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Mechanistic Modelling

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GLOSSARY

Abbreviation / acronym	Description
AE	associative events
Ag	Silver
AO	adverse outcome
AOP	adverse outcome pathways
API	application programming interface
BP	biological process
CC	cellular component
CTD	comparative toxicogenomics database
CV	cross validation
ENMs	Engineered nanomaterials
FC	fold change
GO	gene ontology
GSEA	gene set enrichment analysis
HAEC	human aortic endothelial cells
HAR	high aspect ratio
HUVECs	human umbilical vein endothelial cells
KE	key events
MF	molecular function
MWCNT	multi walled carbon nano tubes
QSAR	quantitative structure-activity relationship
p value	calculated probability
PBMCs	peripheral blood mononuclear cells
PCF	protein corona fingerprint
PVP	poly-vinylpyrrolidine
SA	simulated annealing
SiO_2	silicon dioxide
TiO_2	titanium dioxide
ZnO	zinc oxide

Detailed description of terms: http://www.enanomapper.net/library/enm-dictionary

1. EXECUTIVE SUMMARY

This deliverable describes the mechanistic modelling task which was developed within WP 4 of eNanoMapper project. Three main issues were handled in this task: 1) we initiated a ENMs portal with nanosafety-relevant pathways to WikiPathways, an open database for biological pathways, as a basis for pathway analysis; 2) we established workflows for ENMs data analysis, (mainly transcriptomics data), to reveal the significantly and differently affected biological pathways by a variety of exposure scenarios and ENMs and we added this information to the eNanoMapper database. 3) Additionally, we are further describing the implementation of pathway descriptors, an integration methodology to group omics data for QSAR modelling. A case study is presented for descriptors created with information derived from gene ontologies using a proteomics data set.

2. INTRODUCTION

The purpose of this deliverable 4.4 was to develop strategies to predict potential effects of ENM by taking different mechanisms of action into account. We focused on mechanistic effects of ENMs on biological pathways and modelling of the protein corona effect.

Biological entities do not function alone but in a network of interaction. Gene expression e.g. is highly regulated and the expressed gene regulates the expression of other genes directly or indirectly. These interactions can be drawn in a scheme for better visualization and intuitive understanding of the relation. The oxidative stress pathway for example (Figure 1) visualizes the gene expression as a response to reactive oxygen species and it is immediately clear that reactive oxygen species trigger the induction of antioxidant genes like SOD (1-3) over a cascade of events including MAP kinase.

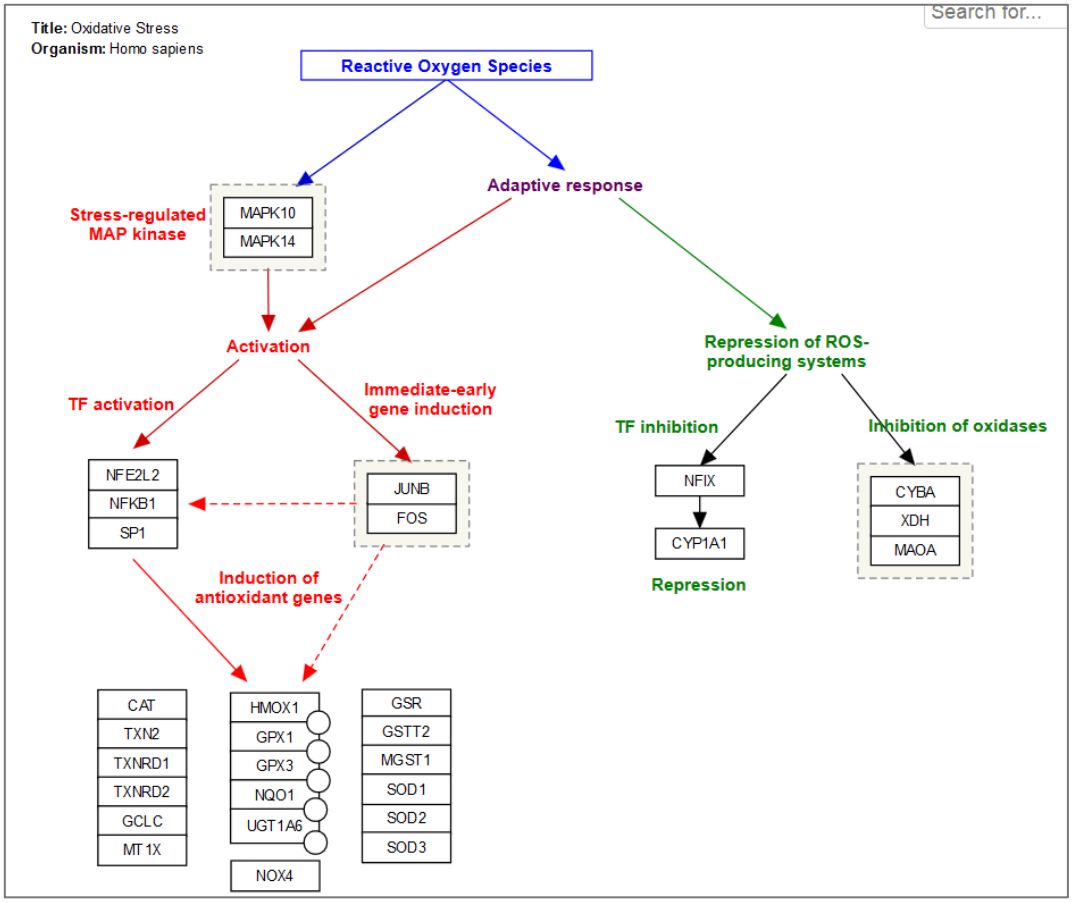

Figure 1: Oxidative stress pathway from WikiPathways (WP408).

Repositories of pathways like this can be used to analyze, quantify and visualize experimental data - transcriptomics, proteomics and metabolomics data. Events like exposure to ENMs trigger changes in gene expression and our hypothesis is that similar ENMs trigger similar biological pathways. This means, that pathway approaches can be used to predict and to classify nanomaterial safety according to their effects on biological systems.

One of the approaches we considered was an enrichment methodology of omics data aiming to integrate biological findings and create descriptors which can then be easily included to QSAR predictive models. Pathway databases, such as the Gene Ontology database, are summarizing information about biology

entities and group genes into meaningful biological groups. These valuable information can be integrated into transcriptomics data to group entities (e.g. genes or proteins) whilst keeping all the information intact. Thus, in this case all data are considered, rather than filtered given the biological question or statistical hypothesis of interest, and grouped. In accordance to well-known Gene Set Enrichment Analysis (GSEA) methods (Subramanian et al., 2005), we have considered those groups of genes that are mostly enriched in the data.

3. MECHANISTIC MODELLING

3.1. MECHANISTIC MODELLING: PATHWAY DATABASE

WIKIPATHWAYS

WikiPathways is an open user curated database for biological pathways [Kutmon 2015, Kelder 2012, Kelder 2009]. A typical biological pathway consists of nodes (genes, gene products or metabolites) and edges (interactions like conversion, stimulation, inhibition, or binding) connecting them with each other (Figure 1 and 2). Every node has an annotation linking it to a database and the information stored there. Over a special tool - BridgeDb - the database information is linked with other databases for the same entity. E.g. in Figure 2 the pop-up window for MAPK10 is shown. MAPK10 was annotated by Entrez Gene identifier (5602) and BridgeDb added the cross-link for MAPK10 on other databases like Ensembl, HGNC, RefSeq, UCSC Genome Browser, UniGene, Gene Ontology and some more. This makes WikiPathways repository multifunctional for a variety of data input.

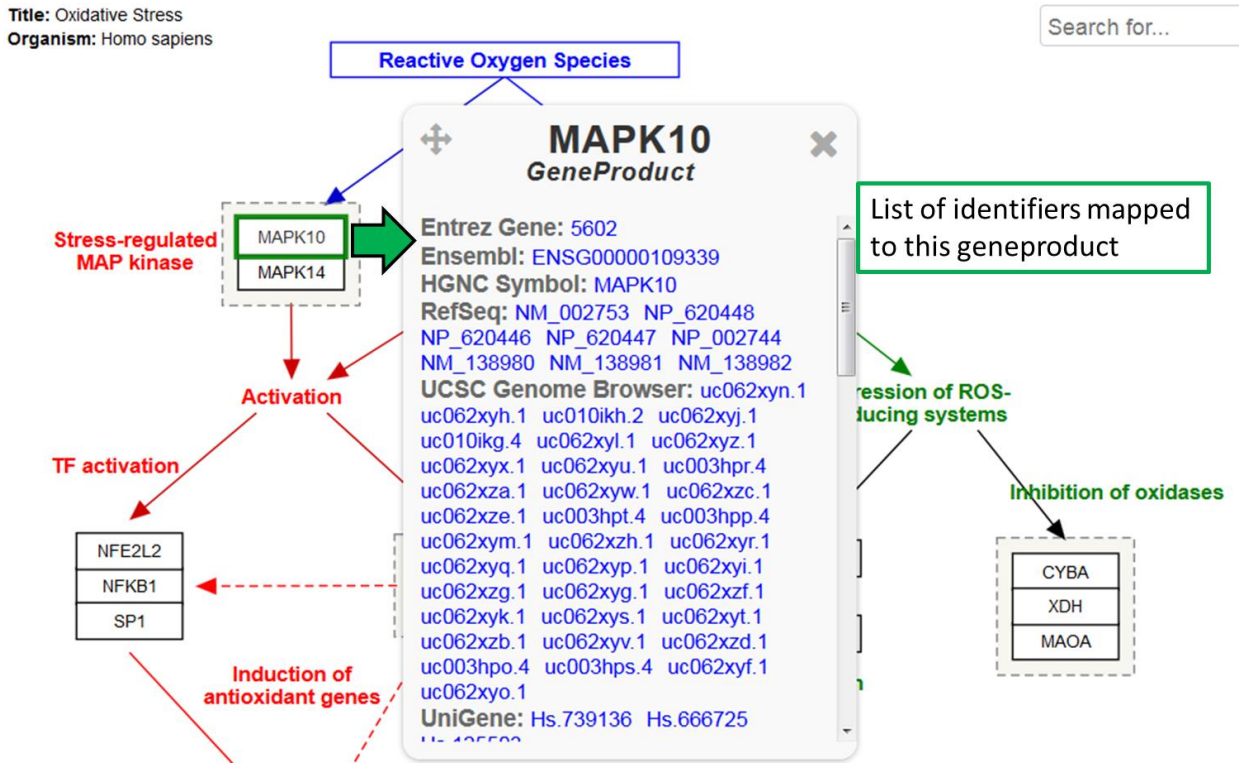

Figure 2: Interactive pathway on WikiPathways. The nodes are annotated with database identifiers e.g. Entrez Gene, and the mapping tool adds the associated identifiers from other databases (e.g. Ensembl) and gene ontology.

