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Immunotoxic and genotoxic effects after short-term inhalation of fibrous nanomaterials

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1 Description of task

Fibrous nanomaterials, including nanocellulose and carbon nanotubes will be evaluated for acute pulmonary immunotoxic and genotoxic effects *in vivo* after short-term inhalation (1-5) days. The immunotoxicological assays include the determination of inflammatory cells and relevant cytokines and chemokines in bronchoalveolar lavage fluid. The genotoxicity assays include the determination of DNA damage by the comet assay in lung cells (pulmonary effect) and micronuclei in bone marrow erythrocytes (systemic effect).

2 Description of work & main achievements

2.1 Summary

The present study was carried out at the Finnish Institute of Occupational Health (FIOH). The study focused on assessing the genotoxic and immunotoxic effects of nanofibrillar cellulose (NFC) materials provided by Stora Enso and UPM-Kymmene, as part of NFC hazard identification. Also a sample of bulk-sized pulp and multiwalled carbon nanotubes (MWCNTs) were included in the study, to obtain comparative data on traditional cellulose and another type of fibrous material.

The term 'nanocellulose' generally refers to cellulosic materials having at least one dimension in the nanometre range. Nanocellulose is the smallest structural unit of a plant fibre and possesses unique properties compared with the bulk material. Nanocellulose is one of the most promising innovations in the forest industry.

All together four gel-form NFC samples (fibril diameter 2-15 nm; length several micrometres), one bulk-sized control material and MWCNTs were included in the study. The *in vivo* model was used to investigate responses 24 h and 28 d after a single administration to the lungs of C57BL/6 female mice. Since the NFCs were provided in gel-form, pharyngeal aspiration was chosen as the most suitable exposure method.

For genotoxicity assessment *in vivo*, the single cell gel electrophoresis (comet) assay was performed on lung cells and bronchoalveolar lavage (BAL) fluid cells and the micronucleus assay on bone marrow polychromatic erythrocytes (NFC doses 10, 40, 80 and 200 µg/mouse). The same mice were utilized to assess the *in vivo* inflammatory effects by investigating the number of inflammatory cells in BAL, mRNA expression levels of relevant cytokines, and histopathological changes in the lungs (NFC doses 10 and 40 µg/mouse). The results obtained with the NFC materials were compared to data on the negative control (phosphate buffered saline, PBS), the bulk-sized cellulose pulp and MWCNTs.

All NFC materials, except one, caused DNA damage in lung or BAL cells, as determined by the comet assay. For one NFC, the effect was dose-dependent in lung cells both 24 h and 28 days after the exposure. The comparative materials, bulk-sized pulp and MWCNTs, were also able to induce DNA damage after 24 h and 28 days. None of the NFCs was shown to possess systemic genotoxic properties as measured by the micronucleus assay in bone marrow.

All tested materials were able to trigger recruitment of neutrophils 24 h after the treatment. The effect was clearly stronger for two of the NFC materials. In addition, the same two NFC materials induced a slight influx of eosino-phils. 28 days after the exposure, no significant increases in the numbers of neutrophils or eosinophils was seen in BAL, indicating that the inflammation had resolved.

The mRNA expression analysis of cytokines supported the results of cytological assessment of the BAL. All materials tested were able to induce an increase in the expression of inflammation-related cytokines 24 h after the exposure. 28 d later, only a minor induction of mRNA expression was detected in response to the NFC treatment. In contrast, with MWCNTs the mRNA level of the studied cytokines still remained elevated 28 days after the exposure.

Histopathological assessment revealed that most of the NFC materials induced a moderate neutrophilia 24 h after the treatment. The neutrophilic response was accompanied by a low number of eosinophils. The bulk-sized pulp did not induce notable influx of inflammatory cells into lung tissue. No notable Periodic Acid-Schiff (PAS) positivity was observed with any of the celluloses, suggesting that the tested materials did not activate mucin-producing goblet cells and thereby caused no elevated mucus production in airways 24 h after the treatment. Although the celluloses appeared to be biopersistent in the lungs during the follow-up time of 28

days after the treatment, no considerable lung tissue reactivity and no pathological changes were observed in response to the celluloses. MWCNTs induced neutrophil and eosinophil recruitment as well as aggregates surrounding the material in the 24-h sampling, and granuloma formation and PAS positivity were observed 28 days later.

In conclusion, the results indicate that, although the tested NFC materials were able to induce DNA damage and inflammatory responses, the mice exposed to the NFCs showed signs of recovery of the inflammation at 28 d. On the contrary, the mice treated with MWCNTs exhibited characteristics of longer term adverse health effects. However, the observation that NFC was biopersistent in the lungs for at least 28 days raises some concern, because an increase in the level of primary DNA damage, although at a low level, was still observed at this time point. If DNA damage is continuously produced during a prolonged time, it might contribute to carcinogenesis. A longer follow-up would be required to better define the fate of the NFC material in the lungs and the duration of the increased level of DNA damage.

2.2 Background of the task

Cellulose is a naturally occurring fibrous material that is generally considered safe. Cellulose pulp is exempted from the REACH regulation because it is considered to cause minimum risk, due to its intrinsic properties (Regulation (EC) 1907/2006). Nanocellulose is one of the most promising innovations in the forest industry. The term nanocellulose generally refers to cellulosic materials having at least one dimension in the nanometre range. Nanocelluloses possess unique properties compared with the bulk material. Nanofibrillated cellulose (NFC), contains both the amorphous and crystalline cellulose domains. As NFC is a fibrous material with a high surface area to volume ratio, there is some concern that such a high-aspect-ratio nanomaterial (HARN) might act similarly to asbestos which is known to cause lung fibrosis and cancer (Donaldson et al. 2011). However, toxicity studies on nanocellulose, especially the fibrillate form, are still very scarce. Studies performed at the FIOH as part of the 7th Framework Programme project SUNPAP showed that NFC administered by pharyngeal aspiration caused an acute inflammatory response and DNA damage in the lungs 24 h after the treatment, although no systemic genotoxic effect were seen in the bone marrow (Catalán et al. 2016a). Therefore, it was considered important to investigate the possible toxicity of NFC materials at an early stage of product development, to enable their safety-by-design. In the present study, the inflammatory and genotoxic effects of NFC were examined in mice, together with bulk-sized cellulose pulp and multiwalled carbon nanotubes (MWCNTs), to learn more about the possible modes of action of NFC.

