Deliverable Report for Grant Agreement Number 760813

Deliverable 2.2

Extended evaluation of repeated-exposure inhalation and intra-tracheal instillation toxicity studies

Due date of deliverable: 31/10/2019
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Lead beneficiary for this deliverable: BASF

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

TABLE OF CONTENTS

1. Description of task

Task 2.2 Extended evaluation of repeated-exposure inhalation toxicity studies; (BASF, NRCWE, RIVM, DTU); M4-22.

As indicated in Task 2.1, PATROLS partners have previously undertaken several (sub)chronic inhalation studies with a variety of ENM as listed in Table 1 (BASF, NRCWE, RIVM, JBRC, LTAP). Tissues from these studies have been stored and are available for further analysis. PATROLS will not conduct further in vivo inhalation studies[1], but instead will utilize the existing data (Task 2.1) and will generate new data on these stored tissues, to maximize use of existing materials. Available histological sections and paraffin embedded/frozen tissues from repeated dose studies identified in T2.1 as well as those with shorter durations performed by PATROLS partners [A,B,C] will be collected and redistributed to appropriate partners for analysis of additional endpoints. In addition, for ENMs with insufficient toxicity data, range finding studies will be performed to identify the exposure level for which no or minimal adverse effects occur. This information is needed for Task 2.4 for which acute exposures should not result in clear toxicity to assess the biodistribution/kinetics. Toxicity will be based on histopathology, cell differentials and LDH and protein levels in BALF after a single exposure using multiple dose levels. Analyses on the collected tissues from previous studies will include visualization of ENM interaction with biological tissues and cells in lung, liver and spleen by 2D (NRCWE) and 3D (HC) dark field microscopy on paraffin embedded tissue and determination of elemental concentrations by inductively coupled plasma mass spectrometry (ICP-MS) in selected frozen or paraffin-embedded samples (150-200 in total in WP2), respectively (NRCWE, DTU). Available frozen tissues will be analyzed for DNA strand break levels (lung and liver tissues) to relate with the WP3 in vitro lung model based genotoxicity assessments (RIVM). Tissue will also be distributed to partners in Task 2.5 (HC+LTAP) for toxicogenomics and other analysis to support identification of mechanistic key events and refinement of AOPs. The results generated in this task will feed into WP3 to enable further hazard endpoint comparison with the in vitro studies to evaluate the performance of the advanced lung models generated. Additionally, the data from this task will be essential to support the IVIVE modelling activities in WP6.

[1] This refers to the fact that no new 90 day studies in this task will be conducted. However, a pilot study for the biokinetic study will be performed in Task 2.4.

A Landsiedel et al. (2014) Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Part Fibre Toxicol, 11, 16.

B Gosens et al. (2016) Organ burden and pulmonary toxicity of nano-sized copper (II) oxide particles after short-term inhalation exposure. Nanotoxicology, 10, 1084-95.

C Geiser et al. (2010) Deposition and biokinetics of inhaled nanoparticles. Part Fibre Toxicol, 7, 2.

2. Description of work & main achievements

2.1. PATROLS internally available tissue samples for further examinations

As stated in the task description, task 2.2 was not supposed to perform new inhalation studies, (unless new data are really required) and rather to collect preserved tissues and redistribute those tissues to the project partners for generation of new data.

BASF was responsible for coordinating the collection and distribution of available histological sections and paraffin embedded/frozen tissues among WP2 partners. In Table 1 of this deliverable, a list of PATROLS internally available tissues from inhalation studies available for further examinations, are summarised. In addition to the inhalation studies, the table also consisted of several intratracheal instillation studies with tier 1 engineered nanomaterials (ENMs) that were performed at NRCWE. As all the inhalation studies were performed a couple of years ago, the sample quality following long-term storage and availability may be not appropriate for some of the endpoints (e.g. genotoxicity) that were mentioned in the task description. This risk that only limited tissues were available from existing *in vivo* inhalation studies, were pointed out by NRCWE in the grant agreement. Therefore, available samples from more recent instillation studies were included in the Table 1.

