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Mode of toxic action of high aspect ratio nanomaterials

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1 Description of task

Task 4.5.1 Biological effects of pulmonary deposition of HARN in rats

Effects of materials among a collection of 20-40 carbon nanotubes and other HARN (WP2A) were tested for biological effects, biodurability and biodistribution after inhalation, intratracheal instillation in lungs (incl. pleura) and intraperitoneal injection) of rodents. The materials were selected to address specific questions on effect of dimension (diameter, length and aspect ratio) biodurability etc. Rats were followed for several months.

Task 4.5.2 Biological effects of inhalation of HARN in rats

Rats were exposed by inhalation during 28 days with a 6 month follow-up with two HARN selected by data from 5.4.1. They will be exposed to 2 concentrations of HARN and there were control groups exposed to filtered air. The pulmonary deposition of NM and the biodistribution was examined over time. Different tests for assessing biodurability (in vitro and in vivo) was validated. Biological effects were determined by gross pathology, microscopy, lung lining fluid cell composition, a range of molecular markers. Genotoxicity was determined by DNA strand breaks, micronuclei and immunohistologic staining.

2 Description of work & main achievements

2.1 Summary

Intracheal instillation

Ten MWCNT were tested by intracheal instillation in female C57BL/6J mice. Lymphocytic aggregates were detected for all MWCNT on day 28 and 92. Using adjusted, multiple regression analyses, inflammation and genotoxicity were related to dose, time and physicochemical properties. The specific surface area (BET) was identified as a positive predictor of pulmonary inflammation on all post-exposure days. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (–OH and –COOH) was predictor of attenuated inflammation on day 28. BET surface area, and therefore diameter, significantly predicted genotoxicity in BAL fluid cells and lung tissue such that lower BET surface area or correspondingly larger diameter was associated with increased genotoxicity.

Effects of the ten MWCNTs and MWNT XNRI-7 were also evaluated after one year but at the single dose 54 µg/mouse. There were no treatment related neoplasms in pleura or lung (but the study was not designed for detecting carcinogenicity). Surprisingly few fibrotic lesions were observed, However, a critical assessment of the literature,we think, indicates the MWCNT induced fibrogenicity has been exaggerated: Diffuse collagen accumulation is not same as asbestos induced fibrosis. Lymphocytic aggregates were consistently observed signifying chronic inflammation. There was great variation in the induction of histopathological changes with the 11 MWCNT with different physico-chemical properties after one year.

Inhalation nose only

Inhalation of the highest concentration of NM-401 (1.5 mg/m^3) led to a massive influx of granulocytic neutrophils 3 days after the end of the exposure; an effect that abated over time but was still statistically significant after 180 days. There were more lymphocytes 3 days post-exposure and the number decreased over time. The sub-acute exposure to the lowest concentration of NM-401 (0.5 mg/m^3) led to a small but statistically significant reduction of the number of macrophages in the BALF 3 and 30 days post-exposure but there were no changes in numbers of neutrophils or lymphocytes.

Inhalation of NM-403 increased the number of neutrophils for both concentrations 3 days after exposure and to a lesser extent at 30 days post-exposure. At day 90 day post-exposure there was no increase in neutrophils in animals that had inhaled 0.5 mg/m^3 of NM-403, but it was not statistically significantly greater after inhalation of 1.5 mg/m^3. NM-401 only induced significant DNA damages in lung cells at the 30 days post-exposure time, whereas the dose of 0.5 mg/m^3 induced statistically significant DNA strand breaks at 30 days only with Fpg and at 180 days.

(The Fpg enzyme detects oxidative damage). Preliminary results from the whole-body inhalation of NRWCE-64 (Carboxylated MWCNT) at 1.5 mg/m^3 indicate no significant change to total BALF cell numbers at 3 and 30 days post-exposure.