The task contributes to the hazard assessment of fibrous nanomaterials, by providing new information about the *in vivo* immunotoxicity and genotoxicity of a novel nanomaterial, NFC, in comparison with a bulk-sized cellulose and a well-studied fibrous nanomaterial, MWCNTs.

As inhalation is considered to be the most relevant route of exposure, the experiments focused on the pulmonary effects in mice *in vivo*. Pharyngeal aspiration was chosen as the most suitable method of exposure, since the NFC materials were provided in gel form. Two sampling times after a single pharyngeal aspiration were used, to gain insight into the relationship between acute (24 h) and longer term (28 d) toxicity of fibrous nanomaterials.

The results were compared with data available on other high aspect ratio nanomaterials (HARNs), to consider possible modes of action. Comparison with *in vitro* toxicity studies, performed with the same materials in WP5, was conducted.

FIOH collaborated with the national coordinator (TUKES), to link the scientific answers to the regulatory questions.

2.3 Description of the work carried out

The work performed at the FIOH focused on assessing the genotoxic and immunotoxic effects of the NFC materials. *In vivo* studies in mice were used to investigate both acute and subacute responses (24-h and 28-d follow up). For genotoxicity assessment *in vivo*, the comet assay was performed on lung cells and broncho-alveolar lavage (BAL) fluid cells and the micronucleus assay on bone marrow polychromatic erythrocytes, after single pharyngeal aspiration to female C57BL/6 mice (doses 10, 40, 80 and 200 µg/mouse). Inflammatory effects *in vivo* were assessed by investigating the influx of inflammatory cells in BAL, mRNA expression levels of relevant cytokines, and histopathological changes in lungs (doses 10 and 40 µg/mouse).

These tests, together with *in vitro* testing performed with the same material under WP5, provide indication, whether the NFC materials studied are able to cause cellular damage or systemic effects. Our study provides new information about the toxicity of NFC and thereby contributes to the hazard assessment of these materials.

2.3.1 Materials

Four NFC materials and one bulk-sized reference material, produced by Stora Enso Oyj and UPM-Kymmene Oyj, were accepted to the core selection of nanomaterials to be used by the NANoREG-project partners (**Table 1**). The samples were delivered in hydrogel form containing 0.79 – 4.30 % of cellulose in water. The NFC dimensions given in the table concern isolate nanofibers, but the NFC materials also contained thicker fibers and fiber bundles.

Table 1. NFC material properties.

Material	MNN Identification code	Supplier	Lot.	Concentration (%)	Length (µm)	Width (nm)	Zeta potential (mV)
NFC 1	NFC Medium Coarse	Stora Enso	130043	1.60	2-50	3-10	-32
NFC 2	NFC Fine	Stora Enso	130042	2.40	2-20	2-15	-15
NFC 3	UPM Biofibrils AS	UPM-Kymmene	10929	0.79	0,5-10	4-10	-25
NFC 4	UPM Biofibrils NS	UPM-Kymmene	1544	1.47	2-20	7-20	-2
Pulp 5	UPM Bleached Birch Pulp	UPM-Kymmene	1742H71	4.30	1 000	30 000	

All cellulose samples were derived from natural wood-based pulp and consisted of glucose, xylose and mannose, hemicellulose content being 17 – 19 %. The materials were produced in pilot scale factories by mechanical and chemical treatment of the bulk-sized pulp. Details of the manufacturing process are classified property of Stora Enso and UPM-Kymmene. Prior to the experiments, the materials were stored in a refrigerator at 4 – 10 °C protected from light. The same sample batches were used in all experiments.

The suppliers made efforts to ensure that the tested NFC materials were prepared as aseptically as possible, and some of the NFC samples tested had been autoclaved during their preparation. Before starting the toxicity studies, the NFC materials were tested for microbial contamination. To exclude the possible effects of contami-nation on the results, the materials were tested for endotoxin content by the Limulus Amebocyte Lysate (LAL) method (Endpoint chromogenic LAL, R160, Charles River Endosafe) as described in ISO 29701: 2010(E). The endotoxin content was determined from 100 µg/ml dispersions of the materials. All four NFC materials contained some endotoxin. US Food and Drug Administration (FDA) has set the acceptable endotoxin limit for sterile water

used in inhalation as 0.5 EU/ml. Two of the celluloses, NFC 1 (1.33 EU/ml) and NFC 2 (1.27 EU/ml), exceeded this limit. It is not known whether endotoxin contamination at this level could interfere with the results of the immunotoxicity assays used in this study. The endotoxin level of the reference material was under the detection limit. In addition to endotoxin testing, the NFC materials were also tested for yeast and mold contamination with a commercially available test kit (MYSK10025, Merck Millipore, Billerica, Massachusetts, USA). No yeast or mold colonies were detected after a 72-h incubation.

Following instructions given by the manufacturer, the materials were suspended by shaking and high-speed vortexing for 10 min. The dispersions were not sonicated to avoid possible changes in material characteristics due to breakage of the cellulose fibres (Johnson et. al. 2009). Also no serum was added to the dispersions. The dispersions were used immediately after the preparation. For the *in vivo* exposure, the cellulose materials were dispersed in phosphate buffered saline (PBS) which was also used as a negative control treatment.

Characterization of the dispersions by dynamic light scattering (DL) was attempted, as required by the Guidance Document, but the high viscosity and fibrous structure of the NFC materials caused the measurements to fail. The morphology of the material dispersions was observed by light microscopy, scanning electron microscope, and transmission electron microscope imaging (**Figure 1**).

