Deliverable Report for Grant Agreement Number 760813

Deliverable 2.3

Effects of subchronic oral exposure to ENM

Due date of deliverable: 31/03/2020
Actual submission date: 31/03/2020

Lead beneficiary for this deliverable: IUF

Dissemination Level:		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
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CO	Confidential, only for members of the consortium (including the Commission Services)	

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1. Description of task

Task 2.3 Evaluation and complementation of Repeated Dose Oral Toxicity Studies; (IUF, KRISS BASF, NRCWE); M1-27

Original DoW: Systemic uptake of ENM following oral exposure is very low, although translocation to and effects in secondary organs have been shown[1,2,3]. ENM predominantly accumulate in the liver and spleen. Assessment of organ burden from stored tissues will be restricted to intestines (port of entry) and liver/spleen (target). The evaluation of studies on oral toxicity will focus on local effects in the intestine, and on possible liver toxicity to align with WP4. Histopathology and enhanced 2D and 3D darkfield imaging of ENM distribution in the GI tract with focus on Peyer's patches, liver and spleen will be performed on tissues from ongoing and pre-existing studies in mice using, among others, amorphous SiO_2, TiO_2 (P25), Ag and CeO_2 ENM, applied in food pellets at concentrations at and below 1% mass (relating to the allowed amount of (nanosize) EU-approved food additives; IUF, NRCWE, BASF). Application type and concentrations will thus reflect realistic exposure scenarios in humans compared to gavage (bolus) applications in which stomach passage time and food-matrix protective effects differ, or applications in drinking water that may lead to dosimetry artefacts due to ENM sedimentation and exposure dissolution. Fresh food pellets will be used to minimise ENM ageing/modification effects as shown e.g. for AgNP[4]. Tissues from available 13-week oral ENM exposure studies (CeO_2 and SiO_2) and further short-term (28 days) studies in mice will be evaluated for markers of oxidative stress, inflammation and genotoxicity (DNA strand breaks/oxidation; IUF) allowing comparison with investigations in WP4 on 3D GIT and liver models. Effects on GI tract microbiome as shown e.g. for AgNP will also be considered (IUF). This allows for connection to WP4 where the same ENM will be tested *in vitro* with advanced GIT models that incorporate

[1] Geraets et al. (2014) Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Part Fibre Toxicol 11 30.

[2] van der Zande et al. (2014) Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Part Fibre Toxicol 11 8

[3][Folkmann et al. (2009) Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Environ Health Perspect 117 703-8

[4] van den Brule et al. (2016) Dietary silver nanoparticles can disturb the gut microbiota in mice. Part Fibre Toxicol 13 38

simulated digestion[5] and gut microbiota-derived metabolites (Task 4.2) as well as to WP5 where microbiome effects are addressed in zebrafish. Data will also be collected from 28-day oral studies of seven ENM conducted in rats; including full histopathology and metabolome analysis[6] (BASF).

In this task one new 90-day oral exposure study in rats (Ag, by gavage) will be executed by KRISS in collaboration with NRCWE and IUF according to OECD TG408. Selected tissues from this new study will be provided to Task 2.5 for transcriptomics-analyses to identify potential novel ENM-exposure and effect markers to feed into the *in vitro* intestinal and liver models in WP4. All this information will be used by WP4 for the selection of cell types and used in WP6 to support the computational modelling activities.

[5] Gerloff et al. (2013) Influence of simulated gastrointestinal conditions on particle-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells. Nanotoxicology 7 353-66

[6] Buesen et al. (2014) Effects of SiO(2), ZrO(2), and BaSO(4) nanomaterials with or without surface functionalization upon 28-day oral exposure to rats. Arch Toxicol 88 1881-906

2. Description of work & main achievements

2.1 PATROLS internally available tissue samples for further examinations

Within WP2 of PATROLS, an overview of all internally available tissues was made. The tissues that were available for the inhalation route of exposure were listed in the Deliverable 2.2 report. Table 1 of the present report lists the available tissues from the oral exposure studies. It includes studies that used ENM application by oral gavage as well as studies in which ENM were provided *ad libitum* via introduction in feed pellets. In addition to these studies, a new 90-day oral exposure study was performed in rats with TiO_2 according to OECD TG408 by KRISS (see section 2.6). Several of the available tissues were used or made available for further analysis in the course of the project, e.g. for histopathology, ENM distribution analysis or marker analyses as described in the next sections.

For the sub-chronic oral studies that were performed in the C57BL/6J mice with CeO_2 (NM212) and SiO_2 (SAS) histopathological evaluation was explicitly determined for the PATROLS-relevant organs, i.e. small and large intestine, liver and spleen (IUF). The mice in these studies were exposed *ad libitum* by application of the ENM in feed pellets at two different concentrations, i.e. 0.1 % and 1% (w/w). Sections were blindly evaluated by an experienced veterinary pathologist using Haemotoxylin and Eosin (H&E) stained sections for these organs and additionally Periodic Acid Schiff (PAS) stained sections for small and large intestine. Results are shown in table 2. Overall, there was no difference between the experimental groups.

Table 1. Available tissues from oral exposure studies performed by PATROLS partners for further analysis

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Intestine	Kidney	Liver	Spleen	Hearts and Kidney
BaSO4 NM-220	BaSO4 (NM-220)	oral gavage, 28days	1	**BASF**	x	x	In block	Preserved tissue	
SAS	SiO_2 amorphous	Oral gavage, 28 days	1	**BASF**	x	x	In block	Preserved tissue	
CeO_2 SiO_2 amorphous	CeO_2-NM212 SiO_2	Oral in feed pellets 1% and 0.1% (w/w) (ad libitum, 3 weeks and 13 weeks)		**IUF**	In block ("Swiss Rolls" of small intestine & colon)	In block & Preserved tissue	In block & Preserved tissue	In block	
CeO_2	Degussa/ Quimidroga	IV, PO with 3 post-exposure follow-up times and one dose of 162µg	1, 28, 180	**NRCWE**	?	√	√	√	√ and lung
TiO_2	Rutile, NanoAmor	IV, PO with 3 post-exposure follow-up times and one dose of 162µg	1, 28, 180	**NRCWE**	?	√	√	√	√ and lung
Ag-PVP TiO_2 p25	Ag-PVP TiO_2 p25	Oral in feed pellets 1 concentration vs controls (ad libitum) 28 days & 28 days + 14 days recovery		**IUF**	In block ("Swiss Rolls" of small intestine & colon)	In block & Preserved tissue	In block & Preserved tissue	In block & Preserved tissue	
Ag-PVP TiO_2 p25	Ag-PVP TiO_2 p25	Oral in feed pellets, 6 concentrations (ad libitum), 28 days		**IUF**	In block ("Swiss Rolls" of small intestine & colon)	Preserved tissue	In block & Preserved tissue	Preserved tissue	

X = H&E-stained slide available, may need to prepare new ones if the quality is not good any more. (x) = H&E-stained slide available, probably no new slides. IV = intravenous administration; PO = Oral administration

√ = In general, NRCWE has histology H&E- and Sirius-stained slides and paraffin blocks from lung, liver, kidney, spleen and heart, and for additional organs for some studies & frozen liver

Table 2. Histopathological evaluation of mice organs after 13 week feeding with SiO_2 or CeO_2 ENM.