Table 1 Available tissues from inhalation or intra-tracheal instillation studies performed by PATROLS Partners for further analysis of additional endpoints

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Lung	Lung-ass. lymph nodes	Liver	Spleen	Kidney &heart
ZnO	ZnO NM-111	inhalation, 5days (STIS)	2, 21	BASF	x	(x)	In block	Preserved tissue	
	NM-111, NM-110	single IT with 3 post-exposure follow-up times and 3 doses	1, 3, 28	NRCWE	√	√	√+Frozen tissue	√	√
	NRCWE-031 ZnO, CAS-No:1314-13-02	single IT with 3 post-exposure follow-up times and 3 doses	1, 3, 28	NRCWE	√	√	√+Frozen tissue	√	√
$BaSO_4$	$BaSO_4$ (NM-220)	inhalation, 5days (STIS)	2, 21	BASF	Preserved tissue	Preserved tissue	Preserved tissue	Preserved tissue	
	$BaSO_4$ (NM-220)	inhalation, 28 days	1, 28,	BASF	X OCT block Deep frozen sample	(x)	X	x	
CeO_2	CeO_2 nano	inhalation, 5days (STIS)	2, 21	BASF	x	(x)	In block	Preserved tissue	

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Lung	Lung-ass. lymph nodes	Liver	Spleen	Kidney &heart
	CeO_2 NM-212, NM-211	inhalation, 5days (rat and mice)	2, 21	BASF	x	(x)	In block	Preserved tissue	
	CeO_2 NM-212	Inhalation, 28days (rat)	1, 28	BASF	X OCT block Deep frozen sample	(X)	In Block	In block	
	CeO_2 NM-212	inhalation 12 month and longer	1	German Government	(x)	(x)	x	x	
	Degussa/Quimidroga	single IT with 3 post-exposure follow-up times and one dose of 162µg	1, 28, 180	NRCWE	√	√	√+Frozen tissue	√	√
TiO_2	TiO_2 T-lite SF™ NM-105	inhalation, 5days (STIS)	2, 21	BASF	x	(x)	In block	Preserved tissue	
	NRCWE-001, -002	single IT with 3 post-exposure follow-up times and 3 doses	1, 3, 28	NRCWE	√	√	√+Frozen tissue	√	√
	UVTitan L181,	single IT with	1, 28, 90	NRCWE	√	√	√+Frozen	√	√

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Lung	Lung-ass. lymph nodes	Liver	Spleen	Kidney &heart
	Boesens Fabrikker	3 post-exposure follow-up times and 3 doses					tissue		
	NRCWE-025 NaBond, 030 NanoAmor,	single IT with 3 post-exposure follow-up times and 3 doses	1, 3, 28	NRCWE	√	√	√+Frozen tissue	√	√
	Rutile, NanoAmor	single IT with 3 post-exposure follow-up times and one dose of 162µg	1, 28, 180	NRCWE	√	√	√+Frozen tissue	√	√
	Anatase, custom synthesis: Spherical 15 and 100 nm, Tube D10 nm L200 nm, Cube 20x20nm,	single IT with 4 post-exposure follow-up times and 3 doses	1, 3, 28, 90	NRCWE	√	√	√+Frozen tissue	√	√
SiO_2	SiO_2 amorphous SAS	inhalation, 5days (STIS)	2, 21	BASF	x	(x)	In block	Preserved tissue	
	100nm and 300nm, methylated, porous	single IT with 2 post-	1, 28	NRCWE	√	√	√+Frozen tissue	√	√

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Lung	Lung-ass. lymph nodes	Liver	Spleen	Kidney &heart
	and non-porous, with and without CuO-doping	exposure follow-up times and 4 doses							
	DQ12	single IT with 2 post-exposure follow-up times and 2 doses	1, 28	NRCWE	√	√	√+Frozen tissue	√	√
	DQ12	single IT with 4 post-exposure follow-up times and 3 doses	1, 3, 28, 90	NRCWE	√	√	√+Frozen tissue	√	√
MWCNT	Mitsui-7	single IT with one dose of 54 µg	1 year	NRCWE	√	√	√+Frozen tissue	√	√
	Mitsui-7	single IT with 3 post-exposure follow-up times and 3 doses	1, 28, 90	NRCWE	√	√	√+Frozen tissue	√	√
	Mitsui-7	single IT with 3 post-	1, 3, 28	NRCWE	√	√	√+Frozen tissue	√	√