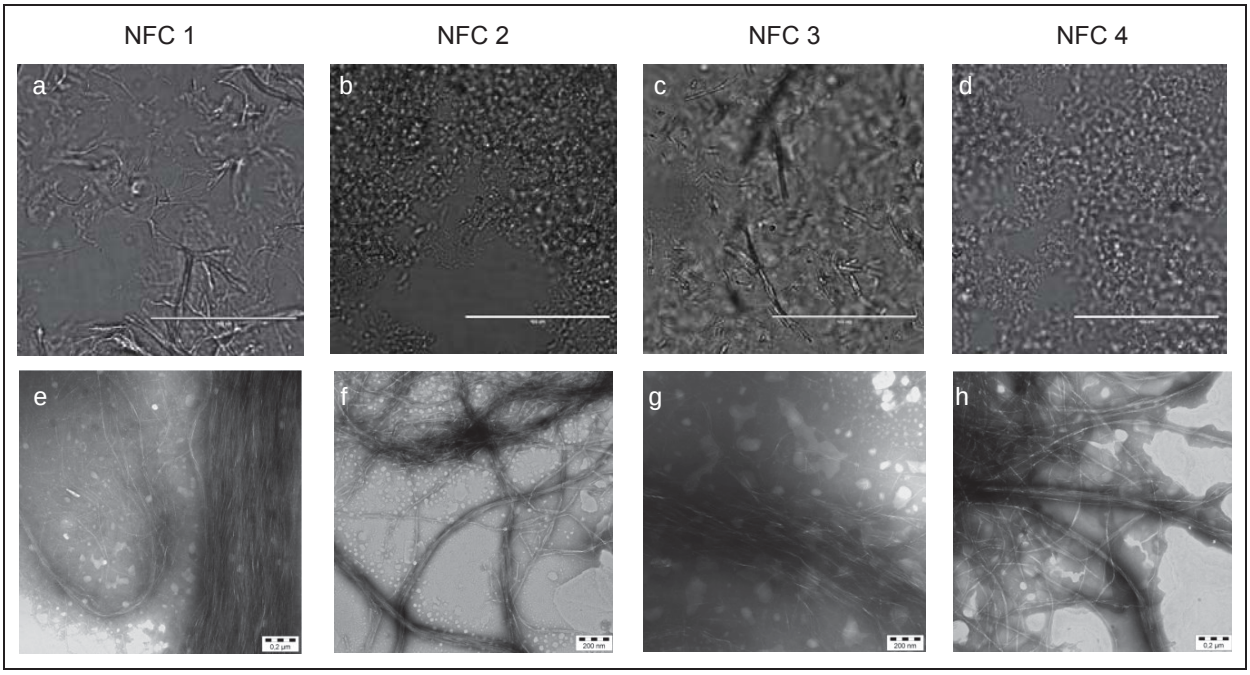

Figure 1. Images of the NFC material dispersions (***a-d***) in phase contrast light microscope (measure bar 400 µm) and (***e-h***) transmission electron microscope (measure bar 200 nm).

MWCNTs (Mitsui-7 MWCNTs) were included in the test series as a reference material. MWCNTs have earlier been shown to induce acute and long-term pulmonary inflammation in mice and DNA damage in lung cells (Rydman et al. 2014; Catalán et al. 2016b). In addition, MWCNTs have a fibrous structure similar to the cellulose materials. MWCNTs were dispersed in PBS (added with 0.6 mg/ml bovine serum albumin) and sonicated 20 min with ElmaSonic S15H (Tovatech LLC South Orange, NJ, USA) at 37 ºC.

2.3.2 Exposure and sampling

As inhalation is considered to be the most relevant route of exposure, the experiments focused on the pulmonary effects in mice *in vivo*. Since the materials were provided in gel-form, pharyngeal aspiration was chosen as the most suitable exposure method. Pharyngeal aspiration is a safe and reliable method for exposing rodents, and the dose delivered into the lungs can be ascertained. This type of exposure can be seen to represent a worst-case scenario, simulating tentative exposure to liquid aerosols.

The *in vivo* experiments were performed in 7-8 week-old C57BL/6 female mice (weight ~20 g). The animals were purchased from Scanbur AB (Sollentuna, Sweden). They were housed in stainless steel cages bedded with aspen chip and were provided with standard mouse chow diet and tap water ad libitum. The environment of the animal room was carefully controlled, with a 12-h dark/light cycle, temperature of 20-21 °C, and relative humidity of 40-45 %. The study was approved by the Animal Experiment Board and the State Provincial Office of Southern Finland. The same animals were used for the immunotoxicity and genotoxicity studies.

NFC dispersions were administered to the mice by a single pharyngeal aspiration using four doses, 10, 40, 80 and 200 µg/mouse (corresponding to 50 µl/mouse of the following NFC dispersions: 0.2, 0.8, 1.6 and 4 mg/ml), for the genotoxicity assessments (6 mice/group) and two doses, 10 and 40 µg/mouse (corresponding to 50 µl/mouse of the following NFC dispersions: 0.2 and 0.8 mg/ml), for the immunotoxicity assessments (8 mice/group). Same dosing was used for the bulk-sized pulp. MWCNTs were tested using three doses, 10, 40 and 80 µg/mouse, for the genotoxicity assessment and two doses, 10 and 40 µg/mouse, for the immunotoxicity assays. The mice were anesthetized with vaporized isoflurane. 50 µl of the particulate suspension was delivered onto the vocal folds under visual control. Immediately after the delivery the mouse nostrils were covered, enforcing the mouse to inspire the instilled suspension.

Samples were collected 24 h after the exposure, to assess acute effects and 28 days later for longer-term effects. For the 28-day experiment, the mice were weighed at the beginning and in the end of the experiment. The treatment with the test materials had no negative effects on the weight gain of the mice. The mice were sacrificed using an overdose of isoflurane. The lungs were lavaged with PBS via the tracheal tube and BAL fluid was collected. The mouse chest was opened, and blood was collected from the vena cava (hepatic vein). The lungs and femurs were collected for further preparations.

2.3.3 Genotoxicity assays

In the present study, genotoxicity was assessed *in vivo* in BAL fluid cells and in the lung cells by the comet assay and in bone marrow erythrocytes by the micronucleus assay. OECD has published guidelines for both assays (OECD 474 and 489) and this guidance was followed.