Shown is the grading of the lesion and the number of animals in brackets.
The following grading has been used: 0 = no findings, 1 = minimal, 2 = slight, 3 = moderate, 4 = severe, 5 = massive.

		control	0.1 % SiO_2	1 % SiO_2	0.1 % CeO_2	1 % CeO_2
Liver		n = 5	n = 6	n = 6	n = 6	n = 6
	Focal inflammatory infiltrated	1 (5)	1 (4), 2(2)	1 (6)	1 (6)	1 (6)
	Focal necrosis	1 (2)	1 (1)	1 (2)	0 (6)	0 (6)
	Increased interstitial cells	0 (5)	2 (1)	1 (1)	1 (1)	1 (1)
	Increased glycogen	0 (5)	0 (6)	0 (6)	2 (2), 3 (1)	0 (6)
	Vacuolation	0 (5)	0 (6)	0 (6)	0 (6)	2 (3)
Spleen		n = 5	n = 6	n = 6	n = 5*	n = 6
	Increased pigment	2 (1)	2 (3)	0 (6)	2 (2)	1 (1), 2 (1)
	Congestion	0 (5)	2 (1)	0 (6)	0 (5)	0 (6)
	Increased extramedullary haematopoiesis	0 (5)	0 (6)	3 (1)	1 (1)	2 (1)
	Increased megakaryocytes	0 (5)	0 (6)	2 (1)	0 (5)	0 (6)

* one sample not evaluable due to embedding artefacts

Small & Large Intestine	Minimal focal inflammatory infiltrates (intra-mucosal) in all specimens. In some cases inflammatory infiltrates in adjacent tissue and pancreas with focal vacuolation (grade 2). Clearly visible goblet cells in PAS stained slides (small intestine +; large intestine +++) Partly mucus on surface. Gut associated lymphoid tissue (GALT) in almost all specimens detectable.

2.2 Enhanced 2D darkfield imaging (NRCWE)

To identify possible cellular interactions of ENM *in vivo*, enhanced darkfield microscopy was performed on tissues from a sub-chronic oral study mice with CeO_2 (NM-212) in feed pellets (1% w/w).

Method. Cytoviva enhanced darkfield hyperspectral system (Auburn, AL, USA) was used for scanning histological sections of small and large intestine and liver. Aggregates of foreign material in tissue originating from CeO_2 exposure is hardly detectable in brightfield microscopy, but show intense light scattering in enhanced darkfield[7,8]. Images were acquired at 40x and 100x on an Olympus BX 43 microscope with a Qimaging Retiga4000R camera for darkfield and a Nikon DS-Fi2 camera for brightfield.

1-2 sections per tissue from 5 animals per group were scanned at 40x magnification. "Swiss roll" sections[9] of small intestine were only partly scanned by scanning across the roll through the middle in two perpendicular scans, thereby including all representative parts of small intestine. All lymphoid tissues/Peyer's patches were located in brightfield and scanned in enhanced darkfield.

Results. In general there were many light scattering artefacts in all samples including controls, which complicated the analysis. For liver and colon, 1 tissue section/animal from 5 animals per group were scanned. Very few nanomaterial-like agglomerates were found in liver and colon of only one animal (not shown), and therefore we cannot conclude if these were artefacts or nanomaterial.

In small intestine, we found light scattering objects in most Peyer's patches/lymphoid tissues in both the control and the CeO_2 exposed group. However, in 3 of 5 CeO_2 exposed animals there appeared to be an increased density of scattering objects in

[7] Modrzynska J, Berthing T, Ravn-Haren G, et al. In vivo-induced size transformation of cerium oxide nanoparticles in both lung and liver does not affect long-term hepatic accumulation following pulmonary exposure. PLoS One 2018; 13(8): e0202477; doi: 10.1371/journal.pone.0202477.

[8] Modrzynska J, Berthing T, Ravn-Haren G, et al. Primary genotoxicity in the liver following pulmonary exposure to carbon black nanoparticles in mice. Part Fibre Toxicol 2018, doi: 10.1186/s12989-017-0238-9

[9] *The Swiss Roll technique enables the (immuno)histological analysis of long segments of the intestine: Segments are slit open longitudinally. Following careful removal of contents the segments are rolled up longitudinally with the mucosa outwards and placed in fixative for subsequent embedding* (Moolenbeek C, Ruitenberg EJ. The "Swiss roll": a simple technique for histological studies of the rodent intestine. Lab Anim. 1981;15:57-59)

the periphery of large lymphoid tissues (Figure 1). Based on standard H&E stain, it was not possible to identify if the material is associated with a specific cell type (Figure 2). A high density of light scattering objects was also found in 2 of 5 CeO_2 exposed animals in what appeared to be adventitia - the outer tissue surrounding the small intestine (Figure 3). However, the objects could also be located in mucus belonging to the lumen of the next layer of small intestine in the Swiss Roll preparation. It requires further imaging to evaluate whether this observation represents an artefact or not.
A few observations of nanomaterial-like agglomerates were found in the small intestine epithelium of one CeO_2 exposed animal (Figure 4). The agglomerates appeared inside absorptive cells or in one case possibly a goblet cell (not shown). A single similar observation was found in one control animal, although with a slightly different appearance in darkfield and brightfield (Figure 4).

In conclusion, the darkfield analysis indicates the presence of foreign material in small intestine, but not in colon or liver, after sub-chronic oral exposure to CeO_2 in feed pellets. Further imaging and chemical analysis is needed to determine if the scattering objects found in Peyer's patches and adventitia or mucus of small intestine is CeO_2 nanomaterial, and if the material is interacting with other cell types than lymphocytes.