Substance	ENM ID	Exposure (type, duration)	Post-exposure follow up (days)	Owner	Lung	Lung-ass. lymph nodes	Liver	Spleen	Kidney &heart
		exposure follow-up times and 4 doses							
	Mitsui-7	single IT with 4 post-exposure follow-up times and 3 doses	1, 3, 28, 90	NRCWE	√	√	√+Frozen tissue	√	√
	NM-402	single IT with 3 post-exposure follow-up times and 3 doses	1, 28, 90	NRCWE	√	√	√+Frozen tissue	√	√
	NM-402	single IT with one dose of 54 µg	1 year	NRCWE	√	√	√+Frozen 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.
IT = intratracheal instillation.
√ = 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 is available for most studies and will be shared with Shareen Doak for mutation assay. Frozen lung tissue has been fully spent for most studies.

2.2. Exchange of tissue samples

The compiled list (Table 1) of available organs and tissues was distributed to all partners of Tasks 2.2, 2.3 and 2.4. Since the list was circulated for the first time in January 2019, NRCWE announced they would analyse particle distribution in lung and liver slices from the 2-year inhalation study with CeO_2 (German government). However this long-term study is on-going, and it is uncertain whether the samples will be available later. NRCWE further requested samples from a 90day (Schwotzer et al. 2017 [1]) inhalation study with nano CeO_2 (NM 212) and $BaSO_4$ identified in task 2.1. This however, was not a study performed by a PATROLS partner; it was conducted by Fraunhofer ITEM. In this study, liver histopathology was not analysed. Several emails were sent to Fraunhofer ITEM to confirm the sample availability. Fraunhofer institute could not confirm if they were able to share the samples because the study was founded by the German Ministry for Education and Research.

While the decision was pending, and the sample availability was uncertain, NRCWE asked for samples from 28-day studies with CeO_2 (NM 212). There were two 28-day inhalation studies with CeO_2 (NM 212), one is from RIVM, the other from BASF. As the RIVM study was older than the BASF study, the samples from the BASF study were preferred for the dark field microscopy.

However, the BASF study is a GLP study. As such, the existing H&E stained slices need to stay at the test facility. BASF has been preparing new samples from the paraffin blocked lung and liver tissues of the 28-day study. The samples comprised the control and high concentration (25 mg/m³ CeO_2) animals, 1 day after termination of the exposure and after a recovery period of 28 days. Histotechnical preparation needs time, the darkfield analysis and results will therefore be reported later on as an update of this Deliverable 2.2 by October 2020, month 34 of PATROLS.

At the beginning of October 2019, the German Ministry for Education and Research agreed to provide liver samples from the Schwotzer et al. 2017[D] study. The livers were preserved in formalin and need to be embedded in paraffin and further processed before they can be distributed. Details of shipment of these samples from Fraunhofer ITEM, Germany to NRCWE are currently under discussion. Moreover, NRCWE need to find a partner for histotechnical preparation and assign budget for the sample preparation, as this was not foreseen in the project. Considering the late commitment of Fraunhofer ITEM and the time for shipment and sample preparation, it was impossible to have any results (liver histopathology and dark field microscopy) within the time frame of Task 2.2. Thus, the examination will be performed by NRCWE and results will be reported as an update of Deliverable 2.2 by October 2020.