Inhalation whole body

To be added after finalizing this part of the task

2.2 Background of the task

Much of the interest in the hazard of high aspect ratio nanomaterials (HARN) stems from occupational and public health problems caused by inhalation of fibers, especially asbestos. One of the major challenges in nanotoxicology is to be able to predict human risk following long term exposure to low exposure levels based on short term studies in animal models using much higher exposure levels. In addition, the internationally recognized and harmonized protocols for toxicity testing favor rats as model, whereas mice are the preferred animal model in more mechanistic studies involving transgenic animals or –omics methods.

MWCNT differ vastly in physico-chemical properties including length, thickness, levels and types of metal impurities, types and levels of surface modifications. To be able to assess the effect of these variations in physicochemical properties, several different CNTs with well-characterized physico-chemical properties should be assessed in the standardized subchronic inhalation test involving several dose levels and time points as well as careful determination of the dosimetry. These sub-chronic inhalation studies are expensive and time consuming and there are presently only two such studies with CNTs available in the scientific literature. However, identification of the physico-chemical properties that drive different toxic effects may also contribute to grouping and ranking of nanomaterials including HARN by enabling ranking of other CNTs relative to the CNTs used in the sub-chronic inhalation studies.

1. To be able to understand which HARN properties that drive biological effects it is necessary to examine a panel of materials with different properties. 20 CNT and 3 nanocelluloses were investigated by intratracheal instillation. This administration deposits the test material into the whole lung and it well distributed. This is a way of delivering controlled amounts and the distribution is independent of the agglomerate-size. This is in contrast to deposition after inhalation which is highly dependent on agglomerate size distribution and the administration is better suited for material comparisons. Within 90 days it is possible to detect acute and persistant inflammation and genotoxicity and evidence of beginning chronic pathology can be detected.

2. For detecting disease it is necessary follow mice over longer times. Mice were therefore instilled with a single dose at early life. The welfare of mice was followed over a year after which they were humanely killed and the lungs were examined for histological changes.

3. Chronic or sub-chronic inhalation studies are the golden standard of testing. Aspiration and intratracheal instillation has been used as a faster and cheaper alternative that allows more control of the delivered dose, thus allowing comparison of toxicity between different test materials. Comparisons between aspiration or intratracheal instillation on one hand and inhalation of CNTs on the other hand have shown that aspiration and instillation gave similar toxicity as inhalation but inhalation gave stronger effects. The purpose of this work is to study the pulmonary toxicological properties of two carbon nanotubes in rats exposed by inhalation. These nanomaterials were selected among a list of 20-30 samples tested in WP 4.5.1 in which the pulmonary toxicity of these carbonaceous compounds was assessed following intratracheal instillation. Rats were exposed by inhalation for 2 x 3 h/day, 5 days/week for 4 weeks.The results obtained in WP 4.5.1 and 4.5.2 would allow comparing the two methods to assess the pulmonary toxicity of carbon nanotubes and help determining whether instillation is a predictive method. In addition, a comparison between whole body and nose-only inhalation methods will be done. NM-401 and NM-403 have been tested by nose only inhalation in rats. Testing has commenced of NRWCE-64 (carboxylated MWCNT) by whole body inhalation and when this is complete NM-401 will also be tested using the whole-body system, to allow comparison with the nose-only results.

The toxic effects of CNT and other HARN are not clear and sparingly systematic data exist on the relationship between dimension such as size and thickness, rigidity, agglomeration state and a range of chemical properties. The NANoREG project uniquely presented opportunities of coupling excellent physico-chemical characterization to toxicological investigation. Lung deposition by inhalation is the critical safety issue for HARN. The complex nature of lung physiology and anatomy necessitates investigation in animals. A systematic investigation of principles for toxicity of high aspect ratio nanomaterials and validation of whether testing principles for fiber materials are suitable was conducted.