The alkaline comet assay was performed to determine, if the cellulose materials produce DNA damage in BAL cells and lung cells of the exposed C57BL/6 mice. PBS, administered by pharyngeal aspiration, was used as a negative control. WC/Co/CP (tungsten carbide cobalt mixture/cyclophosphamide) was used as a positive control for acute genotoxicity. WC/Co mixture (1 mg/mouse) was administered to the mice by pharyngeal aspiration. Small proportion of the collected cells were exposed to hydrogen peroxide (100 µM H_2O_2) *ex vivo* and these cells were used as an internal control to verify the performance of the method.

The cells collected were mounted, embedded in agarose gel, onto a microscopic slide and were then subjected to alkaline conditions (pH>13) to denature DNA and to remove all cellular material other than nuclear DNA (nucleoid). When these gel-mounted nucleoids were placed under electric current in electrophoresis, DNA with strand breaks migrated towards the anode forming a ‘comet tail’. Nucleoids were visualized with a DNA-binding fluorescent dye, ethidium bromide (2 µg/ml in water). The proportion of DNA in the tail was relative to the number of DNA strand breaks caused by the treatment. The percentage of the DNA in comet tail compared with total DNA of the nucleoid was analyzed using a fluorescent microscope and an interactive semiautomated comet counter (Komet 4.0, Kinetic Imaging Ltd.). Two replicate slides per animal were analyzed (50 cells / slide) from coded slides.

For the *in vivo* micronucleus assay, the bone marrow of the exposed mice was collected, and the cells were spun on a microscope slide by cytocentrifugation (Cytospin 3, Shandon Scientific LTD). The cells and micronuclei in them were visualized with May-Grünwald Giemsa staining, which also enabled the distinction of immature, polychromatic erythrocytes (PCEs; stained blue) from the mature, normochromatic erythrocytes (NCEs; stained purple). An automated microscopic analysis system (Metafer 4 v3.10.2, MetaSystems GmbH) was used to select >2000 PCEs per animal, and the proportion of micronucleated cells (MNCs) was confirmed

by micro-scopy. In mouse bone marrow, an increase in the frequency of micronucleated PCEs is an indication of geno-toxicity of the test material.

2.3.4 Immunotoxicity assays

The BAL samples were cytocentrifuged on slides. After drying, the slides were stained with May-Grünwald Giemsa. The number of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) of three high power fields (HPF) per each slide were counted under a light microscope at 40x magnification.

A slice of the left lung was fixed with formalin and embedded in paraffin. Thereafter sections of the paraffin embedded slices were cut and affixed on slides and stained with Hematoxylin and Eosin (H&E), to detect the overall tissue structure, Periodic Acid-Schiff (PAS), to detect goblet cell activation which is an indication of mucus production, and Picro Sirius Red (PSR; 28 d samples only), to detect collagen formation which is related to the development of fibrosis.

Approximately a half of the left lung was collected for mRNA analysis. Tissue pieces were homogenized in a FastPrep FP120 (BIO 101, Thermo Savant) machine, and total RNA was extracted by a liquid-liquid extraction method. Thereafter, the RNA was processed into complementary DNA, and the gene expression analysis was performed by the qualitative real-time polymerase chain reaction (q-RT-PCR) method. The mRNA expression levels of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, the anti-inflammatory cytokine IL-10, the T helper (Th) 2 cytokine IL-13 and the Th1 cytokine IFN-γ were measured in the samples collected 24 h after the NFC exposure. IL-1β, TNF-α, IL-13 and the fibrosis-related cytokine TGF-β were measured in the samples collected 28 d after the treatment.

2.4 Results

Most of the tested materials induced DNA damage, as seen in the comet assay (Figure 2). 24 h after the treatment, the level of DNA damage was increased by NFC 2 in both BAL and lung cells and by Pulp 5 in BAL cells. At 28 d, DNA damage induction was observed for NFC 1, NFC 2 and NFC 4 in BAL cells and for NFC 2, NFC 4 and Pulp 5 in lung cells. The effect was clearly dose-dependent for NFC material 2 in lung cells. NFC 3 did not induced DNA damage. MWCNTs increased DNA damage in lung cells at 24 h and in BAL and lung cells at 28 d. The positive control, WC/Co/CP, induced an increase in DNA damage (mean 12.1; data not shown) in the 24 h samples but not in the 28-d samples, in accordance with fact that it should only have an acute influence.

No clear systemic genotoxic response in the bone marrow was seen in the micronucleus assay (data not shown). As expected, the positive control, WC/Co/CP, increased the frequency of micronucleated PCEs 24 h but not 28 d after the treatment.

All tested materials were able to trigger the recruitment of neutrophils after 24 h. NFC 2 and 4 induced a clearly stronger effect than the other NFCs. In addition, NFC 2 and 4 induced a slight influx of eosinophils, and also a minor lymphocyte infiltration was observed in response to NFC 2 after 24 h. (Figure 3) 28 days after the exposure, only the presence of macrophages and no significant increases in the numbers of other cell types was seen in BAL with any of the tested NFC material or bulk-sized pulp, indicating that the inflammation had resolved.

A decreased number of macrophages was observed 28 d after the MWCNT treatment indicating that the inflammatory reactions induced by this material were continuous and thus the consequences of the exposure are more severe than with NFCs (data not shown).