There are currently (June 2016) 2510 public pathways available. The biggest subgroup is human pathways with 822 pathways covering 6795 unique genes. Furthermore, WikiPathways provides interactive pathways readily available for analysis. The database can be used by tools like PathVisio to analyse

transcriptomics, proteomics or metabolomics data and has an API for access to the data. Pathways can be downloaded in different formats like pathways (GPML), as images (PDF, TIFF), or gene lists.

For eNanoMapper we created six new pathways, uploaded them to the repository and set up a portal for ENMs (Portal:Nanomaterials). These pathways visualize the mechanistic action of ENMs leading to different experimental (or *in vivo*) outcomes. The pathways are the following:

- overview of nanoparticle effects Pathway:WP3287
- nanomaterial induced apoptosis Pathway:WP2507
- nanoparticle triggered autophagic cell death Pathway:WP2509
- nanoparticle triggered regulated necrosis Pathway:WP2513
- nanoparticle-mediated receptor signaling Pathway:WP2643
- lung fibrosis pathway Pathway:WP3624

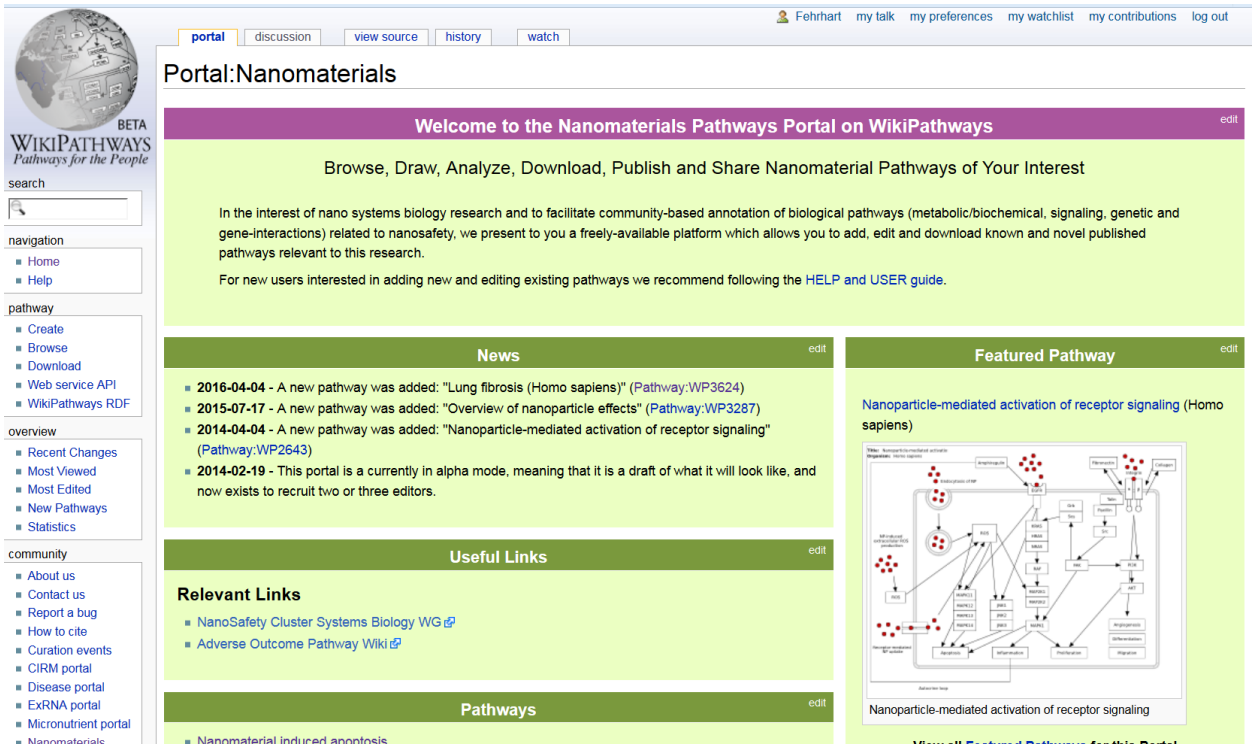

Figure 3: Portal:Nanomaterials on WikiPathways (Portal:Nanomaterials).

TUTORIAL: HOW TO MAKE A PATHWAY

A tutorial was developed on how to make a new pathway using PathVisio software and how to upload it to WikiPathways. The tutorial is available under this link: how-make-a-pathway. It is a straightforward summary of the help and guideline sections of the WikiPathways website and contains the basic steps of creating a pathway:

1. Drawing nodes and edges, e.g. genes, gene products, metabolites or pathways and connecting them with the proper interactions, e.g. conversion, stimulation inhibition or catalysis
2. Adding identifiers to the nodes, e.g. ENSG00000117560 of Ensembl database to FASL (Fas ligand)
3. Adding literature references and a proper pathway description
4. Upload to WikiPathways

THE MAKING OF THE LUNG FIBROSIS PATHWAY

A case study for capturing existing knowledge for a toxicity pathway relevant to nanosafety is presented in this subsection in more detail. Information and omics data related to pulmonary fibrosis and high aspect ratio (HAR) ENMs were extracted and mined from literature and databases. Pulmonary fibrosis is a well-studied human chronic disease, caused by a wide diversity of environmental exposures, and the cumulative number of publications between 1975 and 2010 reached beyond 17 000 papers [Todd 2012]. We first focused our search on reviews and general schemes of fibrosis development and progression, such as the one by Todd et al. (2012). In addition we emphasized reviews discussing the effects of ENMs, particularly high aspect ratio (HAR), such as the review by Vietti et al (2016). We also concentrated on reviews discussing alterations on molecular level related to pulmonary fibrosis or fibrogenic changes following exposure [Todd 2012; Vietti, 2016].

A set of 64 genes with direct evidence of association (either as biomarker or as therapeutic target) with pulmonary fibrosis in the Comparative Toxicogenomics Database (CTD) were listed and used to build the pathway [Grondin 2016]. GeneMANIA [Warde-Farley 2010] was used to identify interaction links between the genes and ConsensusPathDB [Kamburov 2011] was used to identify groups of genes enriched for pathways and functions (KEGG, WikiPathways and Gene Ontologies). This information was then used to build connections and interactions between the different elements of the pathway (genes, pathways and general scheme elements). Finally, the putative AOP (adverse outcome pathway) for pulmonary fibrosis and the associated omics data analysis in Labib et al. (2016) were used to assign AOP events (key events [KEs], associative events [AEs] and adverse outcome [AO]) to the different genes and gene groups in the pathway. The gathered data resulted in an employable WikiPathways for this adverse outcome.

In conclusion, WikiPathways templates are expected to be highly useful for computational analysis of large-scale omics data, serving diversely for: i) gene set enrichment analysis, ii) pathway enrichment analysis among differentially expressed genes, iii) integration into AOP-based testing strategies, iv) application as descriptors in (Q)SAR approaches, and finally, iv) for grouping and read across among ENMs, coupled to identification of specific pathway-activating ENMs.

WikiPathways is species specific. To convert a pathway for a different species it is possible to copy a pathway but it is still necessary to adapt the identifiers for genes and gene products. For the lung fibrosis pathway a homologue pathway was made for mouse (Pathway:WP3632) and another for rat is in preparation.

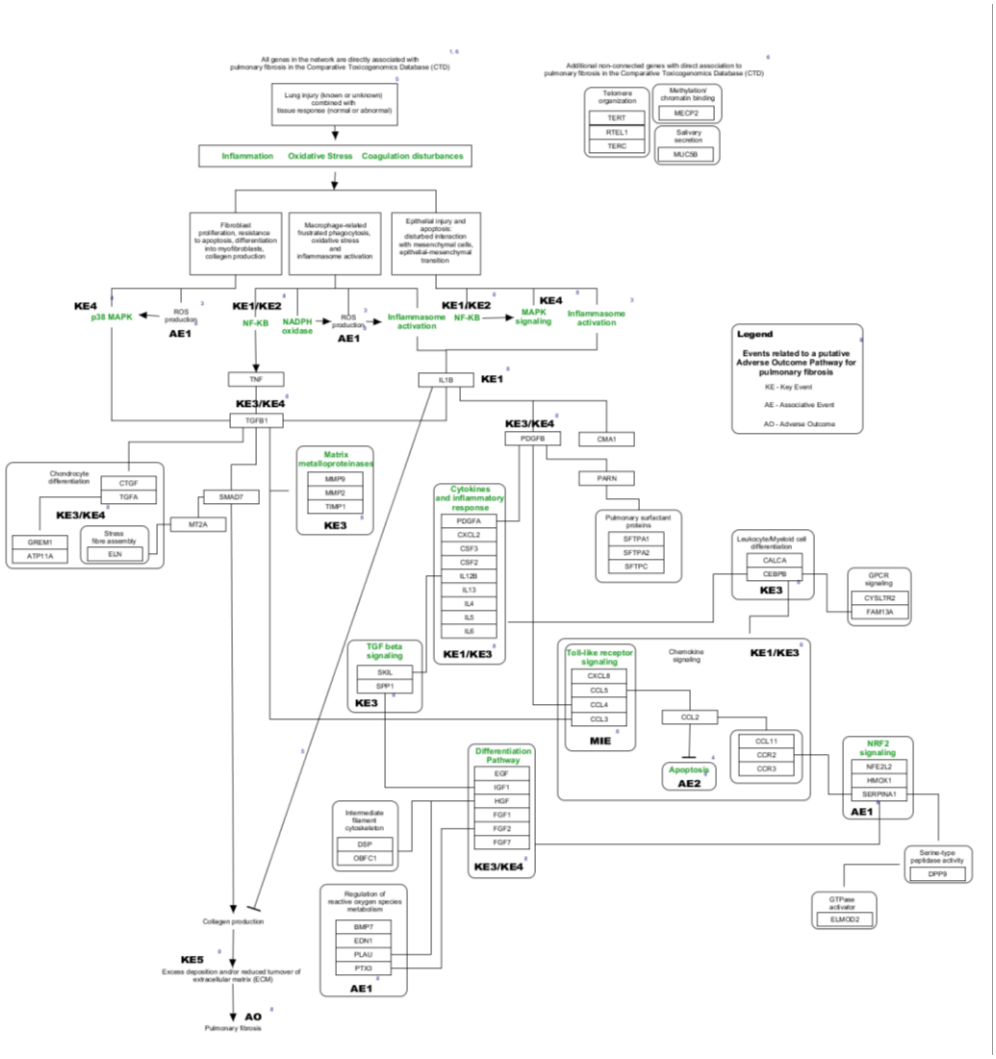

Figure 4: Human lung fibrosis pathway (WP3624).