KRISS has requested tissue samples of lung and liver from NRCWE studies with intratracheal instillation of MWCNT, TiO_2 and CeO_2 for CARS (Coherent Anti-Stock Raman Scattering) imaging and TOF-SIMS (Time Of Flight – Secondary Ion Mass Spectroscopy). This would greatly improve the resolution of particle tissue distribution compared to darkfield imaging and offer chemical verification of the nanomaterial. Sample preparation for KRISS (lung and liver) is ongoing at NRCWE where so far samples from 18 animals have been identified as relevant. The relevant samples come from toxicological mouse studies with single intratracheal instillation of MWCNT, TiO_2 and CeO_2, where particle tissue distribution has already been imaged with darkfield -

D Schwotzer D, Ernst H, Schaudien D, Kock H, Pohlmann G, Dasenbrock, C, Creutzenberg O. (2017) Effects from a 90-day inhalation toxicity study with cerium oxide and barium sulfate nanoparticles in rats. Part Fibre Toxicol 14:23.

1 day, 6 months or 1 year post-exposure. When the complete list of relevant samples has been identified the paraffin blocks of lungs and livers will be shipped to an external collaborator for sectioning before distribution to KRISS. The results will be included in an update of Deliverable 2.2 by October 2020.

2.3. Spleen morphology 180 days after a single intratracheal exposure of mice to CeO_2 nanoparticles (NRCWE)

Introduction

A small fraction of pulmonary deposited nanoparticles (NPs) undergo translocation and primarily accumulates in the liver but also in other secondary organs. In deliverable 2.1, *in vivo* biodistribution studies with several of the selected PATROLS nanomaterials (i.e. CeO_2) reported spleen as an organ that receives an ENM burden after (sub)chronic inhalation or oral exposure. Furthermore, analysis of human biopsy samples from livers and spleens revealed presence of TiO_2 particles in these organs, and the calculated mass concentration (mg/kg organ) of particles in the spleen was higher than in the liver (Heringa et al. 2018 [E]). This indicates that besides liver, spleen is also an important organ for accumulation of nanoparticles in the body. Although we are not developing an *in vitro* model for spleen in PATROLS, it is relevant to identify whether ENMs can be expected to have an effect on spleen.

We have previously demonstrated translocation of CeO_2 NP to the liver following intratracheal instillation (IT) of mice by ICP-MS and enhanced darkfield microscopy (the study is listed in Table 1 and published in Modrzynska et al. 2018 [F]). The study indicated no substantial effects on liver morphology 180 days after IT exposure of mice to CeO_2 NP (unpublished), however spleen was not analysed. The aim of the present investigation was to examine whether IT exposure of mice to CeO_2 NP can affect spleen morphology considering that the spleen is a relevant organ for particle accumulation.

Methods

Spleen samples were collected from C57BL/6 (B6JBOM-F) female mice (N=9/group) after 180 days after IT of a vehicle (nanopure water) or CeO_2 NP (162 µg/animal). The preparation and characterisation of CeO_2 NP was done as described in Modrzynska et al. 2018 [3,G]. Spleen specimens were fixed in 4% neutral buffered formaldehyde solution, embedded in paraffin, sectioned in 4-6 µm slices and stained with haematoxylin and eosin (H&E staining) for histological examination in order to evaluate morphological changes by light microscopy.

Histological examination aimed to detect microscopic changes and was performed by one examiner twice. The first reading of slides was performed with knowledge of treatment groups (so called “open reading” when the control group is compared with

[E] Heringa, M. B., Peters, R. J. B., Bleys, R. L. A. W., van der Lee, M. K., Tromp, P. C., van Kesteren, P. C. E., Bouwmeester, H. (2018). Detection of titanium particles in human liver and spleen and possible health implications. Particle and Fibre Toxicology, 15(1).

[F] Modrzynska J, Berthing T, Ravn-Haren G, Kling K, Mortensen A, Rasmussen RR, et al. (2018). *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 13(8): e0202477.

[G] Modrzynska J, Berthing T, Ravn-Haren G, Jacobsen NR, Weydahl IK, Loeschner K, Mortensen A, Saber AT, Vogel U. (2018). Primary genotoxicity in the liver following pulmonary exposure to carbon black nanoparticles in mice. Part Fibre Toxicol 15:2.

the treatment group exposed to CeO_2 NP). This is done because the examiner needs to know which animals have been treated so that the background information can be sifted out from true treatment effects. The second reading was blind, which means without knowledge of which samples were from the control or the treatment group. All changes were recorded and findings were reported as "present". While reading the slides attention was paid to the presence or absence of microscopical changes described by Suttie (2006)[H] and using a modified "check list" for potential histologic changes in this organ proposed by Elmore (2006)[I].