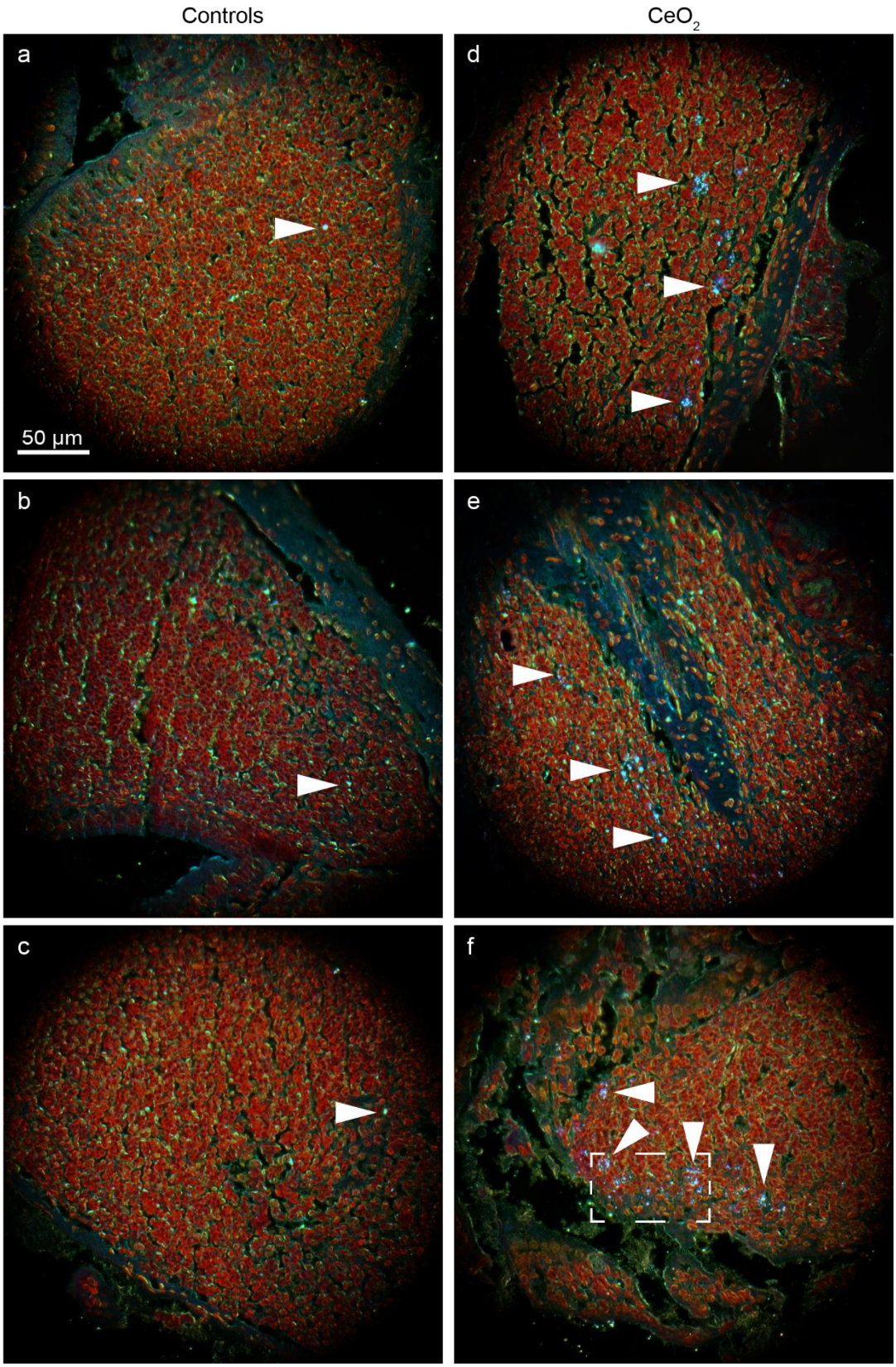

Figure 1. *Lymphoid tissue (red) in murine small intestine after subchronic oral exposure to CeO_2 in feed pellets. (a-c) Control group. (d-f) CeO_2 group. A higher density of foreign material (white arrowheads) is found in the periphery of large lymphoid tissues in the CeO_2 group. f) The marked area is shown at higher magnification in figure 2. Enhanced darkfield microscopy, H&E stain.*

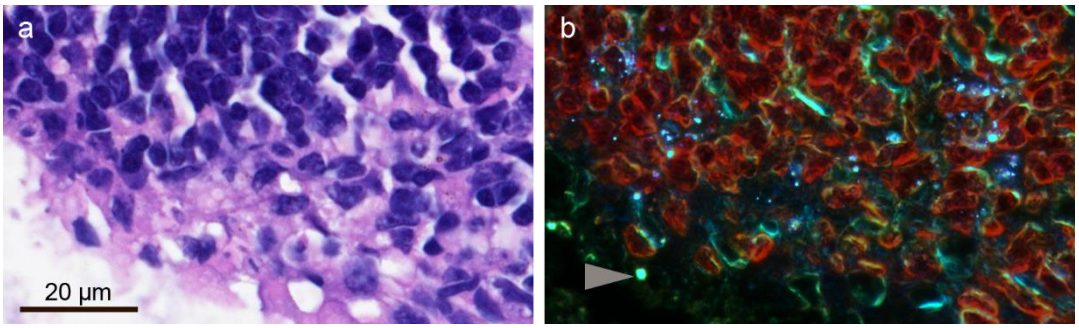

Figure 2. *Periphery of large lymphoid tissue in murine small intestine after subchronic oral exposure to CeO_2 in feed pellets. Brightfield (a) and enhanced darkfield (b) microscopy at higher magnification of the marked area in figure 1f. Foreign material is barely detectable as brown spots in brightfield (a) and appears white and particle-like in darkfield (b). Grey arrowhead marks a common light scattering artefact (light green) from sample preparation.*