3.2. MECHANISTIC MODELLING: CASE STUDIES

In this chapter we present several case studies of the nanomaterial pathway analysis workflow and one case study for the Chipster bioinformatics workflow based on tutorials 2,3,4,5 (please see subsection 3.5) which provide detailed instructions on how to perform mechanistic modelling using nanomaterial transcriptomics data. The workflows proposed in the tutorials were used as a standard except for pathway analysis, where the desktop version of PathVisio was used instead of the web tool. The datasets are summarized in Table 1. The experimental information is shortly given as type of *in vitro* or *in vivo* study and the nanomaterial which it was exposed to. The data is either publically available from previously published studies or was provided by project partners or other research institutes. The accession number for the dataset and the original publication are listed in Table 1.

Table 1: Summary of data used for nanoparticle pathway analysis.

Experiment	Data source	published in
human intestine cell line (Caco-2) exposed to Ag nanoparticles	GSE62253	Boehmert et al. 2014
human bronchial epithelial cell line (BEAS-2B) exposed to MWCNT	E-GEOD-63552 E-GEOD-63559	Nymark et al. 2015
human aortic endothelial cells exposed to TiO_2 nanostructured surface	E-GEOD-17676)	Peng et al. 2010
human hepatic stellate cells exposed to ZnO	GSE60159	Osmond-McLeod et al. 2014
human T-cell line (Jurkat) exposed to ZnO	GEOD39330	Tuomela et al. 2013
human umbilical vein endothelial cells (HUVECs) exposed to fullerenol	E-GEOD-3364	Yamawaki et al. 2006 Smeets et al. (in preparation)
Human small alveolar epithelial cells (SAE)	GSE42067	Tilton et al, 2014
in vivo Ag nanoparticle feeding study with rats, analysis of liver transcriptome	RIKILT (Wageningen, NL)	unpublished data
in vivo Ag nanoparticle feeding study with rats, analysis of PBMCs transcriptome	RIKILT (Wageningen, NL)	unpublished data
in vivo SiO_2 nanoparticle feeding study with rats, analysis of liver transcriptome	RIKILT (Wageningen, NL)	van der Zande et al. 2014
in vivo SiO_2 nanoparticle feeding study with rats, analysis of gut transcriptome	RIKILT (Wageningen, NL)	van der Zande et al. 2014

PATHWAY ANALYSIS OF AG ENMS EFFECTS ON HUMAN CACO-2 CELLS

The aim of this study was to investigate the changed pathways in a human intestinal cell line model after being exposed to low and high doses of Ag nanoparticles and Ag solution. The significant changes of gene expression were calculated using ArrayAnalysis.org and used for performing pathway statistics using PathVisio. The different experimental groups were compared to the untreated (culture medium) control group. A pathway was considered as changed if the number of differentially expressed genes was higher than 3, z-score higher than 1.96 and the change was significant ($p < 0.05$).

Low dose ENMs and Ag solution did not reveal changes in biological pathways. High dose of Ag nanoparticles showed changes in metal ion homeostasis (namely Zinc and Copper homeostasis). Several oxidative stress, apoptosis and inflammation pathways were changed: NRF2 pathway, Quercetin and Nf-kB/ AP-1 Induced Cell Apoptosis, and Oxidative Stress.

While low doses of Ag ENMs and the tested concentration of Ag ion solution had no relevant effect on biological pathways of Caco-2 cells, high dose of Ag ENMs activated several oxidative stress, apoptosis

and inflammation related pathways. Additionally, metal homeostasis pathways were affected possibly due to compensation reaction of the cells.

Cell model: human intestinal epithelial (Caco-2) cells
Nanoparticle: Ag ENMs in high (25 µg/ml) low (2.5 µg/ml) dose or AgNO solution vs. control
Pathways changed: Oxidative stress, inflammation, metal homeostasis (Table 2)

Because of clarity the extensive pathway analysis tables of the following are given in the appendix.

PATHWAY ANALYSIS OF MULTI-WALLED CARBON NANOTUBES ON GENE EXPRESSION IN BRONCHIAL EPITHELIAL BEAS 2B CELLS

One class of the most commercialized ENMs is multi-walled carbon nanotubes (MWCNTs). Previous studies have indicated that MWCNTs have possible toxic effects on human cells, such as inflammation, production of reactive oxygen species and apoptosis. The aim of this study was to determine the effect of low doses of MWCNTs on gene expression in bronchial epithelial BEAS 2B cells using a systems biology approach. An existing suitable dataset was chosen on ArrayExpress. This dataset was analyzed with a systems biology approach. Pathway analysis was done with PathVisio. In response to the nanomaterial, 567 genes were found to be differentially expressed in the pathway analysis (LogFC < -0.25 OR LogFC > 0.25 AND P-value < 0.05). A broad range of pathways were affected. Notable is the amount of neurological involved pathways. Extension of the pathways with miRNAs and TFs in the analysis showed that miRNAs are less likely to be involved in the regulation of gene expression after exposure to MWCNTs. MWCNT induced changes in gene expression in a broad range of biological processes, including neurological processes, inflammation inhibition and a response to reactive oxygen species.

Cell model: human bronchial epithelial BEAS 2B cells
Nanoparticle: MWCNTs
Pathways changed: Neurological processes, inflammation inhibition, and response to reactive oxygen species (Annex Table 1)

Table 2: Summary of changed pathways for high dose Ag nanoparticle treated Caco-2 cells (cut off: logFC [1]).

Pathway	positive (r)	measured (n)	total	%	z-score	p-value
Zinc homeostasis	10	30	39	33.33%	6.1	0
NRF2 pathway	20	122	145	16.39%	4.63	0
Quercetin and Nf-kB/ AP-1 Induced Cell Apoptosis	5	15	27	33.33%	4.31	0
Triacylglyceride Synthesis	6	24	37	25.00%	3.77	0.002
Farnesoid X Receptor Pathway	5	19	21	26.32%	3.59	0.003
Oxidative Stress	6	26	32	23.08%	3.52	0.002
Fatty Acid Omega Oxidation	4	14	16	28.57%	3.43	0.004

Transcriptional activation by NRF2	4	14	20	28.57%	3.43	0.008
Copper homeostasis	8	46	58	17.39%	3.1	0.009

PATHWAY ANALYSIS OF NANOSTRUCTURED TiO_2 SURFACES IN HUMAN AORTIC ENDOTHELIAL CELLS

In this genomics study, the research goal was to find differentially expressed genes in primary human aortic endothelial cells (HAECs) after growing the cells for 24 hours on a TiO_2 nanotube array with different diameter sizes (30nm and 100nm).
The dataset (E-GEOD-17676) containing the microarray gene expression data was provided by the online genomic database ArrayExpress. ArrayAnalysis was used for statistical analysis. PathVisio was used for pathway visualization and pathway analysis.
Statistical analysis revealed that the different diameter sizes of the nanotubes did not have a lot of effect on the amount of gene expression. Pathway analysis showed that mainly genes in pathways involved in inflammatory and proliferative processes were significantly changed, having a positive effect on proliferation and decreasing the inflammatory genes and proteins. Network analysis did identify a decreased expression of the transcription factor E2F1, also involved in apoptotic and inflammatory pathways.
Results suggest that TiO_2 nanotubes in an array mesh with a diameter size of 100 nm have an anti-inflammatory and pro-proliferative effect. Future research in the field of proteomics and biomedicine is needed to validate these findings and expand them further.

Cell model: human aortic endothelial cells
Nanomaterial: TiO_2 nanostructured surface
Pathways changed: anti-inflammatory and pro-proliferative pathways (Annex Table 2)

PATHWAY ANALYSIS OF ZINC OXIDE EFFECTS ON GENE EXPRESSION OF HUMAN HEPATIC STELLATE CELLS AND IMMUNE-COMPETENT CELLS

Zinc oxide (ZnO) is a nanoparticle used in many products such as sunscreens, cosmetics and biomedical instruments. Although it's widespread use, there are still some concerns about the toxicity of ZnO nanoparticles. There are some studies performed on cytotoxicity of ZnO in human cells using cell viability assays, but the number of studies done on gene expression remains limited. Therefore we investigated the effects of ZnO nanoparticles on human hepatic stellate cells and immune-competent cells on a pathway level using gene expression data. We used two different datasets to perform pathway analysis and network analysis. The first dataset studied the use of a surface coating on the cytotoxicity in human hepatic stellate cells. The second dataset investigated the cytotoxicity of nine modified ZnO nanoparticles in Jurkat cells, HMDM and MDDC cells. We found in the first dataset that the cells treated with the uncoated ZnO nanoparticles have a lot more differentially expressed genes than in the cells treated with the coated ZnO nanoparticles. Pathway analysis showed that for many genes there is an opposite effect in gene expression between the two groups. Furthermore, some genes are only up- or downregulated by the uncoated ZnO nanoparticles. In the second dataset, we found a dose-dependent effect of ZnO-4 treatment on the gene expression in Jurkat cells. In Jurkat cells and MDDC cells the number of differentially expressed genes decreases over time, but in HMDM cells this increases. Pathway analysis showed that genes involved in the immune response are activated. In both datasets, genes that encode for metallothioneins are upregulated. Whether this is a direct effect of ZnO or the free radicals formed by

ZnO needs to be investigated. We also observed many differentially expressed genes that encode for proteins with cysteine elements. This study showed that ZnO treatment has an effect on the gene expression in human immune-competent cells and hepatic stellate cells. With the use of pathway and network analysis the effect on gene expression can be visualized and interpreted in a broad way.