Results

The observations collected during histological examination are summarized in Table 2. Both in the vehicle controls and CeO_2 NP exposed mice the distinction between the red and white pulp was clear (Figure 1 a and b). In the red pulp (RP) matured erythrocytes were prominent. Other cells observed in the RP were lymphocytes but also granulocytes and megakaryocytes. Macrophages containing pigment and/or apoptotic debris were also present (Figure 2). In the white pulp there was no difference between the control and CeO_2 NP exposed mice regarding the total area evaluated on a cross section, in the size of periarteriolar lymphoid sheaths (PALS) or of marginal zone and of lymphoid follicles. There was no difference in observed apoptotic lymphocytes between the control and treated animals. An intragroup variation in presence of pigment/pigmented macrophages was noted in vehicle controls: in spleen of three animals, there was less pigment than in 6 other spleens. The small amount of intracytoplasmic pigment within the RP macrophages is a common background finding in rodents. It is typically found in the RP but is also seen in the marginal zone and/or lymphatic sheaths on the white pulp (WP) (Figure 3, a and b), and also in the capsule or trabeculae. The pigment can be haemosiderin (from macrophage engulfment of damaged or aged erythrocytes), ceroid/lipofuscin, melanin or test related articles. At present, no attempt was made to examine whether the pigment in the treated group could contain also CeO_2 NP translocated from lungs. In addition, several megakaryocytes were observed in one of the vehicle controls as compared to the other vehicle controls and to the exposed mice.

[H] Suttie AW. (2006) Histopathology of the spleen. Toxicol Pathol 34(5): 466-503.

[I] Elmor SA (2007) Enhanced histopathology of the spleen. Toxicol pathol 34(5): 648-655.

Table 2: Overview of typical histological changes in the spleen and their presence or absence 180 days post-exposure of mice either to a vehicle (nanopure water) or to CeO_2 NP.

Type of lesion	Vehicle control (N=9)		CeO_2 NP (N=9)	
	Present	Not present	Present	Not present
White pulp Increased/decreased size	0	9/9	0	9/9
White pulp follicles Increased/decreased germinal centre	0	9/9	1 (2)	8/9
Red pulp Increased/decreased size	0	9/9	0	9/9
Increased number of megakaryocytes in parenchyma	1 (1)	8/9	0	9/9
Decreased number of pigmented macrophages	3 (1)	6/9	0	9/9
Apoptotic lymphocytes	1 (1)	8/9	1(1)	8/9
Fibrosis	0	9/9	0	9/9
Necrosis	0	9/9	0	9/9

Grading system when applied: (0) – normal; (1) – minimal, (2) – mild, (3) – moderate, (4) – marked.