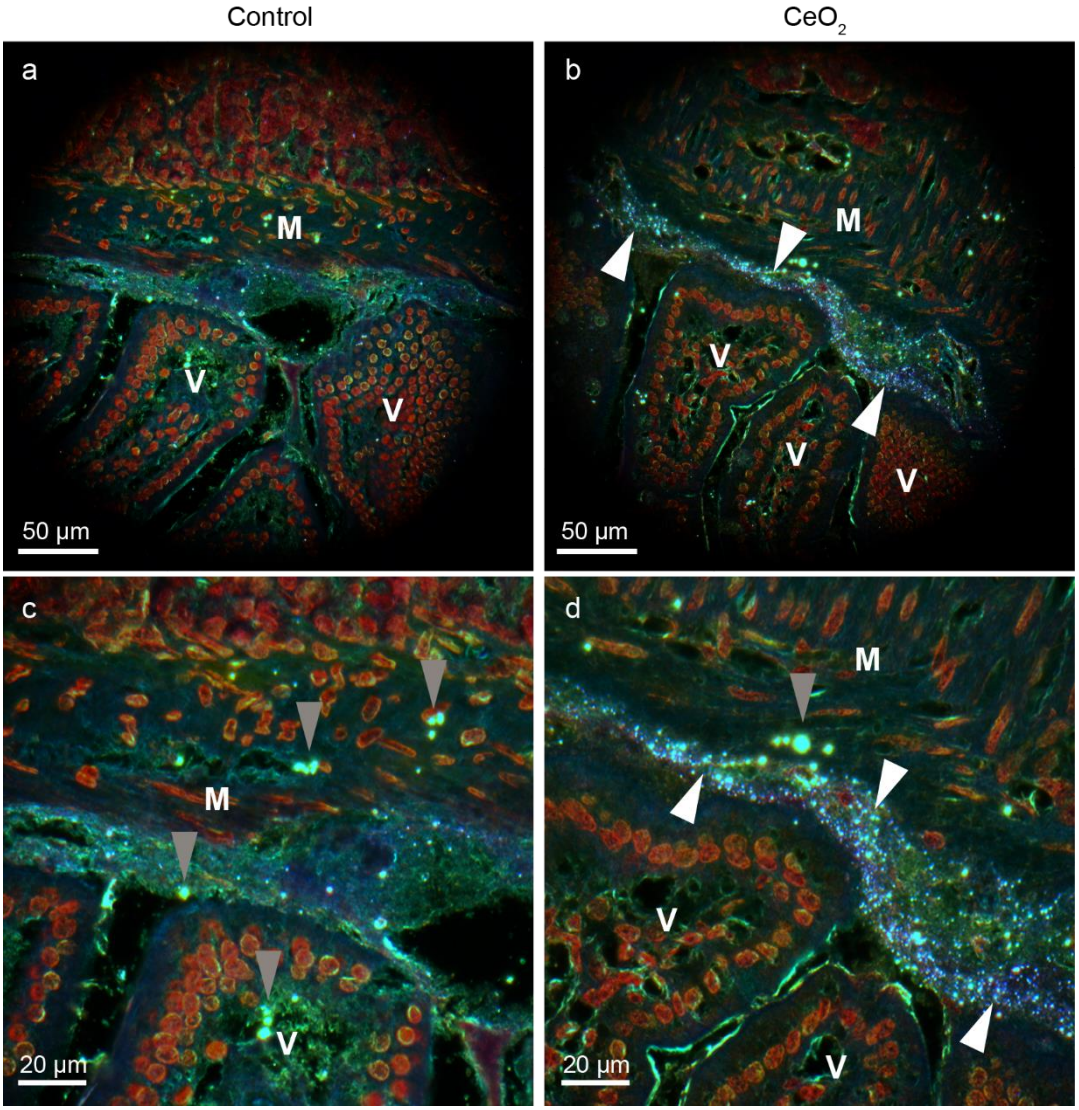

Figure 3. *Foreign material between layers of murine small intestine in Swiss Roll. a) Control tissue, with higher magnification in c). b) CeO_2 group, with higher magnification in d). A higher density of light scattering material (white arrowheads) is seen in CeO_2 exposed tissue either in adventitia below muscular tissue (M), or in mucus above villi (V) of mucosa. Grey arrowheads mark common light scattering artefacts (light green) from sample preparation. Enhanced darkfield microscopy, H&E stain.*

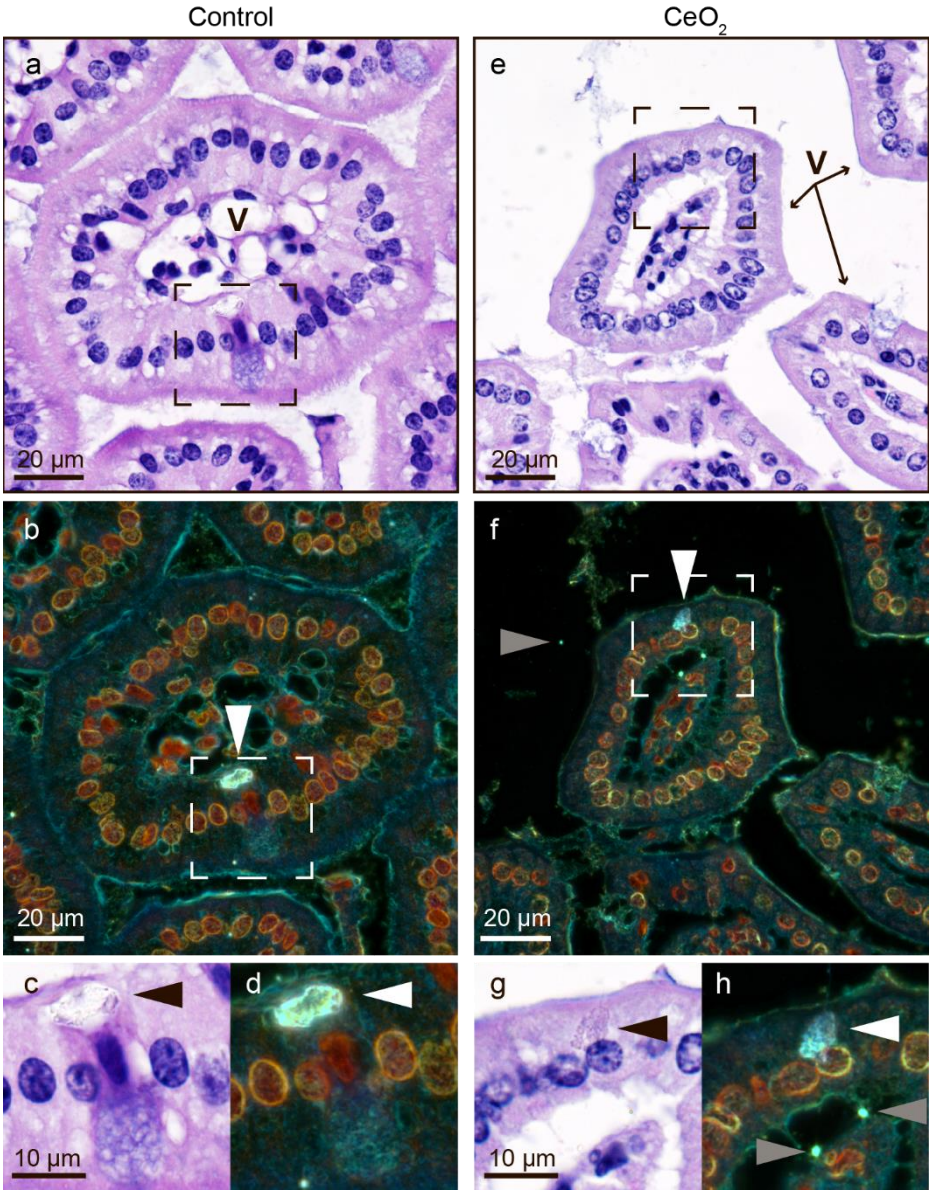

Figure 4. *Foreign material agglomerates (white and black arrowheads) in murine small intestine epithelial cells after subchronic oral exposure to feed pellets without CeO_2 (a-d) or with 1% w/w CeO_2 (e-h). Transverse sections of villi (V). Marked areas in a) and b) are shown at higher magnification in c) and d). Marked areas in e) and f) are shown at higher magnification in g) and h). Grey arrowheads mark common artefacts (light green) from sample preparation. Brightfield (a, c, e, g) and enhanced darkfield microscopy (b, d, f, h). H&E stain.*

2.3 Oxidative stress, genotoxicity, inflammation and mucin markers (IUF)

2.3.1 Oxidative stress, inflammation and mucin gene expression analyses

For comparison and further development of the *in vitro* GIT models in WP4, mRNA expression analyses were conducted by qRT-PCR from available mouse intestine tissues from 21 day exposure studies with CeO_2 and SiO_2 and 28-day studies with Ag-PVP and TiO_2 (the mice were exposed via the introduction of ENM in feed pellets). As markers of inflammation and oxidative stress, the following genes were selected: TNF-α, IL-1β, IL-6, Mip-2, Kc and Hmox-1. Furthermore, given the importance of the mucus barrier in intestinal homeostasis, also a panel of mucin genes was identified for mRNA expression analysis in the intestine tissues, i.e.: Muc1, Muc2, Muc5AC, Muc13 and Muc20. The same set of mucin genes has also been evaluated *in vitro* in the context of the advanced GIT model development in WP4 (IUF).