Cell model: human hepatic stellate cells
Nanoparticle: 4 different types of coated and uncoated ZnO nanoparticles
Pathways changed: immune response, metallothionins (Annex Table 3 and 4)

Cell model: immune-competent cells
Nanoparticle: 4 different types of coated and uncoated ZnO nanoparticles
Pathways changed: apoptosis, signalling pathways (insulin, NKF) (Annex Table 5)

PATHWAY ANALYSIS OF FULLERENOL EFFECTS IN HUMAN VASCULAR ENDOTHELIAL CELLS (FROM PAPER IN PREPARATION)

The water soluble derivative of the ball-shaped C_{60} fullerene nanoparticle, fullerenol ($C_{60}(OH)_{24}$) has been proposed for use in future clinical treatments because of its advantageous properties. Obviously, before it is actually put on the market, it is important to assess and understand its possible toxic effects. Upon intravenous injection of fullerenol, the particles will encounter vascular endothelial cells, which provide a barrier between the blood and tissues all across the cardiovascular system. Endothelial cells play a crucial role in maintaining blood vessel homeostasis and are essential in preventing atherosclerosis. Here, we aim to unravel the toxicological effects of fullerenol on human umbilical vein endothelial cells (HUVECs) and to investigate underlying biological mechanisms.
Methods: In our analysis we combined previously published microarray data from a study by Yamawaki et al. (2006), obtained from ArrayExpress under the accession number E-GEOD-3364 with pathway and transcription factor data. In the original experiment, HUVECs were stimulated with 100 µg/ml $C_{60}(OH)_{24}$. PathVisio and pathways from WikiPathways were used to perform pathway analysis.
Results: We show that in response to treatment with fullerenol, the gene expression profile of vascular endothelial cells is altered with increases in expression of genes relevant for intracellular production, collection and preservation of cholesterol. This response involves the activation of the SREBF1 and SREBF2 transcription factors, which are known to regulate intracellular lipid concentrations, including those of sterols such as cholesterol.
Conclusions: If our findings with respect to cholesterol synthesis also occur at concentrations occurring in vivo after fullerenol application, disruption of this process might worsen atherosclerotic onset. Because of this potential effect on cholesterol metabolism, clinical use introducing fullerenols into the vascular system of humans prone to developing atherosclerosis would require evaluation of cholesterol levels as part of targeted toxicological studies.

Cell model: human umbilical vein endothelial cells (HUVECs)
Nanoparticle: fullerenol ($C_{60}(OH)_{24}$)
Pathways changed: intracellular production, collection and preservation of cholesterol (Annex Table 6)

PATHWAY ANALYSIS OF NANOSTRUCTURED SILICA EFFECTS ON RAT LIVER AND JEJUNUM (SUBCHRONIC TOXICITY STUDY)

Transcriptomics data from a published paper by van der Zande et al. (2014) “Sub-chronic toxicity study in rats orally exposed to nanostructured silica” was reanalysed for the purpose of obtaining knowledge on the mode of action of nanostructured silicas. The below information is partly retrieved from the abstract of the paper from van der Zande et al.:

BACKGROUND:
Synthetic Amorphous Silica (SAS) is commonly used in food products and drugs. Part of the consumer intake of silica is in the nano-size range (i.e. 5-200 nm) which can be up to 43% of the total silica content. Concerns have been raised about the possible adverse effects of chronic exposure to nanostructured silica.
METHODS:
Rats were orally exposed to 100 (low), 1000 (medium) or 2500 (high) mg/kg bw/day of SAS, or to 100 (low), 500 (medium) or 1000 (high) mg/kg bw/day of NM-202 (a representative nanostructured silica for OECD testing from JRC) for 28 days, or to the highest dose of SAS or NM-202 for 84 days. RNA was retrieved from the liver after 28 days and 84 days of exposure to both materials. From the latter period also RNA was retrieved from the jejunum of the exposed rats. Differentially expressed gene levels were then evaluated including overrepresentation analysis of biological pathways. The pathway over-representation analysis was performed in PathVisio using the WikiPathways repository.
RESULTS:
Extensively characterization of both SAS and NM-202 as pristine materials, but also in the feed matrix and gut content of the animals, and after in vitro digestion, was performed and is available in the paper from van der Zande et al. From this paper they concluded that exposure to SAS or NM-202 did not result in clearly elevated tissue silica levels after 28-days of exposure. However, after 84-days of exposure to SAS, but not to NM-202, silica accumulated in the spleen. Biochemical and immunological markers in blood and isolated cells did not indicate toxicity, but histopathological analysis, showed an increased incidence of liver fibrosis after 84-days of exposure, which only reached significance in the NM-202 treated animals. This observation was accompanied by a moderate, but significant increase in the expression of fibrosis-related genes in liver samples. With the additional overrepresentation analysis also other biological processes and pathways were found to be affected. Also comparisons were made between the different doses and exposure periods of SAS and NM-202.

Type of study: *in vivo*
Animal model: rat
Type of tissue: liver and jejunum
Nanoparticles analysed: Synthetic Amorphous Silica (SAS) and NM-202 (a representatively nanostructured silica for OECD testing from JRC)
Pathways: hardly any change at low doses, some changes in biotransformation, statin pathway and calcium regulation (of cardiac cells) for medium and high doses (see Annex Tables 8-17 for individual doses and exposure periods)

PATHWAY ANALYSIS OF EFFECTS OF SILVER IONS AND SILVER PARTICLES ON RAT LIVER AND PBMCS (SUB-CHRONIC TOXICITY STUDY)

Transcriptomics data from a study conducted by van der Zande et al., described in the publication from 2012 “Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure., was analyzed for the purpose of a better understanding of the effect of silver ions and particles on the rat liver. Parts of the information below are from the paper of Van der Zande et al.:

BACKGROUND: Silver ENMs are mainly used for their bactericidal effects and are therefore widely used worldwide in a number of consumer products. Also within the food and feed area the use of silver nanoparticles is growing. The antibacterial effects of silver particles are suggested within literature to be caused by the interaction with bacterial membranes. These effects are expected to become larger with decreasing particle size. These properties however also make them potentially harmful to humans. Silver ions are biologically more active than the nanoparticles themselves.

METHODS: Rats were orally exposed for 28 days AgNP <to 20 nm non-coated (NM300K = JRC reference material), or <15 nm PVP-coated silver nanoparticles ([Ag] = 90 mg/kg body weight (bw)), or AgNO(3) ([Ag] = 9 mg/kg bw), or carrier solution only. At day 29 rats were sacrificed, and RNA was extracted from liver and PBMCs. In addition full characterisation of the nanoparticles has been done using TEM and DLS to verify the size distribution of the manufacturer. Also distribution, elimination and toxicity were monitored.
RESULTS: The results from the van der Zande et al. paper are: Silver was present in all examined organs with the highest levels in the liver and spleen for all silver treatments. Silver concentrations in the organs were highly correlated to the amount of Ag(+) in the silver nanoparticle suspension, indicating that mainly Ag(+), and to a much lesser extent silver nanoparticles, passed the intestines in the silver nanoparticle exposed rats. In all groups silver was cleared from most organs after 8 weeks post-dosing, but remarkably not from the brain and testis. Using single particle inductively coupled plasma mass spectrometry, silver nanoparticles were detected in silver nanoparticle exposed rats, but, remarkably also in AgNO(3) exposed rats, hereby demonstrating the formation of nanoparticles from Ag(+)in vivo that are probably composed of silver salts. Biochemical markers and antibody levels in blood, lymphocyte proliferation and cytokine release, and NK-cell activity did not reveal hepatotoxicity or immunotoxicity of the silver exposure. In conclusion, oral exposure to silver nanoparticles appears to be very similar to exposure to silver salts. However, the consequences of in vivo formation of silver nanoparticles, and of the long retention of silver in brain and testis should be considered in a risk assessment of silver nanoparticles. With the additional overrepresentation analysis of the transcriptomics data after 28 days of exposure in liver and PBMCs also other biological processes and pathways were found to be affected. Also comparisons were made between the different doses and exposure periods of $AgNO_3$, PVP and NM300K.

Type of study: *in vivo*
Animal model: rat
Type of tissue: liver and PBMCs
Nanoparticles analysed: non-coated AgNP <20 nm (NM300K - JRC reference materials), PVP-coated AgNP <15 nm and $AgNO_3$ (silver ions).
Pathways: Biotransformation, electron chain, translation factors, apoptosis, cell cycle, calcium regulation and toll-like receptor signaling (Annex Tables 18 - 23 for individual tissue, nanoparticle and exposure)

CASE STUDY USING THE CHIPSTER-BASED BIOINFORMATICS WORKFLOW ON TRANSCRIPTOMICS DATA FROM HUMAN SMALL AIRWAY EPITHELIAL CELLS (SAE) EXPOSED TO TITANIUM DIOXIDE NANOBELTS AND MULTI-WALLED CARBON NANOTUBES

Part of a transcriptomics data set published by Tilton et al (2014) was used to study the effects of titanium dioxide nanobelts (TiO_2 NB) and multi-walled carbon nanotubes (MWCNT) on human small airway epithelial cells (SAE). Details on the characterization of the nanomaterials can be found in the original study and the raw data is available in the Gene Expression Omnibus (GEO) database (GSE42067). The cells were exposed to 10 and 100 µg/ml of the nanomaterials for 1 and 24 hours with 3 replicates for each condition. RNA samples were hybridized to Affymetrix HT HG-U133a 2.0 (=hgu133av2 in Chipster) microarrays. The .CEL files for the 30 samples (24 treated + 6 controls) were brought into Chipster for analysis according to the tutorial. The data was normalized, filtered and analyzed by various methods, including i) clustering by hierarchical and principle component analysis (PCA) methods, ii) statistical analysis to identify differentially expressed genes, iii) gene ontology (GO) enrichment analysis and iv) various gene network and pathway-based analyses using freely available online tools, including ConsensusPathDB, PathVisio and WikiPathways.
The results indicated overall that the two nanomaterials elicit similar effects at the early time point (1h), which later diverge into more nanomaterial-specific effects at 24h. The effect of TiO_2 NBs was stronger

at 24h than that of MWCNTs. Dose-response effects could be seen for both nanomaterials at 1h and the effect was retained for TiO_2 NB, but not for MWCNTs, indicating a potential agglomerating effect leaving less free fibers available for the cells to interact with over time or alternatively an adaptive response over time by the cells exposed to MWCNTs (Figure 1). The low dose was further analyzed for GO enrichment at both time points and indicated changes in RNA metabolic processes at 1h and MAPK cascades at 24h for MWCNTs, while TiO_2 NBs induced changes in primary metabolic processes at 1h and immune response at 24h. Finally, the effects of TiO_2 NB were visualized in the WikiPathways "Nanoparticle mediated activation of receptor signaling" using PathVisio (Figure 2).

These conclusions largely align with the findings in the original study, confirming the use of this standardized workflow (as presented in the tutorial "How to use Chipster for Bioinformatics Analysis of Nanomaterial-based Omics Data") as capable of reproducing analyses done by other groups using other methods.