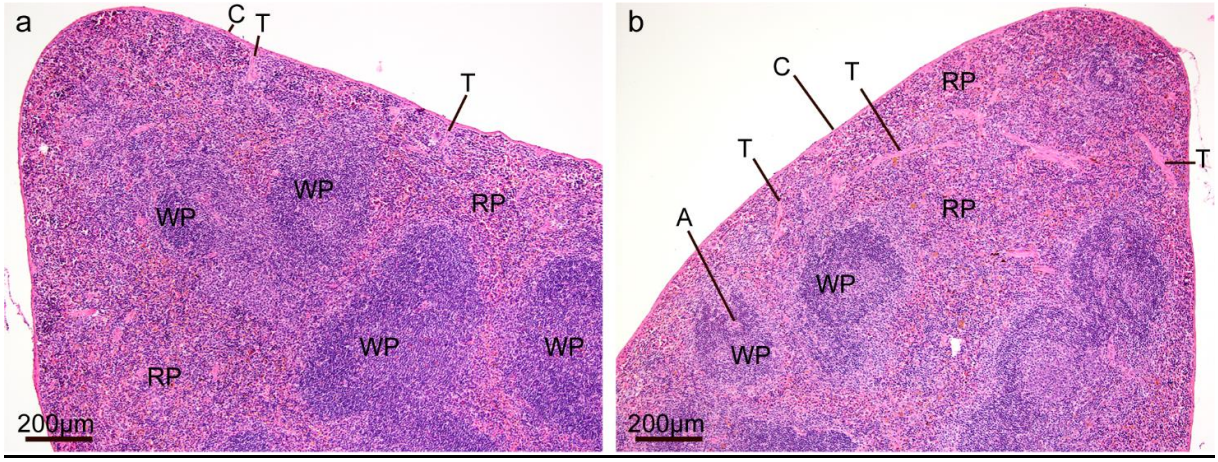

Figure 1: Normal structure of spleen 180 days post-exposure to a vehicle (nanopure water) [1a] or to CeO_2 NP [1b]. C: a fibro-elastic outer capsule; T: trabeculae (a supportive connective tissue sheath); WP: white pulp; RP: red pulp. H&E staining.

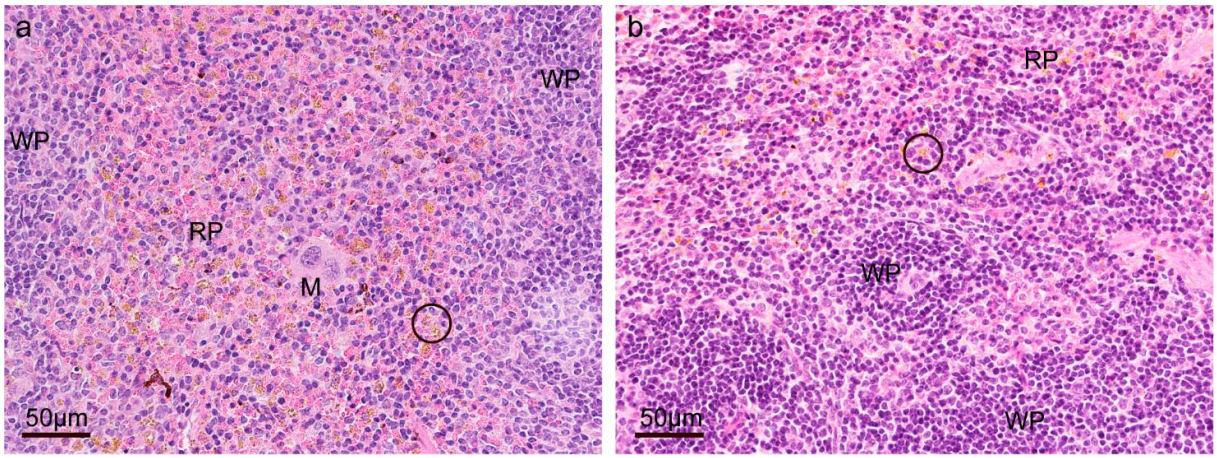

Fig 2: Pigmented macrophages (orange) in red pulp (RP) of spleen 180 days post-exposure to a vehicle (nanopure water) [3a] or to CeO_2 NP [3b]. A few examples of pigmented macrophages are marked with circles. M: megakaryocyte. H&E staining.