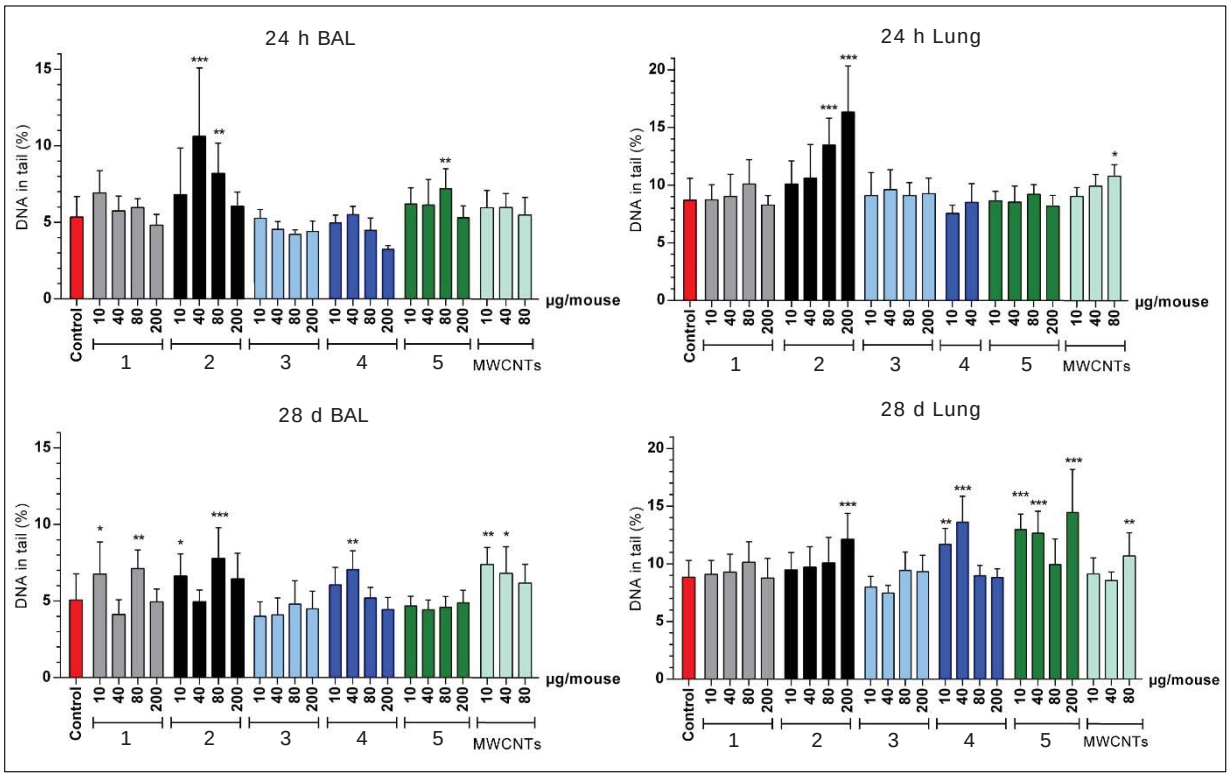

Figure 2. *In vivo* comet assay (percentage of DNA in comet tail; mean ± SD). Nanofibrillated cellulose (NFC) material 2 induced a dose-dependent effect. Materials 1, 4, 5 and MWCNTs induced a non-dose-dependent effect. ***p<0.001, **p<0.01, *p<0.05; one-way ANOVA. The positive control, tungsten carbide cobalt mixture/ cyclophosphamide, induced an increase in DNA damage (mean 12.1; data not shown) in the 24 h samples. The internal control (H2O2) control induced a 2-fold increase in DNA damage in both 24-h and 28-d series (data not shown).

Histopathological assessment revealed that NFC 2 induced a mild to moderate neutrophilia at the lower dose tested (10 µg/mouse) and a moderate to high neutrophilia at the higher dose (40 µg/mouse) 24 h after the treatment. The neutrophilic response was accompanied by a low number of eosinophils. NFC 4 triggered a similar, mild to moderate neutrophilic inflammation in lung tissue with the presence of a few eosinophils. NFC 3, on the other hand, induced a very mild reactivity at the higher dose, whereas NFC 1 and Pulp 5 did not induce a notable influx of inflammatory cells into lung tissue 24 h after the treatment. No notable PAS positivity was observed with any of the materials, suggesting that the tested materials did not activate mucin-producing goblet cells and thereby caused no elevated mucus production in the airways after 24 h. MWCNTs induced neutrophil and eosinophil recruitment in lung tissue as well as cell aggregates surrounding the material after 24 h.

28 days after the treatment, no considerable lung tissue reactivity was observed in response to the celluloses, except for a minor PAS positivity in response to treatment with NFC 4. The histological findings supported the BAL cell counting results, indicating that the observed inflammation induced by the cellulose materials resolved after 28 d. In contrast to the cellulose materials, granuloma formation and PAS positivity were observed in response to MWCNT exposure after 28 d.