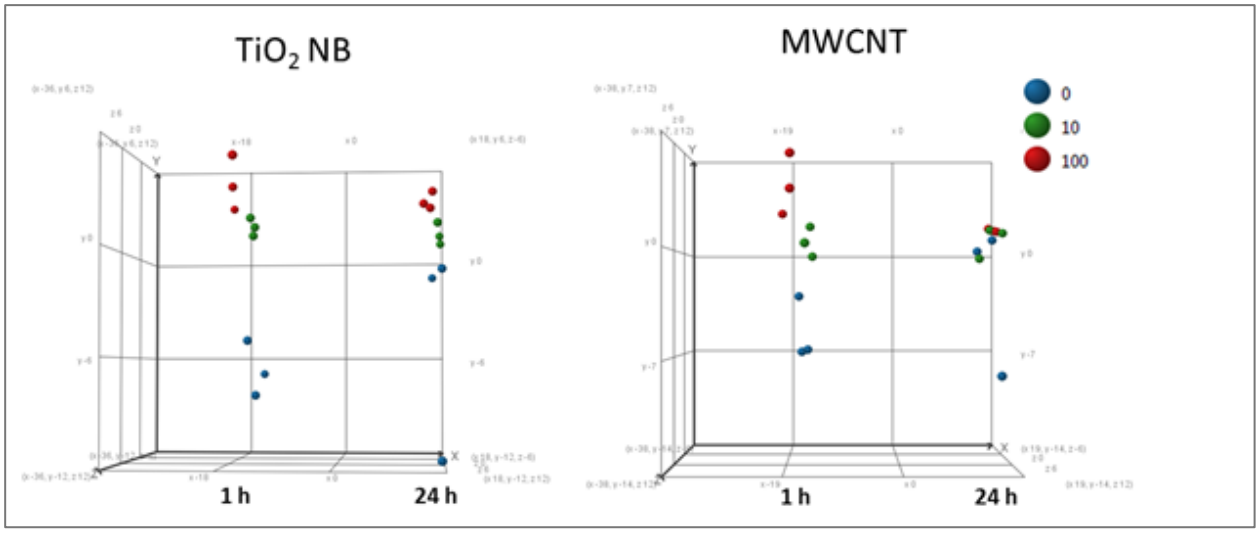

Figure 5. PCA plots based on dose-response effects (i.e. statistically differentially expressed genes showing dose-response effects) in human small alveolar epithelial cells following 1h and 24h exposures to titanium dioxide nanobelts (TiO_2 NBs) and multi-walled carbon nanotubes (MWCNT).

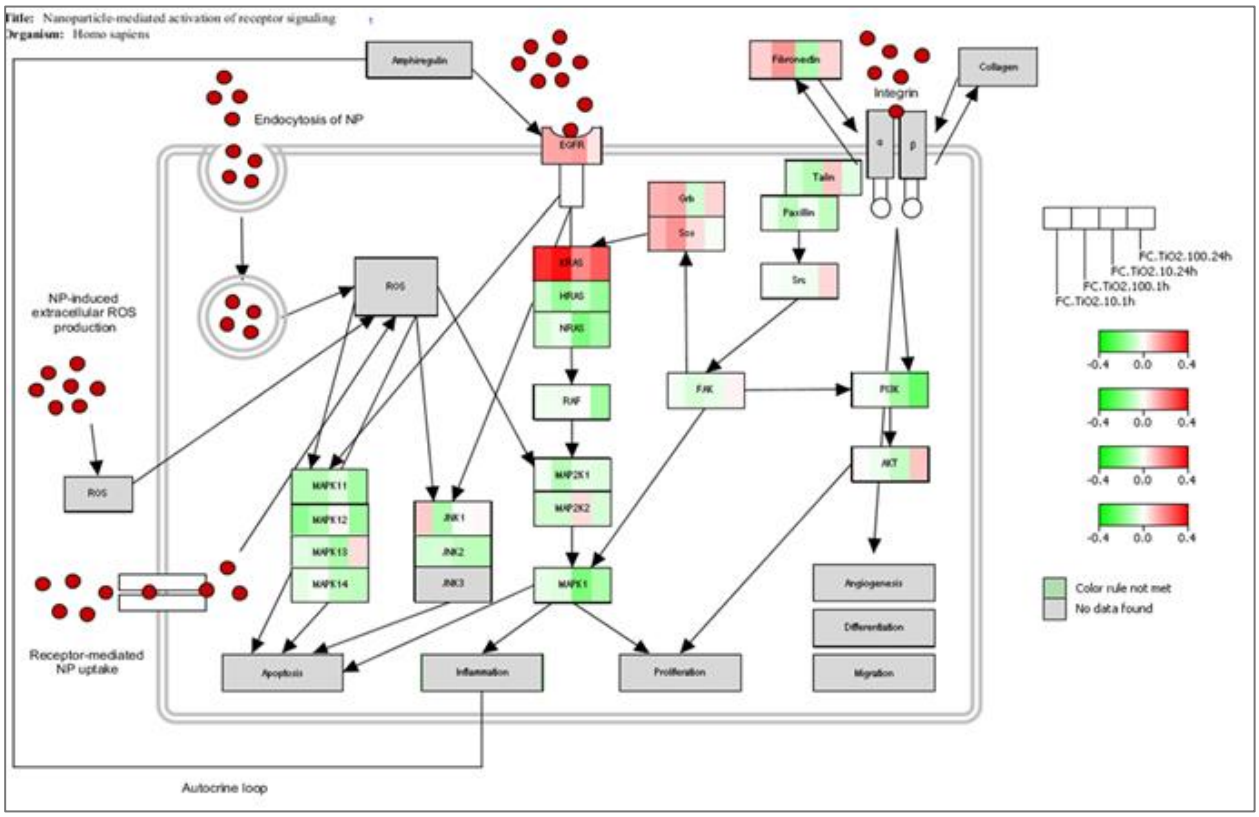

Figure 6. Effects of titanium dioxide nanobelts (TiO_2 NBs) in human small alveolar epithelial cells visualized in the WikiPathways "Nanoparticle mediated activation of receptor signaling".

DISCUSSION

The affected pathways, identified using the standardized workflows in the ArrayAnalysis.org and PathVisio-based tutorials, varied largely between case studies (Table 3), which was expected since: 1. different biological systems (lung epithelial cells, PBMCs, liver tissue) react differently to nanoparticles and to stress in general. Inflammation, immune response and signaling pathways are observed in multiple experiments while other pathways seem to be more cell type or nanoparticle specific. HUVECs are found to upregulate cholesterol pathways if confronted with fullerenols while Caco-2 cells increase their metal homeostasis pathways to deal with Ag nanoparticles. 2. The experimental setup, namely amount, exposure and type of ENM is also known to result in different outcomes. While Caco-2 cells respond strongly to high doses of Ag nanoparticles (25 mg/ml) there are no significantly changed pathways found for low dose of Ag nanoparticles (2.5 mg/ml) or equivalent amounts of Ag ion solution.
The results based on the case study using the Chipster-based tutorial were comparable to the results obtained in the original study, confirming its usefulness to perform large cohesive bioinformatics studies across several data sets.

Due to the variety of experimental approaches it is difficult to compare them directly. To make broader conclusions and predictions larger cohesive data sets are needed and they need to be analyzed in a cohesive manner. Nevertheless, these case studies show that the bioinformatics workflows may serve as a basis - a training set - for modelling. The strength of this approach is the standardized workflow to analyze (or reanalyze) the existing data to provide information for follow-up studies and safety recommendations. Using open-source, freely available, curated and documented web-tools (with help of our tutorials) coordinated cohesive bioinformatics analyses are possible and researchers from different fields are able to give input for the pathway analysis database. This meets exactly the eNanoMapper mission of standardization and harmonization.

Table 3: Summary of affected pathways, identified using the standardized workflows in the ArrayAnalysis.org and PathVisio-based tutorials, for different nanoparticles and *in vitro/in vivo* systems.

Cell type	Exposure	Nanoparticle	Pathways (main changed pathways)
In vitro studies			
human intestine cell line (Caco-2) (differentiated)	**25 mg/mL** silver nanoparticles in serum-free cell culture medium for **24 h**	**Ag**PURE 10% (w/w) silver stabilized with 4% (w/w) polyoxyethylene glycerol trioleate and 4% (w/w) polyoxyethylene (20) sorbitan monolaurate (Tween 20). **15 nm**	Oxidative stress, inflammation, metal homeostasis (Table 2)
human bronchial epithelial cell line (BEAS-2B)	0.25 and 2 mg/cm^2 MWCNT for 1, 4, 6, 12, 24 and 48 h	MWCNT, 74 nm x 5.4 µm	Neurological processes, inflammation inhibition, response to reactive oxygen species (Annex Table 1)
human aortic endothelial cells	growing on nanostructured surface for **24 h**	TiO_2 30 nm and 100 nm nanotube arrays **(surface structure!)**	Anti-inflammatory and pro-proliferative pathways (Annex Table 2)
human hepatic stellate cells	**30 µg/mL** ZnO nanoparticles in cell culture medium for **24 h**	Z- COTE, Nanosun P99/30 (both uncoated ZnO), and Z-COTE HP1 (coated with triethoxycaprylylsilane and Z-COTE MAX coated with a dimethoxydiphenylsilane / triethoxycaprylylsilane crosspolymer **25 - 44 nm**	immune response, metallothionins (Annex Table 3 and 4)
human T-lymphocyte cell line (Jurkat)	**10 µg/ml** in cell culture medium for **6 or 24 h**	ZnO-1 (uncoated ZnO nanoparticles), 14.7 nm	Apoptosis, signalling pathways (insulin, NKF) (Annex Table 5)
human umbilical vein endothelial cells (HUVECs)	**100 µg/m**l in cell culture medium for **24 h**	fullerenol ($C_{60}(OH)_{24}$), 7.1 nm diameter	intracellular production, collection and preservation of cholesterol (Annex Table 6)
In vivo studies (rat)			
liver tissue	oral exposure of rats to **100, 1000 or 2500 mg/kg**	SiO_2 Synthetic Amorphous Silica (SAS, **7 nm**) or NM-202 (**10 - 25 nm**)	hardly any change with low dose, some changes in biotransformation and

	bw/day of SAS, or to **100, 500 or 1000 mg/kg bw/ day** of NM-202 for **28 days**		statin pathway with high and medium dose (Annex Tables 8 - 17)
gut (jejenum) tissue	oral exposure of rats to **100, 1000 or 2500 mg/kg bw/day** of SAS, or to **100, 500 or 1000 mg/kg bw/ day** of NM-202 for **28 days**	SiO_2 Synthetic Amorphous Silica (SAS, **7 nm**) or NM-202 (**10 - 25 nm**)	hardly any change with low dose, calcium regulation was affected by high dose of NM-202 (Annex Tables 8 - 17)
liver tissue	oral exposure of rats to **90 mg/kg** body weight for **28 days**	non-coated Ag NP **<20 nm** (NM300K - JRC reference materials), PVP-coated AgNP **<15 nm** and AgNO3 (silver ions).	Biotransformation and apoptosis (NM300K), Cell cycle, calcium regulation and toll-like receptor signaling (PVP-coated AgNP) (Annex Tables 18 - 23)
PBMCs	oral exposure of rats to **90 mg/kg** body weight for **28 days**	non-coated Ag NP **<20 nm** (NM300K - JRC reference materials), PVP-coated AgNP **<15 nm** and AgNO3 (silver ions).	Biotransformation and electron chain (NM300K), translation factors (PVP-coated) (Annex Tables 18 - 23)

3.3. PATHWAY DESCRIPTORS: OMICS DATA INTEGRATION

We have considered advanced clustering algorithms and their application on binary Gene Ontology (GO) matrices, derived by integrative meta-analysis of omics data, aiming to predict the cell association of ENMs and partition them into groups based on their protein corona fingerprint (bio-signature) similarity. As already introduced in D4.2 section 5, we have created a workflow where estimated groups of omics data are summarized to produce readily biology interpreted descriptors. Results are presented for the protein corona fingerprint (PCF) data as it appeared in Walkey et al. (2014) and by employing clustering methods to the relevant GO membership matrix. The suggested method is potentially also applicable to transcriptomics data for investigation of mechanistic associations with toxicity endpoints. The main steps of the workflow are the following:

1. Extract Uniprot ids from PCF data and translate them to Entrez Gene ids, also subtracting duplicates or null values.
2. Perform Gene Set Enrichment Analysis (GSEA) as introduced by Subramanian et al. (2005) and implemented in R GOstats package, to estimate the statistically significant GO terms (hypergeometric p-value≤0.05).
3. Cluster the binary membership matrix showing memberships of proteins to GO ids, by employing either bi-clustering (R iBBiG package) or hierarchical clustering (R vegan package).