Results of the analyses from the intestine tissues of the orally exposed mice are shown in Figure 5. No significant effect on the expression of any of the investigated genes was observed after exposure to TiO_2. After exposure to Ag-PVP, the gene expression of Muc2 was significantly downregulated by fold change 0.81. For none of the other investigated genes, a significant effect on the expression was found after Ag-PVP exposure. After exposure of mice to CeO_2, a significant 2-fold downregulation of the Muc1 expression was observed. The expression of none of the other investigated genes changed significantly upon CeO_2 exposure. SiO_2 exposure caused no significant effect on the ileal expression of any of the investigated genes. A high variation of the fold changes were primarily observed for mice exposed to SiO_2.

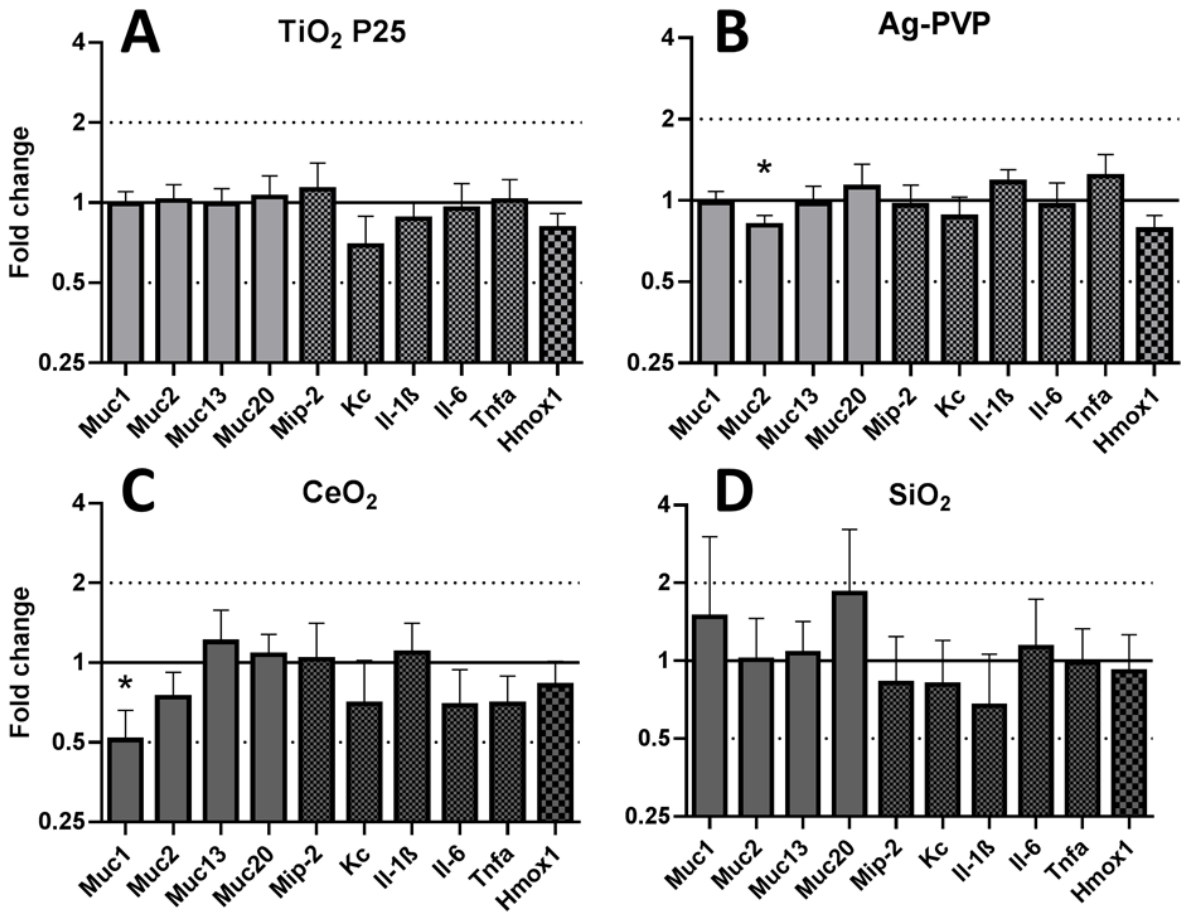

Figure 5. Gene expression of mucins, cytokines and Hmox1 in murine ileal tissue after ENM exposure*. C57BL6/J mice were fed with feed pellets containing either no additive, 1.0% TiO_2 P25 (A) or 0.2% Ag-PVP (B) for 28 days, or 1.0% CeO_2 (C) or 1.0% SiO_2 (D) for 21 days before sacrificing. The relative gene expression of Muc1, Muc2, Muc13, Muc20, Mip-2, Kc, Il-1β, Il-6, Tnfα and Hmox1 was assessed by qRT-PCR and calculated using the ΔΔCT method. Results were normalised to non-exposed mice as negative control and Rplp0 as reference gene. Depicted fold changes with SEM were derived from the average and SEM of ΔΔCT-values of n≥5 independent experiments (t-test; *p<0.05).*

2.3.2 DNA damage

For the investigation of genotoxicity following oral exposure to ENM, tissues were analysed from mice that were exposed to Ag-PVP and TiO_2 ENM for 28 days via introduction in the feed pellets at five different treatment concentrations, with 1 % and 0.2 % as highest concentrations for TiO_2 and Ag, respectively. Body and organ weights were determined but revealed no obvious treatment related effects. *In vivo* genotoxicity was determined for both ENM by the alkaline comet assay in the intestinal epithelial cells upon their isolation from freshly collected intestines. The fresh analysis of DNA damage avoids potential artificial induction of additional damage by freezing/thawing. No significant DNA damage was observed for both ENM (See Figure 6).