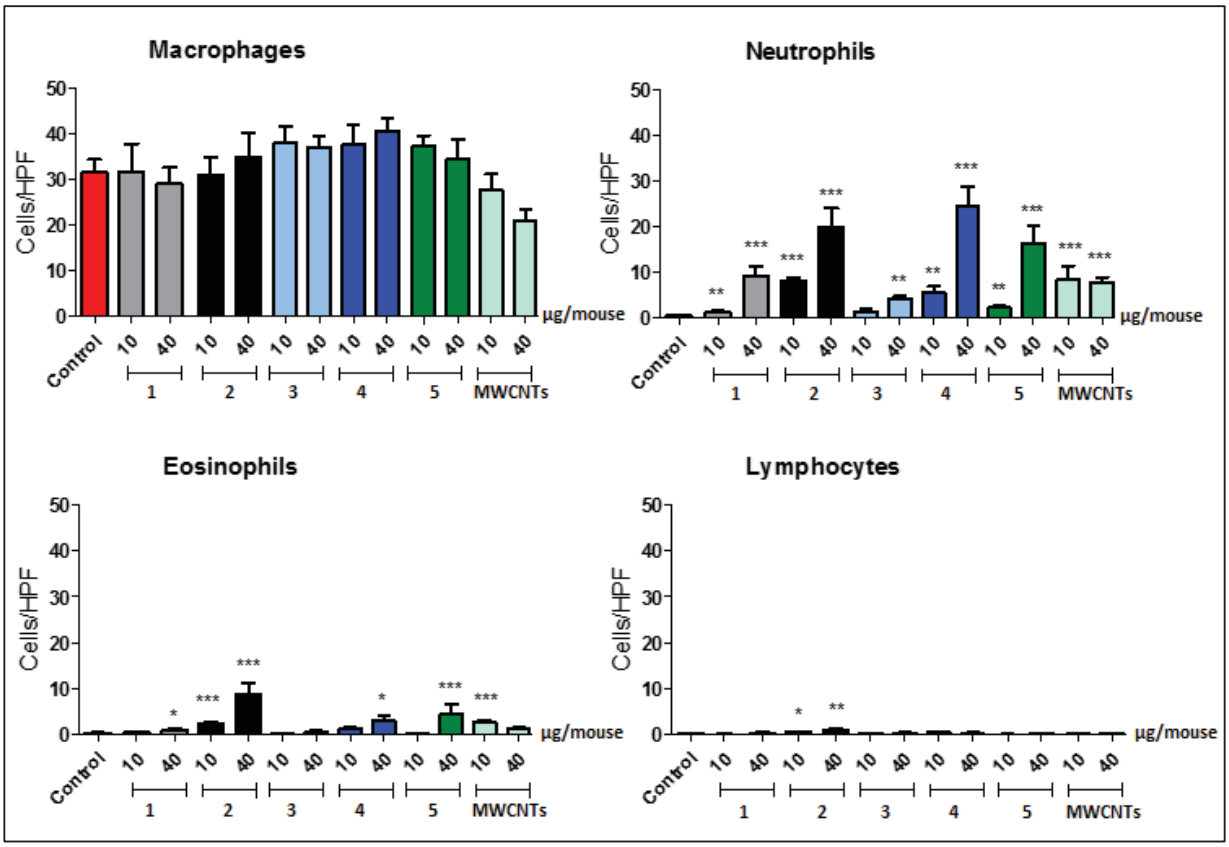

Figure 3. Recruitment of inflammatory cells in bronchoalveolar lavage (BAL) fluid 24 h after the exposure. Values presented as mean ± SEM. All tested materials were able to trigger the recruitment of neutrophils and all except NFC 3 also the recruitment of eosinophils in the BAL fluid. *p<0.05, **p<0.01, ***p<0.001.

To evaluate the fate of the material, its presence was assessed in the lungs with exonuclease (EXG) stain that specifically identified cellulose. The EXG staining method was developed at the National Research Centre for the Working Environment (NRCWE) in Denmark, who kindly provided us the protocol and the stain. We applied the technique to the slides of our NFC study, and the paper describing the method was published together with NRCWE (Knudsen et al. 2015). The EXG staining revealed that the cellulose was still present in the lungs after 28 d (Figure 4). Thus, it seemed that NFC is biopersistent in the lungs for at least 28 days. Distribution of the cellulose materials in the lungs appeared to depend on the size of the fibres, but all materials studied were biopersistent for the 28-day observation period.

Data obtained from mRNA expression analysis supported the results of the cytological and histological assessments (Figure 5). NFC 2 induced the strongest response, as it triggered the expression of a wide range of cytokines including IL-1β, TNF-α, IL-6, IL-10 and IL-13 after 24 h. NFC 1 triggered a significant elevation of IL-1β and IL-6 expression. NFC 3 was able to induce an increased expression of IL-6 and IL-13, while NFC 4 caused the expression of IL-1β, TNF-α, IL-6 and IL-13 cytokines. The bulk-sized reference material (Pulp 5) was able to trigger mRNA expression of IL-1β and IL-6, suggesting that also bulk-sized cellulose was able to cause pro-inflammatory responses. Compared with MWCNTs, the mRNA expression levels induced by NFCs were lower, indicating milder toxic properties for the NFCs than MWCNTs.