4. Use the above clusters to summarize proteins and build the new set of descriptors, called GO descriptors. For example, Descriptor 1 is an average of the relative abundances of the proteins found to cluster together in cluster 1 estimated in step 3.

Additionally, a stochastic search Simulated Annealing (SA) algorithm was built to estimate the optimal set of GO descriptors or equivalently the optimal partition of the binary membership GO matrix in terms of maximizing the coefficient of determination R2 accuracy of a 10-fold cross-validation (CV) performance scheme for neural network model in the test set. This is an important addition since a unique data set is returned which is in fact the best performing data set given. The steps followed for this simulated annealing search are the following:

1. Set minimum and maximum thresholds for the number of clusters (each cluster should contain at least five proteins). We have also set a threshold to the maximum number of clusters equal to 1/10 of the genes included in the data.
2. Suggest a new data partition by either merging two randomly selected clusters with probability 1/3, or split them into three clusters with probability 2/3. In the latter case the split is performed by either bi-clustering or hierarchical clustering.

The new workflow was tested with the original PCF data sets as they are described in section 5.3 of D4.2 and introduced in [Walkey 2014], namely the 129x84 gold PCF data set and the filtered 76x84 gold PCF data set. Moreover we have considered the data sets presented in a follow-up publication by Liu et al. (2015), who suggested a 11x84 PCF dataset when considering a linear regression model and a 9x84 PCF data set when applying a non-linear support vector regression model. The above data sets are denoted by PCF129, PCF76, PCF11, and PCF9, respectively. Throughout this analysis we have considered the GO Molecular Functions (MF) ontology as being most relevant to PCF data. The subgroup Cellular Compartment (CC) was in this case deemed less relevant, since the corona is formed by proteins in serum, which are mainly extracellular. Similarly, the GO subgroup Biological Process (BP) was also considered less relevant, since the interpretation of enriched biological processes among these proteins becomes more difficult. The proteins in a corona are not actively involved in processes, but rather affect the uptake of the NP.

As seen in the Table 4, GO descriptors are performing well in terms of predicting the cellular association of proteomics data, which emphasizes the flexibility of the model being able to retain the whole data set and also perform well in QSAR modelling due to the small number of descriptors considered. Particularly the set of 6 descriptors derived by hierarchical clustering, denoted by GOdescrH6, is the best set in terms of both 10-fold and 4-fold CV. The performance accuracy in the test set for PCF9 and the GOdescrB4 dataset (4 GO descriptors derived by our biclustering method) are comparable, where PCF9 is the best performing set amongst the PCF datasets. More results and biological interpretation of the findings are reported in Tsiliki et al. (2016). In a sense our methodology is folding the PCF data set to be included in QSARs and unfolding for biological interpretations of the findings, which also offers a readily available interpretation since each GO descriptor is being 'formed' by a set of GO terms. Biological interpretation of the GO descriptors indicated that many of the descriptors shared several GO ids (albeit based on different proteins), but also contained specific unique GO ids, such as phosphatidylcholine binding, scavenger receptor activity and phospholipid binding. Interestingly, "calcium ion binding" was shared among all GO descriptors and strongly linked to the most influential descriptor, GOdescr4, which had the highest weight of importance in the model (100%, as compared to 0 for the least important GO descriptor). Calcium signaling as well as the calcium binding acute phase C-reactive protein (CRP), which

contributed to the importance of GOdescr4, have been shown to be strongly linked to opsonization and thereby phagocytosis, i.e. cell association [Nunes 2010, Bodman-Smith 2002].

In conclusion, generation of biological descriptors as the ones described above allow for more mechanism-based (quantitative) structure activity-relationships ([Q]SARs), provide a framework to include omics data into QSAR modelling, and a method for grouping ENMs according to their mechanistic characteristics.

Table 4: R^2 CV values for gold protein corona fingerprint data sets and GO descriptors, for the PCF data. Next to the name of the data sets is the number of descriptors they consist of. The best performing model is reported for the GOdescrH6 data set.

Dataset	R^2_{10CV}	R^2_{10CV}test	R^2_{4CV}	R^2_{4CV}test
PCF129	0.6526	0.7132	0.5985	0.7186
PCF76	0.7553	0.6277	0.7209	0.7001
PCF11	0.8664	0.7634	0.8550	0.7634
PCF9	0.7903	0.8644	0.7681	0.8022
GOdescrH6	0.6995	**0.8969**	0.6947	**0.8731**
GOdescrB4	0.6188	0.8511	0.5975	0.8656

3.4. DISSEMINATION AND TEACHING

DISSEMINATION

CONFERENCE

1. UM (Lars, Egon) participated in the NanoSysBio symposium organized by NanoSolutions in Stockholm, Nov 2015 and presented eNanoMapper as tool to support systems biology.

POSTERS

2. Penny Nymark, Linda Rieswijk, Friederike Ehrhart, Nina Jeliazkova, Georgia Tsiliki, Vesa Hongisto, Pekka Kohonen, Haralambos Sarimveis, Chris Evelo, Roland Grafström, Egon Willighagen. Applying “Big Data” for handling nanomaterials read across and adverse outcome studies. 2nd Nanosafety Forum for Young Scientists, Visby, Gotland, Sweden, 2016. Oral presentation.
3. Dimitra-Danae Varsou, Georgia Tsiliki, George Drakakis, Penny Nymark, Pekka Kohonen, Charalampos Chomenidis, Philip Doganis, Roland Grafström, Haralambos Sarimveis. Exploring correlations patterns on toxicity omics data. 2nd Nanosafety Forum for Young Scientists, Visby, Gotland, Sweden, 2016. Poster Presentation.

4. Pekka Kohonen, Penny Nymark, Vesa Hongisto and Roland Grafström. Predictive Toxicogenomics Space (PTGS) – an omics-based tool for predictive toxicity testing. 2nd Nanosafety Forum for Young Scientists, Visby, Gotland, Sweden, 2016.
5. Vesa Hongisto, Penny Nymark, Johannes Hattara, Pekka Kohonen and Roland Grafström. Multi-readout in vitro high-throughput screening analyses serve efficiently for evaluation and ranking of nanomaterial toxicity under diverse testing protocols. 2nd Nanosafety Forum for Young Scientists, Visby, Gotland, Sweden, 2016.

PORTAL:NANOMATERIALS ON WIKIPATHWAYS

As mentioned above, a Portal about nanomaterial related pathways was installed at WikiPathways. This portal offers direct link to all nanomaterial related pathways on WikiPathways.

PUBLICATIONS

1. Georgia Tsiliki, Penny Nymark, Pekka Kohonen, Roland Grafström and Haralambos Sarimveis. Enriching nanomaterials omics data: an integration technique to generate biological descriptors. *Submitted to Nat Nanotechnology*.
2. Penny Nymark, Linda Rieswijk, Friederike Ehrhart, Nina Jeliazkova, Georgia Tsiliki, Vesa Hongisto, Pekka Kohonen, Haralambos Sarimveis, Chris Evelo, Roland Grafström, Egon Willighagen. "Big data"-driven nanosafety: pathways for read across and adverse outcome studies. *In preparation*
3. Bart Smeets, Lars MT Eijssen, Friederike Ehrhart, Chris T Evelo, Egon L Willighagen. Fullerenol affect cholesterol metabolism in human vascular endothelial cells at a transcriptional level. *In preparation*

TRAINING

TUTORIALS

For this deliverable in total six tutorials were developed:

1. How to use PathVisio to make a pathway which can be uploaded to Wikipathways database: how-make-a-pathway
2. How to use AffyQC to do quality control and pre-processing with raw transcriptomics data: tutorial affyqc-web-tool
3. How to use the statistics tool of ArrayAnalysis.org to calculate fold change, logFC, average expression and significance of change of pre-processed data (from AffyQC) tutorial statistics-analysis
4. How to do pathway analysis using the pathway analysis tool of ArrayAnalysis.org tutorial pathway-analysis
5. How to use Chipster to perform data quality control, pre-processing and analysis of transcriptomics data tutorial chipster

   Processing of large and diverse sets of information and metadata towards mechanism-based QSAR modelling is in also being developed. Import of physico-chemical and endpoint toxicity data into Chipster has been developed to enable integration with omics data. A further extension of the Chipster tutorial is being planned. Those tutorials are available on the eNanoMapper website in the tutorial registry http://www.enanomapper.net/enm-tutorials.