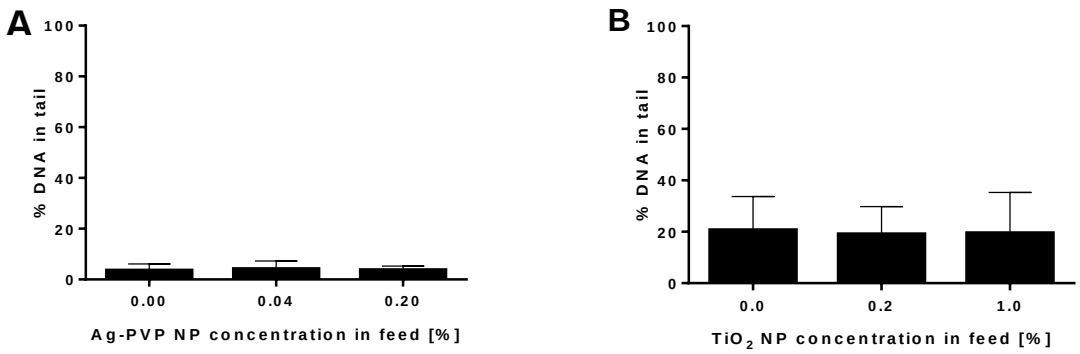

Figure 6. *DNA damage in isolated murine colon epithelial cells after 28-day feeding studies exposing mice to (A) Ag ENM (0.04 or 0.2 % w/w in feed) and (B) TiO_2 ENM (0.2 or 1 % w/w in feed) (Average ± SD of n=3 animals per treatment group)*

In relation to these DNA damage analyses, the colon tissues from the ENM-exposed mice were analysed for mRNA expression of the DNA base excision repair (BER) genes OGG-1, APE-1 and XRCC-1 as well as the oxidative stress marker gene HO-1 (See Figure 7). In line with the results from the *in vivo* comet assay analyses, no changes in the expression of these genes was found in association with oral exposure to the Ag-PVP or TiO_2 ENM.

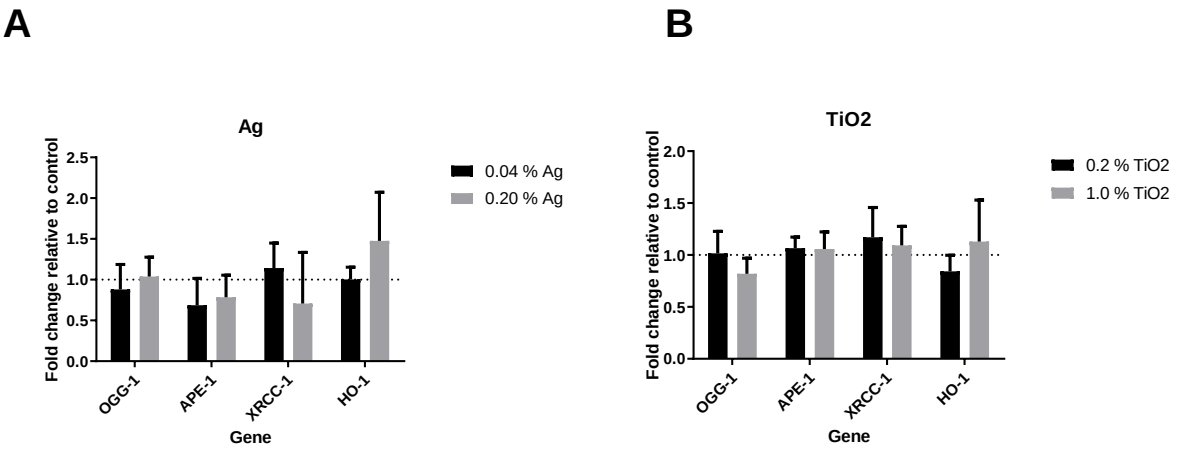

Figure 7. *Gene expression in murine colon tissue after 28-day exposure to (A) Ag-PVP and (B) TiO_2 ENM (Average ± SD of n=5)*

2.4 Analysis of ENM effects on mouse microbiome after oral exposure (IUF)

Data of effects of oral ENM exposure on mouse microbiome were used to obtain information on the need to include microbiome-mimicking compounds/metabolites in the advanced GIT models (PATROLS Task 4.2). In addition, these data are of use for the cross-species comparisons (e.g. mice [WP2] versus Zebrafish [WP5]). The microbiome analysis was performed using next generation sequencing (NGS) on DNA from faecal samples obtained from mice that had been exposed to CeO_2 and SiO_2 (21 days feeding studies) as well as in mice exposed to Ag-PVP and TiO_2 (28 days feeding). For this, the V3- and V4-regions of the bacterial 16S rDNA was enriched and sequenced using Illumina-Platform. For all four ENM (i.e. CeO_2, SiO_2, Ag-PVP, TiO_2) a detailed analysis of microbiome changes has been performed. As an example, here the results of the α-diversity are shown in relation to oral exposure to CeO_2 (NM212) and SiO_2 (SAS) in Figure 8. The α-diversity is a measure of the intrinsic diversity of each individual sample. Besides the richness, the evenness was investigated in the form of Shannon entropy and Simpson's index (See Figure 8).

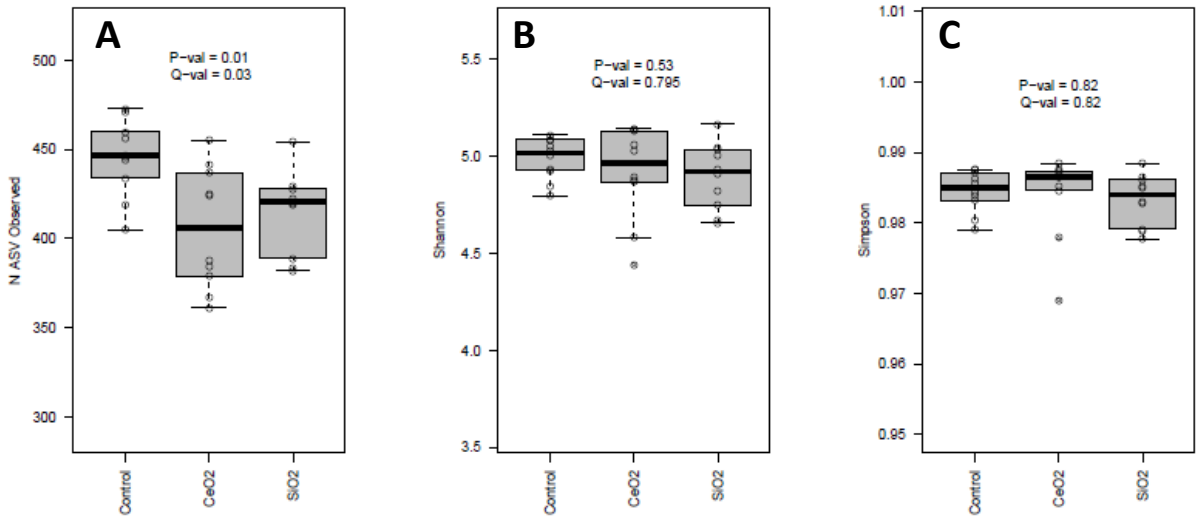

Figure 8, α-diversity of the intestinal microbiome of mice after treatment with CeO_2 or SiO_2. The microbiome data were obtained from female C57BL6/J mice fed with feed pellets containing either no additive, 1.0% CeO_2 or 1.0% SiO_2 (n=10 each) for 21 days before sacrificing (A-C). The V3 and V4 region of the bacterial 16S rDNA isolated from stool were sequenced. The richness (A), Shannon entropy (B) and 1 - Simpson's index (C) were calculated.