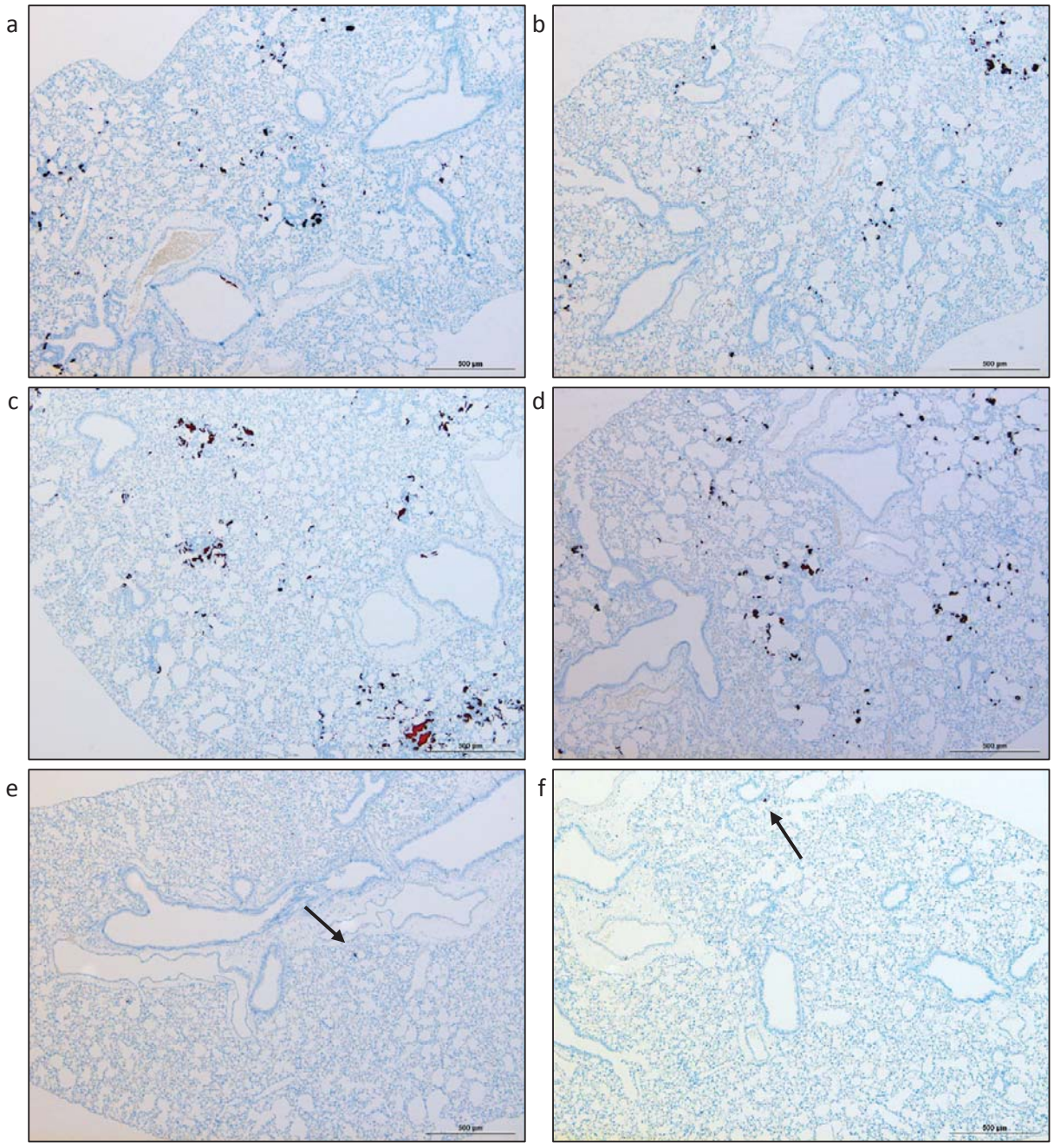

Figure 4. EXG stained lung tissue sections 24 h or 28 d after exposure to 40 µg of (a) NFC 2 (24 h), (b) NFC 2 (28 d), (c) NFC 4 (24 h), (d) NFC 4 (28 d), (e) Bulk-sized Pulp 5 (24 h) (f) Bulk-sized Pulp 5 (28 d). NFC can be seen as black/red dots (pointed by arrows in e and f). The amount of cellulose material in the lungs does not seem to be lower after 28 d than 24 h. The images are shown with a 500-µm scale bar.

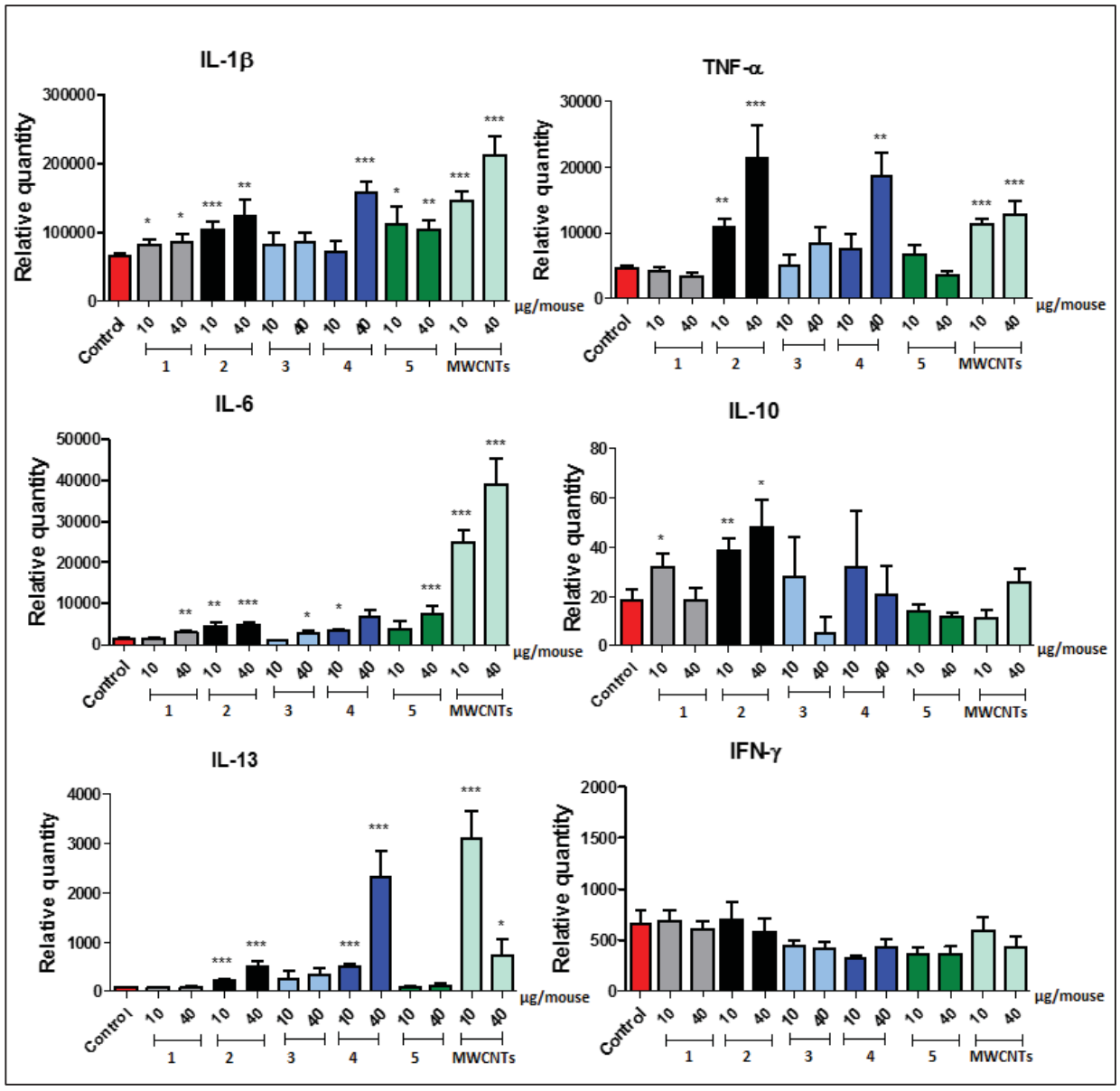

Figure 5. mRNA expression of relevant inflammatory cytokines 24 h after exposure to nanofibrillated celluloses NFC 1, 2, 3 and 4, bulk-sized Pulp 5, and multiwalled carbon nanotubes (MWCNTs). Values presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

When the mRNA expression levels of IL-1β, TNF-α, IL-13 and TGF-β were assessed 28 days after the exposure (**Figure 6**), none of the cellulose materials induced the expression of these cytokines, except for a minor induction of IL-13 by NFC 1 and 4. These results supported the cytological and histological findings, suggesting that the inflammation observed after 24 h mostly resolved by day 28. In contrast, MWCNT-induced inflammation seemed to continue still after 28 d, as judged by the elevated mRNA expression of TNF-α and IL-13, in accordance with the inflammatory response observed in the cytological and histological assessments.