6. How to calculate omics descriptors tutorial GO descriptors

SAFERNANODESIGN SUMMER SCHOOL (ESI, ARCHAMPS)

SaferNanoDesign is a one-week intensive course about aspects of nanosafety and safe design of nanomaterials for all states of life cycle.
UM contributed a lecture and a practical about using biological and nanoparticle databases and analysis of transcriptomics data using pathway analysis with the following content:

1. Databases

The main goal is to gain understanding on the content of online biological databases, like NCBI and Ensembl and nanomaterial specific databases like nanowerk and eNanoMapper. Skills will be developed to search, interpret and integrate the information available in online databases. Ultimately, this will help to better understand the function and structure of a gene and/or protein in its role in a biological process or a disease and help to get information about nanomaterials.
In molecular biology, DNA, RNA and protein are the three biomolecules that are the most important information carriers. The information they are carrying can be transferred from DNA to RNA (transcription) and from RNA to proteins (translation). Transcription occurs in the nucleus where the gene is transcribed into a messenger RNA (mRNA). Thereafter, the mRNA moves from the nucleus into the cytoplasm where the translation of the mRNA into the protein occurs.
Databases contain valuable information on the biomolecules described above and they are constantly adapted based on new insights. To use the database content properly it is important to know 1) which databases are available and 2) how to find and interpret the content.
Databases:
Ensembl http://www.ensembl.org
NCBI http://www.ncbi.nlm.nih.gov/
Gene Ontology http://geneontology.org/
eNanoMapper http://data.enanomapper.net

2. Pathway analysis

The main goal of the pathway analysis part of this course is to understand what biological pathways are and how they can be used in biomedical research to analyze and visualize experimental data.
Pathways are intuitive visual representations of biological processes usually consisting of a series of actions among molecules that lead to a certain product or change in a cell. Cells are constantly receiving cues from both inside and outside the body, which are prompted by things like injury, infection, stress or food. To react and adjust to these cues, cells send and receive signals through biological pathways. The molecules that make up biological pathways (genes, proteins and metabolites) interact with signals, as well as with each other, to carry out their designated tasks. The most common pathways are metabolic, gene regulation and signaling pathways. Researchers have discovered many important biological pathways through laboratory studies, however many pathways remain to be found. It will take years of research to identify and understand the complex connected among all of the molecules in all biological pathways, as well as to understand how these pathways work together.
There are several different online resources to find biological pathways. One commonly used, community curated pathway database is WikiPathways (http://www.wikipathways.org).
Advances in measuring technologies (high-throughput sequencing, gene/protein profiling techniques) enable a comprehensive monitoring of a biological system. Independent from the technology used, analysis of high-throughput data typically yields a list of differentially expressed genes. This list is extremely useful in identifying genes that may have roles in a given experiment, but it also often fails to provide mechanistic insights into the underlying biology. Pathway analysis provides three main features:

1. It simplifies the analysis by grouping long lists of individual genes into smaller sets of related genes or proteins. Instead of investigating thousands of genes, the complexity is reduced to just several hundred pathways.
2. Additionally, the approach puts the data into a clear biological context and identifying active pathways that differ between two conditions can have more explanatory power than a simple list of different genes or proteins.
3. Lastly, pathway analysis tools often allow the visualization of the experimental data on the pathway diagrams which provide an intuitive, visual representation of the changes in the selected process.

3. Software

PathVisio http://www.pathvisio.org

4. CONCLUSION

In this deliverable we described the work performed in the eNanoMapper project related to:

- creating pathways to serve as repository for quantitative (statistics) and qualitative (visualization) pathway analysis. These pathways are publicly available at WikiPathways (Portal:Nanomaterials) and most of them already belong to the curated/featured edition of WikiPathways.
- establishing workflows on how to do transcriptomics data analysis of cells and tissue exposed to a variety of different nanomaterials and exposure scenarios.
- drawing first conclusions on which general and specific biological pathways are affected by certain ENMs and exposure scenarios.
- adding this pathway analysis data to the eNanoMapper database.
- demonstrating the use of pathway enrichment analysis to toxicity omics data by grouping ENM data and interpreting the biological findings
- emphasizing the need of omics data in the nanosafety field to facilitate grouping of ENMs and the development of novel descriptors for QSAR modelling
- Preparing tutorials and teaching sessions to educate the community on how to use the above tools.

As mentioned before, due to the high variability of nanomaterials, biological systems and exposure scenarios the amount of data is currently not enough to make more than preliminary statements about which biological pathways are affected by a certain class of nanoparticles. But we showed that this approach is functional and will be extended with more data in the future.

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6. ANNEXES

6.1. GRADUATION THESIS

- The effect of Multi-walled carbon nanotubes on gene expression in bronchial epithelial BEAS 2B cells. Marloes Poort, FHML, Maastricht University (August 2015) – Supervisor: Dr L Eijssen, B Smeets, Dr E Willighagen
- The effect of different sized TiO_2 nanotubes on the gene expression in human aortic endothelial cells. Stan van Roij, FHML, Maastricht University (August 2015) – Supervisor: Dr L Eijssen, B Smeets, Dr E Willighagen
- The effect of zinc oxide on gene expression in human hepatic stellate cells and immune-competent cells. Tessa Lebbink, FHML, Maastricht University (August 2015) – Supervisor: Dr. S Coort, B Smeets
- Exploration of biological information related to toxicity: application to nanoparticle proteomics data (in Greek - includes English summary). Alexandra Roussi, School of Chemical Engineering, NTUA (September 2015) - Supervisor: Prof H Sarimveis
- Bioinformatics analysis of toxicity omics data. Dimitra-Danae Varsou, School of Chemical Engineering, NTUA (To be submitted in September 2016) - Supervisor: Prof H Sarimveis

6.2. PATHWAY ANALYSIS RESULTS

Annex Table 1: Summary of changed pathways for MWCNT treated BEAS 2B cells (cut-off LogFC [0.25])

Pathway	Z-score
Biogemic Amine Synthesis	3.14
Globo Sphingolipid Metabolism	2.8
Trans Sulfuration Pathway	2.7
TarBase Pathway	2.57
Dopamine Metabolism	2.5
Matrix Metalloproteinases	2.5
Fluoropyrimidine Activity	2.33
Selenium Metabolism and Selenoproteins	2.31
Arylamine metabolism	2.3
Neurotransmitter Clearance in the Synaptic Cleft	2.3
Urea Cycle and metabolism of Amino Groups	2.08
IL-17 Signaling Pathway	2.02

Vitamin B12 Metabolism	2.02
Neural Crest Differentiation	1.99

Annex Table 2: Summary of changed pathways for TiO_2 nanotube treated human aortic endothelial cells (cut-off LogFC [0.58])

Pathway	z-score
Diclofenac Metabolic Pathway	4.43
Toll-like receptor signaling pathway	4.16
IL1 and megakaryocytes in obesity	4.14
Eicosanoid synthesis	3.47
Regulation of toll-like receptor signaling pathway	3.14
Nicotine metabolism	2.98
TGF Beta Signaling Pathway	2.93
Differentiation Pathway	2.71
Nuclear Receptors	2.71
miR-targeted genes in adipocytes - TarBase	2.46
Blood Clotting Cascade	2.43
Glucuronidation	2.43

Annex Table 3: Pathways affected in hHSC cells by the coated ZnO NP (HP1 and MAX) measured after 24 hours. (cut-off LogFC [1])

HP1	
Pathway	**z-score**
Trans-sulfuration and one carbon metabolism	6.29
Eicosanoid Synthesis	6.28
Selenium Micronutrient Network	6.00

Folate Metabolism	5.07
Vitamin B12 Metabolism	4.65
MAX	
Trans-sulfuration and one carbon metabolism	6.84
Eicosanoid Synthesis	6.81
Selenium Micronutrient Network	5.64
Cytokines and Inflammatory Response	5.47
Prostaglandin Synthesis and Regulation	4.89

Annex Table 4: Pathways affected in hHSC cells by Nanosun and Z-COTE measured after 24 hours. (cut-off LogFC [1])

Nanosun	
Pathway	**z-score**
miR-targeted genes in lymphocytes - TarBase	5.22
miR-targeted genes in muscle cell - TarBase	5.15
miR-targeted genes in epithelium - TarBase	4.77
miR-targeted genes in squamous cell - TarBase	4.45
TSH signaling pathway	3.35
Z-COTE	
miR-targeted genes in muscle cell - TarBase	4.33
TSH signaling pathway	4.07
miR-targeted genes in lymphocytes - TarBase	4.06
miR-targeted genes in epithelium - TarBase	3.78
RB in Cancer	3.64

Annex Table 5: Pathways affected in Jurkat cells by ZnO-1 measured after 6 and 24 hours. The pathways were calculated with logFC ≥ 1 or logFC ≤ -1, number of affected genes ≥ 3, and P-value ≤ 0.05.

Pathway	**z-score**

Jurkat ZnO1 (6h)	
Apoptosis Modulation and Signaling	5.48
Oncostatin M Signaling Pathway	4.77
Hypertrophy Model	4.77
Quercetin and Nf-kB/ AP-1 Induced Cell Apoptosis	4.61
Apoptosis	4.58
Jurkat ZnO1 (24h)	
NRF2 pathway	4.88
TGF Beta Signaling Pathway	3.87
TSH signaling pathway	3.35
Insulin Signaling	3.26
MAPK Signaling Pathway	3.19

Annex Table 6: Pathways affected in HUVECs cells by fullerenol. (cut-off FC [1.5])

Pathway	**z-score**
Cholesterol Biosynthesis	12.16
SREBP signaling	8.38
SREBF and miR33 in cholesterol and lipid homeostasis	5.51
Statin Pathway	4.88
GPCRs, Class C Metabotropic glutamate, pheromone	4.27
Fatty Acid Biosynthesis	3.78
Neurotransmitter Release Cycle	3.39
Differentiation Pathway	3.08
Dopamine metabolism	3.06
Focal Adhesion	2.98

Annex Table 7: Summary of changed pathways for high dose Ag nanoparticle treated Caco-2 cells (cut off: logFC [1])

Pathway	z-score
Zinc homeostasis	6.1
NRF2 pathway	4.63
Quercetin and Nf-kB/ AP-1 Induced Cell Apoptosis	4.31
Triacylglyceride Synthesis	3.77
Farnesoid X Receptor Pathway	3.59
Oxidative Stress	3.52
Fatty Acid Omega Oxidation	3.43
Transcriptional activation by NRF2	3.43
Copper homeostasis	3.1

6.3. PATHWAY ANALYSIS RESULTS FROM DATA PROVIDED BY OTHER RESEARCH INSTITUTES

Analysis of nanostructured silica effects on rat liver and jejunum (sub-chronic toxicity study)

Data was provided by van der Zande, RIKILT, Wageningen University, Wageningen, the Netherlands

Annex Table 8: Pathways affected by a low dose of SAS measured at day 29 in rat liver (cutoff, logFC>0.58 or logFC<-0.58 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Fatty Acid Biosynthesis	2	18	35	11,11%	4,88	0,000
Beta Oxidation Meta Pathway	2	28	32	7,14%	3,75	0,017
Fatty Acid Beta Oxidation	2	30	85	6,67%	3,59	0,013
Type II interferon signaling (IFNG)	2	30	35	6,67%	3,59	0,015

Annex Table 9: Pathways affected by a medium dose of SAS measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive	measur	total	%	Z Score	p-value

	(r)	ed (n)				(permuted)
Statin Pathway	3	16	29	18,75%	5,13	0,000
PKA-HCG-Glycogen Syntase	3	34	44	8,82%	3,11	0,017
Cholesterol metabolism	2	20	40	10,00%	2,78	0,017
Oxidative Stress	2	24	28	8,33%	2,43	0,046
Cytokines and Inflammatory Response (BioCarta)	2	25	28	8,00%	2,35	0,059
Nuclear receptors in lipid metabolism and toxicity	2	26	40	7,69%	2,28	0,030