Beyond the aforementioned parameters, the β-diversity was computed according to weighted UniFrac analysis. Differences of abundance between control and exposure groups were then studied in detail on the taxonomic levels of phylum, family and genus. The results and conclusions of these analyses are not included in this deliverable report in view of its "PUBLIC" dissemination level.

2.5 Data from a 28 oral exposure study in rats (BASF)

Seven different nanoparticles were administered by gavage over a period of 4 weeks to groups of 5 male and 5 female Wistar rats. The test materials were:

1. SiO_2·PEG: Levasil® 200 with covalent surface functionalization with a low-molar-mass silane having a PEG end group with a molecular weight of 500 g/mol (PEG-500), imparting some steric stabilization
2. SiO_2·phosphate: Levasil® 200 with covalent surface functionalization with a low-molar-mass silane having a negatively charged phosphonate end group on a flexible,short C3-linker
3. SiO_2·amino: Levasil® 200 with covalent surface functionalization with a low-molar-mass silane having a positively charged amino end group on the same C3-linker
4. ZrO_2 acrylate: ZrO_2 with acrylate surface modification having a strongly negative electrical charge and imparting steric stabilization
5. ZrO_2·TODS: ZrO_2 with trioxadecanoic acid (TODS) surface modification having a slightly positive electrical charge
6. $BaSO_4$: reference material NM-220
7. SiO_2·naked: SiO_2 without surface modification (Levasil ® 200)

The test substances were tested in two sub-studies A and B. The sub-study A comprised the first four substances and a concurrent control group, whereas the sub-study B includes substances 6 and 7, as well as a concurrent control group.

The four amorphous SiO_2 and the two ZrO_2 were delivered as suspension, whereas BaSO4 was delivered as powder. To achieve uniform substance preparations, all substances were suspended in phosphate buffered saline (PBS) containing 1 g/L bovine serum albumin. The substance preparations contained 10 % test substances by weight. The rats were administered daily by oral gavage (1000 mg/kg body weight). The control animals received equivalent amount of PBS containing bovine serum albumin.

Food consumption, water consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Before the start and end of the administration period, the administration period feces of animals of all test groups were

sampled.

Clinicochemical and hematological examinations as well as urinalyses were performed towards the end of the administration period. After the administration period all rats were sacrificed and assessed by gross pathology, followed by histopathological examinations. The following organs and tissues were examined by histopathology:

1. All gross lesions
2. Cecum
3. Colon
4. Duodenum
5. Heart
6. Ileum
7. Jejunum
8. Kidney, left
9. Liver
10. Mesenteric lymph node (one half)
11. Mesenteric lymph node (second half)
12. Peyer's patches
13. Rectum
14. Spleen
15. Stomach (forestomach and glandular stomach)

Metabolome analysis with MetaMap®Tox methodology. As described by van Ravenzwaay et al. (2007)[10] and Kamp et al. (2012)[11], EDTA-K3 blood samples of all rats taken on study day 28 were analyzed in regard to their metabolite profiles upon metabolite extraction by a proprietary method: GC–MS and LC–MS/MS were applied for broad profiling and hormone measurement. The method resulted in 225 semi-quantitative analytes, 171 of which are chemically identified and 54 are structurally unknown. Analysis of the recorded metabolite profiles was performed making use of the MetaMap®Tox database (van Ravenzwaay et al.2012)[12].

Results. There were no clinical signs of toxicity. Bodyweight development, food and water consumption were not impaired. Parameters of clinical chemical and hematological parameters in blood were either comparable with the concurrent control

[10] van Ravenzwaay B, Cunha GC-P, Leibold E, Looser R, Mellert W, Prokoudine A, Walk T, Wiemer J (2007) The use of metabolomics for the discovery of new biomarkers of effect. Toxicology Lett 172:21–28

[11] Kamp H, Strauss V, Wiemer J, Leibold E, Walk T, Mellert W, Looser R, Prokoudine A, Fabian E, Krennrich G, Herold M, van Ravenzwaay B. Reproducibility and robustness of metabolome analysis in rat plasma of 28-day repeated dose toxicity studies.Toxicol Lett 215:143–149

[12] van Ravenzwaay B, Herold M, Kamp H, Kapp MD, Fabian E, Looser R, Krennrich G, Mellert W, Prokoudine A, Strauss V, Walk T, Wiemer J (2012) Metabolomics: a tool for early detection of toxicological effects and an opportunity for biology based grouping of chemicals-from QSAR to QBAR. Mutat Res 746:144–150

groups, or within historical controls. Examination of urine did not show any treatment-related changes.
Sub-study A (Table 3 and 4): All findings noted were either single observations or they were equally distributed between control and treatment groups. All findings were considered to be incidental or spontaneous in origin and without any relation to the test substance treatment. Sub-study B (Table 5): In the female animals of the BaSO4 test group, minimal to slight inflammatory cell infiltrates in the submucosa of the glandular stomach were observed. All other findings were either single observations, or they were equally distributed between the control and treatment groups. Therefore, all observations were considered to be incidental or spontaneous in origin and unrelated to the treatment.

At a dose level of 1,000 mg/kg, none of the tested NMs had a biologically relevant impact on the plasma metabolome pattern of rats. When compared against the control animals on a significance level of $p < 0.05$, in both male and female animals, the number of significantly changed endogenous metabolites, was below, or at the false positive rate for all particles tested and were assessed as “statistical variance” of the metabolome analysis (Table 6). Using the pattern ranking, i.e., matching the metabolome of the compounds with pre-defined patterns of metabolite changes, which are associated with adverse effects, there were no common, consistent matches with any pattern for any of the investigated substances (data not shown).

Table 3 *Histological findings in male animals of the sub-study A with four surface-modified amorphous SiO_2 and two surface-modified ZrO_2*

	control	SiO_2 PEG	SiO_2 phosphate	SiO_2 amino	ZrO_2 acrylate	ZrO_2 TODS
	n =5	n =5	n =5	n =5	n =5	n =5
Glandular stomach	n =5	n =5	n =5	n =5	n =5	n =5
Metaplasia, basal cells	0	0	0	1	0	0
Dilation of glands	1	1	0	0	1	0
Kidney	n =5	n =5	n =5	n =5	n =5	n =5
Infiltration lymphoid	0	0	0	0	3	0
Cast tubular	0	0	0	0	1	0
Cyst(s)	0	0	0	0	1	0
Liver	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Necrosis, (multi)focal	0	0	0	0	1	0
Peri-/ vasculitis	0	1	0	0	1	1
Fatty change, (multi)focal	0	1	0	0	0	0
Infiltration, lymphoid	5	5	5	5	5	5
Cecum	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Parasites in lumen	0	1	1	0	0	0
Prostate	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Infiltration, lymphoid	1	0	0	0	1	0
Seminal vesicle	n = 0	n = 0	n = 0	n = 0	n = 2	n = 0
	-	-	-	-	2	-