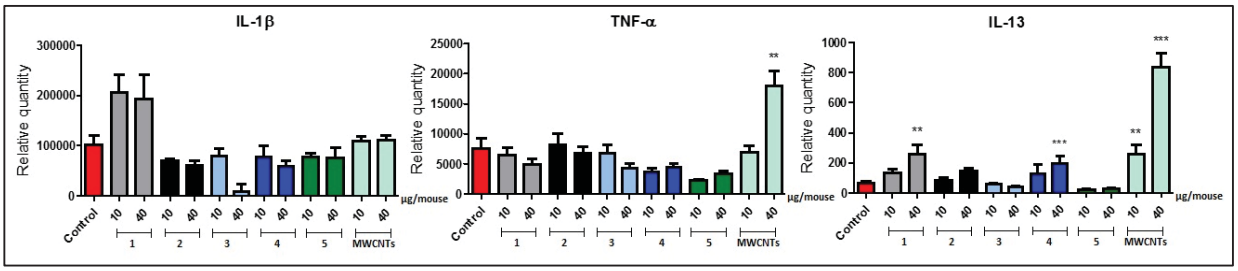

Figure 6. mRNA expression of relevant inflammatory cytokines 28 d after exposure to nanofibrillated celluloses NFC 1, 2, 3 and 4, bulk-sized Pulp 5, and multiwalled carbon nanotubes (MWCNTs). Values presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

2.5 Evaluation and conclusions

The toxic effects observed differed among the four NFCs studied, but effects were also seen with the bulk-sized cellulose studied. As concerns inflammatory effects, the mice exposed to the NFC materials and the bulk-sized cellulose showed signs of recovery of inflammation during the 28-d study period, while the MWCNT-treated mice exhibited characteristics of long-term adverse health effects.

Based on the results of the present study, it seems that exposure through the respiratory tract to NFC can cause acute inflammatory responses, which, however, are not anymore present 28 d later. Although NFCs appeared to be biopersistent during this follow-up time, they induced no pathological changes observable by histological examination of the lungs. In this sense, they markedly differed from MWCNTs which still showed clear inflammatory responses 28 d after the exposure and also produced various alterations in the histology of the lungs.

All NFC materials, except one, caused DNA damage in lung or BAL cells, as determined by the comet assay. For one NCF, the effect was dose-dependent in lung cells both 24 h and 28 days after the exposure. The comparative materials, bulk-sized pulp and MWCNTs, were also able to induce DNA damage after 24 h and 28 days. None of the NFCs or comparative materials was shown to possess systemic genotoxic properties, as measured by the micronucleus assay in bone marrow.

The outcome of the *in vivo* toxicity tests was not consistently predicted by the *in vitro* toxicity studies reported in WP5. None of the NFCs or the bulk-sized pulp were genotoxic *in vitro* and only one of the NFCs was able to induce inflammatory cytokines *in vitro*. This comparison suggested that the mechanisms responsible for the effects observed *in vivo* are not fully present in the *in vitro* cell systems used.

In the present study, exposure via the respiratory route was chosen to mimic a tentative worst-case-scenario, where liquid nanocellulose is aerosolized in the atmosphere during the manufacturing process and inhaled by workers. As NFC production is presently in an experimental phase, it is presently not known how realistic this kind of exposure scenario could be in the future production of NFC. Possible exposure routes may include, in addition to inhalation of aerosols or dry fibres, also dermal and oral exposure.

Toxicological data on nanocelluloses are still scarce, which limits comparison with existing literature. Although NFC is a HARN, nanocellulose fibres are flexible and tangled. It has been suggested that this type of fibres are less harmful than long, rigid, needle-like nanofibers, such as the MWCNTs we used as a reference material which in many ways resemble asbestos. Also the findings of the present study suggest that MWCNTs have more severe adverse health effects than the tested NFCs.

As the work was started, based on the agreement between FIOH, TUKES and the company partners Stora Enso and UPM-Kymmene, at project month 1 and mostly completed by the end of year two, no Guidance Documents or SOPs were readily available. Due to the nature of the material, the dispersion protocol provided by the NANoREG Consortium could not be followed, but instead the NFC materials were dispersed according to instructions from the manufacturer. Sonication was not applied, because it might have broken the cellulose fibres and thus altered the properties of the materials. In addition, the dispersions were not analysed with DLS, as it was found to be unsuitable method for viscous fibre materials.

In conclusion, the results indicate that, although the tested NFC materials were able to induce DNA damage and inflammatory responses at 24 h, the mice exposed to the NFCs showed signs of recovery of the inflammation at 28 d. On the contrary, the mice treated with MWCNTs exhibited characteristics of longer term adverse health effects. However, the observation that the NFCs, and the bulk-sized pulp, were biopersistent in the lungs for at least 28 days raises some concern, because some increase in the level of primary DNA damage was still observed at this time point. If DNA damage is continuously produced during a prolonged time, it might contribute to carcinogenesis. A longer follow-up would be required to better define the fate of the NFC material in the lungs and the duration of the increased level of DNA damage.

2.6 Data management

Data logging has been completed. The data obtained on genotoxicity and immunotoxicity have been entered in to the online database. The data have also been uploaded to CIRCABc system.

3 Deviations from the work plan

The studies were conducted according to the DoW. All experiments were completed as scheduled.

4 References / Selected sources of information (optional)

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5 List of abbreviations (optional)

Explained in the text.

Annexes (optional)

None