Annex Table 10: Pathways affected by a high dose of SAS measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Metapathway biotransformation	3	128	144	2,34%	3,08	0,012
Nuclear receptors in lipid metabolism and toxicity	2	26	40	7,69%	5,29	0,000
Retinol metabolism	2	33	49	6,06%	4,62	0,006

Annex Table 11: Pathways affected by a high dose of SAS measured at day 85 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
p53 signal pathway	2	26	35	7,69%	4,62	0,010
Retinol metabolism	2	33	49	6,06%	4,01	0,003
p53 pathway	2	41	47	4,88%	3,50	0,015
Proteasome Degradation	2	46	60	4,35%	3,25	0,021
Cell cycle	2	71	89	2,82%	2,39	0,063

Annex Table 12: Pathways affected by a high dose of SAS measured at day 29 in rat jejunum (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Oxidative Stress	5	24	28	20,83%	4,13	0,001
DNA Replication	5	36	41	13,89%	2,96	0,002
Translation Factors	5	36	48	13,89%	2,96	0,003
TGF Beta Signaling Pathway	5	41	52	12,20%	2,61	0,011
Endochondral Ossification	5	51	67	9,80%	2,05	0,043
Type II interferon signaling (IFNG)	4	30	35	13,33%	2,54	0,017
GPCRs, Class B Secretin-like	3	20	23	15,00%	2,45	0,028
Ovarian Infertility Genes	3	23	31	13,04%	2,16	0,040
Brain derived neurotrophic factor	2	10	11	20,00%	2,53	0,032
Nucleotide GPCRs	2	10	14	20,00%	2,53	0,016
GPCRs, Class C Metabotropic glutamate, pheromone	2	12	15	16,67%	2,19	0,052
Keap1-Nrf2	2	12	16	16,67%	2,19	0,041
Homologous recombination	2	13	14	15,38%	2,04	0,043

Annex Table 13: Pathways affected by a low dose of NM-202 measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
G1 to S cell cycle control	6	59	67	10,17%	5,70	0,000
Cell cycle	6	71	89	8,45%	5,04	0,000
GPCRs, Class A Rhodopsin-like	6	197	236	3,05%	1,97	0,030
ATM Signaling Pathway	4	23	26	17,39%	6,47	0,000
Fatty Acid Omega	3	10	13	30,00%	7,62	0,000

Oxidation						
Nucleotide GPCRs	2	10	14	20,00%	4,95	0,005
Genetic alterations of lung cancer	2	25	28	8,00%	2,78	0,038
p53 signal pathway	2	26	35	7,69%	2,70	0,018
Retinol metabolism	2	33	49	6,06%	2,25	0,035
DNA Replication	2	36	41	5,56%	2,10	0,061
Cytoplasmic Ribosomal Proteins	2	38	89	5,26%	2,00	0,065

Annex Table 14: Pathways affected by a medium dose of NM-202 measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Insulin Signaling	6	137	158	4,38%	2,24	0,019
MAPK signaling pathway	6	140	161	4,29%	2,18	0,021
G1 to S cell cycle control	4	59	67	6,78%	2,83	0,013
DNA Replication	3	36	41	8,33%	2,90	0,020
Renin - Angiotensin System	3	47	60	6,38%	2,32	0,035
Urea cycle and metabolism of amino groups	2	13	59	15,38%	3,62	0,004
Eicosanoid Synthesis	2	18	35	11,11%	2,92	0,010
Nuclear receptors in lipid metabolism and toxicity	2	26	40	7,69%	2,22	0,027

Annex Table 15: Pathways affected by a high dose of NM-202 measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)

IL-5 Signaling Pathway	2	63	69	3,17%	2,52	0,045
G Protein Signaling Pathways	2	77	96	2,60%	2,15	0,077

Annex Table 16: Pathways affected by a high dose of NM-202 measured at day 85 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Myometrial Relaxation and Contraction Pathways	6	120	158	5,00%	2,42	0,026
MAPK signaling pathway	6	140	161	4,29%	2,00	0,030
Endochondral Ossification	5	51	67	9,80%	4,04	0,001
Fatty Acid Omega Oxidation	3	10	13	30,00%	6,36	0,001
Prostaglandin Synthesis and Regulation	3	30	37	10,00%	3,16	0,006
Type II interferon signaling (IFNG)	3	30	35	10,00%	3,16	0,012
Proteasome Degradation	3	46	60	6,52%	2,22	0,035
Blood Clotting Cascade	2	18	21	11,11%	2,78	0,020
Inflammatory Response Pathway	2	27	31	7,41%	2,03	0,080

Annex Table 17: Pathways affected by a high dose of NM-202 measured at day 29 in rat jejunum (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Calcium Regulation in the Cardiac Cell	5	121	153	4,13%	2,67	0,015
Alpha6-Beta4 Integrin Signaling Pathway	3	53	66	5,66%	2,72	0,030
ATM Signaling Pathway	2	23	26	8,70%	3,04	0,021
Monoamine GPCRs	2	29	41	6,90%	2,58	0,024

Analysis of effects of silverions and silverparticles on rat liver and pbmcs (sub-chronic toxicity study)

Data was provided by van der Zande, RIKILT, Wageningen University, Wageningen, the Netherlands

Annex Table 18: Pathways affected by NM300K (uncoated AgNP) measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Metapathway biotransformation	5	93	144	5,38%	1,98	0,036
Apoptosis	4	67	83	5,97%	2,00	0,039
Nuclear Receptors	3	34	38	8,82%	2,53	0,022
Striated Muscle Contraction	3	34	37	8,82%	2,53	0,018
Glucuronidation	2	9	35	22,22%	3,96	0,005
Fatty Acid Biosynthesis	2	17	35	11,76%	2,59	0,012
Genetic alterations of lung cancer	2	24	28	8,33%	1,96	0,053

Annex Table 19: Pathways affected by AgNO3 (Ag ions) measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Cell cycle	10	71	89	14,08%	4,28	0,000
Inflammatory Response Pathway	4	27	31	14,81%	2,81	0,014
Glutathione metabolism	3	19	45	15,79%	2,57	0,016
ATM Signaling Pathway	3	22	26	13,64%	2,26	0,039
p53 signal pathway	3	25	35	12,00%	1,99	0,036
Glucocorticoid Metabolism	2	6	8	33,33%	3,60	0,005
Estrogen metabolism	2	9	29	22,22%	2,74	0,018
Keap1-Nrf2	2	12	16	16,67%	2,19	0,057

Serotonin and anxiety	2	12	16	16,67%	2,19	0,039

Annex Table 20: Pathways affected by PVP (PVP-coated AgNP) measured at day 29 in rat liver (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Cell cycle	6	71	89	8,45%	3,79	0,000
Calcium Regulation in the Cardiac Cell	6	130	153	4,62%	2,05	0,029
Toll-like receptor signaling pathway	4	67	93	5,97%	2,24	0,040
Beta Oxidation Meta Pathway	3	26	32	11,54%	3,38	0,016
Fatty Acid Beta Oxidation	3	28	85	10,71%	3,20	0,009
Nuclear receptors in lipid metabolism and toxicity	3	29	40	10,34%	3,12	0,009
p53 pathway	3	39	47	7,69%	2,46	0,028
Keap1-Nrf2	2	12	16	16,67%	3,53	0,008
EBV LMP1 signaling	2	15	22	13,33%	3,05	0,017
Hedgehog Signaling Pathway	2	16	22	12,50%	2,92	0,016
Eicosanoid Synthesis	2	17	35	11,76%	2,79	0,011
Glutathione metabolism	2	19	45	10,53%	2,58	0,030

Annex Table 21: Pathways affected by NM300K (uncoated AgNP) measured at day 29 in rat PBMCs (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Metapathway biotransformation	6	124	144	4,84%	3,10	0,004
Electron Transport Chain	4	69	112	5,80%	2,95	0,006

GPCRs, Other	4	71	76	5,63%	2,88	0,008
Oxidative phosphorylation	3	40	65	7,50%	3,12	0,007
Cytoplasmic Ribosomal Proteins	3	42	89	7,14%	3,01	0,008
Id Signaling Pathway	3	49	51	6,12%	2,67	0,022
Aflatoxin B1 metabolism	2	6	13	33,33%	6,40	0,001
Estrogen metabolism	2	13	29	15,38%	4,11	0,011
Mitochondrial LC-Fatty Acid Beta-Oxidation	2	16	25	12,50%	3,61	0,005
p53 signal pathway	2	28	35	7,14%	2,45	0,037
Fatty Acid Beta Oxidation	2	31	85	6,45%	2,27	0,022

Annex Table 22: Pathways affected by AgNO3 (Ag ions) measured at day 29 in rat PBMCs (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Prostaglandin Synthesis and Regulation	6	31	37	19,35%	6,73	0,000
Relationship between glutathione and NADPH	5	54	98	9,26%	3,69	0,001
Spinal Cord Injury	5	95	110	5,26%	2,18	0,030
Eicosanoid Synthesis	4	19	35	21,05%	5,77	0,000
Oxidative Stress	4	27	28	14,81%	4,62	0,000
Complement and Coagulation Cascades	4	56	63	7,14%	2,65	0,012
Selenium Micronutrient Network	3	23	89	13,04%	3,67	0,000
p53 pathway	3	44	47	6,82%	2,19	0,043
Estrogen metabolism	2	13	29	15,38%	3,34	0,013
Hedgehog Signaling Pathway	2	19	22	10,53%	2,57	0,043

Signal Transduction of S1P	2	23	25	8,70%	2,21	0,047
ATM Signaling Pathway	2	24	26	8,33%	2,13	0,058
Folic Acid Network	2	24	103	8,33%	2,13	0,019

Annex Table 23: Pathways affected by PVP (PVP-coated AgNP) measured at day 29 in rat PBMCs (cutoff, logFC>0.26 or logFC<-0.26 and p-val<0.05, >2 genes changed)

Pathway	positive (r)	measured (n)	total	%	Z Score	p-value (permuted)
Translation Factors	4	42	48	9,52%	3,57	0,007
VEGF-receptor Signal Transduction	3	25	32	12,00%	3,64	0,003
Notch Signaling Pathway	3	33	45	9,09%	2,98	0,010
Cytoplasmic Ribosomal Proteins	3	42	89	7,14%	2,44	0,014
ATM Signaling Pathway	2	24	26	8,33%	2,27	0,053
p53 signal pathway	2	28	35	7,14%	1,99	0,062