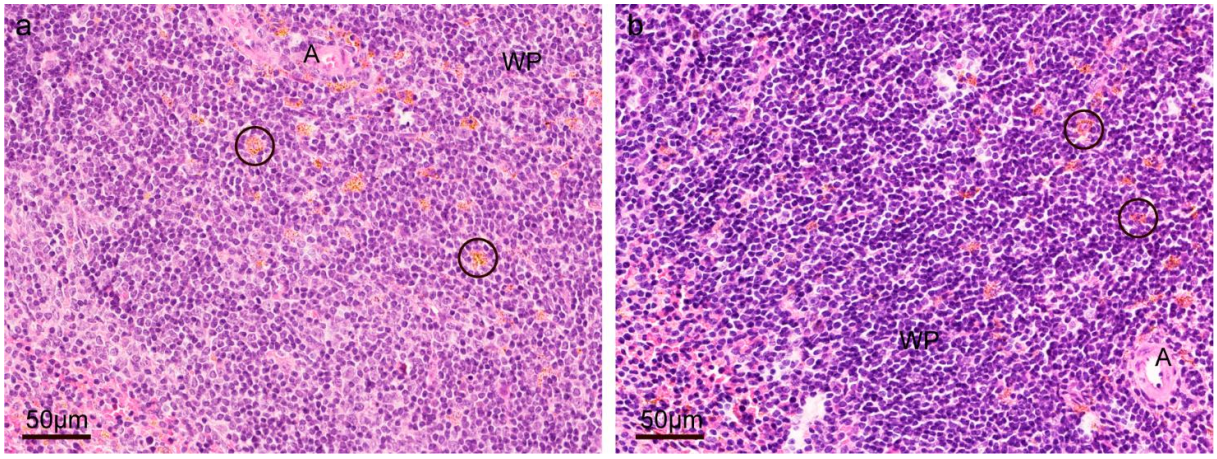

Fig 3: Pigmented macrophages (orange) in white pulp (WP) of spleen 180 days post-exposure to a vehicle (nanopure water) [2a] or to CeO_2 NP [2b]. A few examples of pigmented macrophages are marked with circles; A: arteriole. H&E staining.

Conclusion
No treatment related histological changes were recorded in spleens from mice treated with a single dose of 162 µg CeO_2 NP/animal and terminated 180 days post exposure.

3. Deviations from the Workplan

The compiled list (Table 1) of available organs and tissues was distributed to all partners of Tasks 2.2, 2.3 and 2.4. Since the list was circulated for the first time in January 2019, NRCWE announced they would analyse lung and liver slices from the 28-day and 90 day inhalation studies with nano CeO_2 (NM 212) using dark field microscopy and histopathology analysis, which has not has been done before. KRISS announced they would perform CARS/Dark field/TOF-SIMS in samples from the instillation studies with MWCNT, TiO_2 and CeO_2 performed by NRCWE.

As stated in the previous section, the H&E stained slices of the 28-day study with CeO_2 (NM 212) are being prepared currently at BASF. The samples of the 90-day Inhalation study with CeO_2 (NM 212) will be available soon. Once they are sent to NRCWE, they

are going to be processed histotechnically and are going to be examined to ensure they are of appropriate quality for additional analysis. The first reason for the delay was that agreement was required from the owner of the 90-day study to share the sample with PATROLS. The second reason was that all samples must be histotechnically prepared as in new studies, to overcome the restrictions associated with samples generated under GLP.

Due to the unforeseen obstacles in obtaining samples from existing inhalation studies, NRCWE has examined available spleen samples from one of their own instillation studies and reported the analysis outcomes within this deliverable.

Due to high workload, NRCWE did not manage to send KRISS the samples on time. Therefore, KRISS could not analyse them on time. This work will be performed and reported in an **update of deliverable 2.2 in October 2020**.

In the description of work, it was indicated that DTU would undertake ICP-MS analysis on selected frozen or paraffin-embedded samples. However, the quality of the identified samples, which have been stored for long periods of time is of concern. DTU has therefore decided to perform ICP-MS examinations on fresh tissues obtained from the new studies performed in WP2, Task 2.4 instead. As the DTU budget for ICP-MS would not allow more than 150 to 200 samples, they have decided it is more valuable to give fresh samples higher priority. The results will be reported in Task 2.4, as part of Deliverable 2.4.

The preserved samples identified and located through the Task 2.2 review were not appropriate for genotoxicity or multi-omic examinations. Thus, no samples were requested by the project partners.

As the planned examinations will be reported in an update of Deliverable 2.2, the delay does not have an effect on the overall results of the project.

4. Performance of the partners

There is some delay of planned examinations within Task 2.2. The time for logistics was under estimated during the planning phase. All partners are committed to performing all examinations as soon as samples were prepared and will report them in the updated deliverable 2.2.

5. Conclusions

The Steering Board deems this deliverable to be fulfilled satisfactory