator of risk, and should be further investigated for the safe design of NPs.

New biomarkers related to nanomaterial-related effects and a comparison with already established biomarkers as well as nano-specific positive and negative controls need to be further addressed.

List of Websites:

http://www.nanotest-fp7.eu