Table 4 *Histological findings in female animals of the sub-study A with four surface-modified amorphous SiO_2 and two surface-modified ZrO_2*

		control	SiO_2 PEG	SiO_2 phosphate	SiO_2 amino	ZrO_2 acrylate	ZrO_2 TODS
		n =5	n =5	n =5	n =5	n =5	n =5
Glandular stomach		n =5	n =5	n =5	n =5	n =5	n =5
	Dilation of glands	2	0	0	1	0	0
Kidney		n =5	n =5	n =5	n =5	n =5	n =5
	Infiltration, lymphoid	0	0	0	0	3	0
	Mineralization medulla	5	4	5	4	2	2
	Scar(s), cortical	0	0	0	0	1	0
Liver		n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
	Peri-/ vasculitis	0	1	0	0	1	1
	Infiltration, lymphoid	5	5	5	5	5	5

Table 5 *Histological findings in male and female animals of the sub-study B with $BaSO_4$ and amorphous SiO_2*

	male			female		
	control	**$BaSO_4$**	**SiO_2 naked**	**control**	**$BaSO_4$**	**SiO_2 naked**
	n =5	n =5	n =5	n =5	n =5	n =5
Glandular stomach	n =5	n =5	n =5	n =5	n =5	n =5
Inflammatory infiltration	3	3	2	0	4	0
Edema	0	0	0	0	1	0
Dilation of glands	0	0	0	2	0	0
Infiltration, lymphoid	2	0	0	0	1	0
Kidney	n =5	n =5	n =5	n =5	n =5	n =5
Tubles basophilic	0	1	1	3	5	3
Cyst(s), cortical	0	0	1	1	0	1
Dilation, tubular	1	1	0	1	0	0
Dilation, renal pelvis	0	1	0	0	0	0
Mineralization medulla (m)f	0	0	0	4	5	2
Liver	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Necrosis, (multi)focal	1	0	0	0	0	0
Peri-/ vasculitis	1	0	1	0	0	0
Infiltration, lymphoid	5	5	5	5	5	5

Table 6 *Numbers of significantly changed endogenous metabolites in rat plasma ($p < 0.05$)*

Test substance (1000 mg/kg bw/day)	Sex	Numbers of up-regulated endogenous metabolites	Numbers of down-regulated endogenous metabolites
SiO_2 PEG	Male	7	1
	female	4	7
SiO_2 phosphate	Male	7	0
	female	4	1
SiO_2 amino	Male	4	1
	female	11	5
ZrO_2 acrylate	Male	3	0
	female	3	1
ZrO_2 TODS	Male	7	1
	female	2	2
SiO_2 naked	Male	2	11
	female	3	20
$BaSO_4$	Male	4	1
	female	1	4

2.6 New 90-day oral exposure study in rats according to OECD TG408 (KRISS)

The study was conducted to investigate the potential toxicity profile of the test item, food-grade TiO_2 (E171), in Sprague-Dawley rats after a 13-week of oral gavage at dose 0, 10, 100 and 1000 mg/kg once a day and to assess the reversibility of any effects during a 4-week recovery period. 10 animals/sex/group, were assigned to main groups, and 5 animals/sex/group were assigned to vehicle control and 1000 mg/kg recovery groups, respectively. Mortality and clinical observations, body weight and food consumption measurement, ophthalmology, clinical pathology (hematology, clinical chemistry and urinalysis), organ weights measurement, macro- and microscopic findings were performed and evaluated. The data analysis for this study (including LOAEL/NOAEL evaluation) is complete, and a study report provided to the PATROLS consortium. The results and conclusions of the study are not included in this deliverable report, because of its “PUBLIC” dissemination level. Selected tissues from the study have been made available for omics analysis in Task 2.5 and outcomes will be used to support computational modelling activities of WP6.

3. Deviations from the Workplan

Key aspects that were described in the original DoW but not conducted are listed below with justification:

1. BASF has provided data from oral studies in rats, in the workplan by mistake "mice" instead of "rats" was written.
2. No 3D darkfield analysis of tissues was performed, this method was originally offered by Health Canada. NRCWE performed 2D darkfield analysis
3. For PATROLS, KRISS performed a 90-days study according to OECD TG 408 with TiO_2 instead of Ag [This change has been reported in the interim report with appropriate justification and approved by commission].

In addition, regarding specific subtasks that can be defined from the original DoW, the level of achievements is summarised in Table 7.

Table 7 *Overview of achievements in relation to initial DoW*

Workplan	Achieved
Subtasks 1 and 2: Histopathology and darkfield imaging of ENM distribution in the GI tract with focus on Peyer's patches, liver, spleen.	Histology provided by BASF for 28 days studies in rats and by IUF for 13 weeks study with SiO_2 and CeO_2 including intestine, liver and spleen. 2D darkfield: Small and large intestine of CeO_2 (NM212) exposed mice were screened by 2D darkfield for the presence particles. Further imaging and chemical analysis are needed to verify CeO_2 in Peyer's patches and adventitia or mucus of small intestine, and if the material interacts with other cell types than lymphocytes.
Subtask 2 and 3: Studies on oral toxicity; focus on local effects in intestine, and on possible liver toxicity to align with WP4.	Alignment with WP4 was achieved by side-by-side comparison (IUF) of effects of 4 types of ENM (Ag-PVP, TiO_2, SiO_2, CeO_2) on the induction of oxidative stress, inflammation and mucin gene expression in mouse intestines and in triple GIT model (WP4).
Subtask 4: Effects on GI tract microbiome considered	Actually performed from available stool specimens for 4 ENM (IUF) and data fed into WP4 and WP5 (T5.3/3.5/4.5 linkage)
Subtask 5: Data from 28-day oral studies of seven ENM in rats; including full histopathology and metabolome analysis	Done by BASF.
Subtask 6: New 90-day oral exposure study in rats according to OECD TG408 (study done by KRISS with TiO2).	According to the original DoW selected tissues from this new study would be provided to Task 2.5 for transcriptomics-analyses to identify potential novel ENM-exposure/effect markers for WP4. However, as histopathology evaluation merely indicated the absence of effects, so far only liver tissues from the study have been sent for selected marker analysis to align with WP4 (HWU), and input to WP6 (modelling) is deemed less relevant. In addition, KRISS has planned a new 90-day oral exposure study with CeO_2, as advised by WP6 and steering board.

4. Performance of the partners

All partners involved in this task provided significant contributions. Active discussions during telco's and face-to-face meetings as well as the exchange of protocols, methods and tissue samples supported the task achievements. Experiments performed in this specific task have already resulted in several (poster) presentations at scientific conferences (Listed on the PATROLS website, project library: https://www.patrols-h2020.eu/news-events/project-library/) and we anticipate further dissemination in the form of peer reviewed publications.

5. Conclusions

The Steering Board deems this deliverable to be satisfactorily fulfiled.