2.3 Description of the work carried out

1. Ten commercial MWCNT supplied in three groups of different dimensions, with one pristine and two/three surface modified in each group were tested deposited in lungs of female C57BL/6J mice (7-8 mice per dose and time) by intratracheal instillation of 0, 6, 18 or 54 mg/ mouse. (Jacobsen et al Particle and Fibre Toxicology 6:2, 2009). Pulmonary inflammation (neutrophil influx in bronchoalveolar lavage (BAL)) and genotoxicity were determined on day 1, 28 or 92. Histopathology of the lungs was performed on day 28 and 92. (Poulsen et al Nanotoxicology 10:1263-1275, 2016, Knudsen et al in preparation)

2. In this work we studied the outcome in mice one year after pulmonary exposure to 11 (the same) different MWCNTs by intratracheal exposure. We evaluated lung histopathology, genotoxicity in the secondary organs liver and spleen. Groups of 10 C57BL/6J mice HARN exposed mice were followed for an extended period in order to more fully understand the long term outcome and relevance of lesions associated with the exposure to HARN. We assessed the long term pulmonary toxicity for 11 different MWCNT following intratracheal instillation in mice. A single dose of 54 µg/mouse was delivered by intratracheal instillation and the animals were killed after 1 year. The chosen dose corresponds to three times the total deposited dose at the suggested occupational exposure limit of 1 µg/m3 assuming a pulmonary deposition of 10%, 40 hour working week during 40 years, but ignoring pulmonary clearance.

3. The purpose of this work is to study the pulmonary toxicological properties of two carbon nanotubes in rats exposed by inhalation. These nanomaterials were selected among a list of 20-30 samples tested in WP 4.5.1 in which the pulmonary toxicity of these carbonaceous compounds was assessed following intratracheal instillation. The results obtained in WP 4.5.1 and 4.5.2 would allow comparing the two methods to assess the pulmonary toxicity of carbon nanotubes and help determining whether instillation is a predictive method. In addition, a comparison between whole body and nose-only inhalation methods will be done. NM-401 and NM-403 were tested by nose only inhalation in rats. Testing has commenced of NRWCE-64 (carboxylated MWCNT) by whole body inhalation and when this is complete NM-401 will also be tested using the whole-body system, to allow comparison with the nose-only results. Aerosols were generated with an acoustic generator (McKinney et al, 2009, Inhalation Toxicology, 21(12), 1051-63) and delivered simultaneously to 32 animals maintained in nose-only contention tubes placed in 4 9-port nose-only inhalation chambers (EMMS, UK). Concomitantly, 32 rats were exposed in a similar way to filtered air. The aerosol and filtered air were conditioned at a temperature of 22 ± 2°C and a relative humidity of 55 ± 10 % in order to respect animal physiological needs. Thirteen-week old female Sprague-Dawley rats were exposed by inhalation 2x3 h/day, 5 days/week for 4 weeks to either filtered humidified air or 0.5 and 1,5 mg/m^3 of each carbon nanotube samples aerosols. 3, 30, 90 and 180 days after the end of exposure, animals from control and CNT-exposed groups (8 rats per exposure condition and per post-exposure time) were euthanized and tissue samples were collected.

2.4 Results

1. Using adjusted, multiple regression analyses, inflammation and genotoxicity were related to dose, time and physicochemical properties. The specific surface area (BET) was identified as a positive predictor of pulmonary inflammation on all post-exposure days. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (–OH and –COOH) was predictor of lowered inflammation on day 28. BET surface area, and therefore diameter, significantly predicted genotoxicity in BAL fluid cells and lung tissue such that lower BET surface

area or correspondingly larger diameter was associated with increased genotoxicity . (Poulsen et al Nanotoxicology 10:1263-1275, 2016, Knudsen et al in preparation). This study provides information on possible toxicity driving physicochemical properties of MWCNT. The results may contribute to safe-by-design manufacturing of MWCNT, thereby minimizing adverse effects. Pulmonary inflammation (neutrophil influx in bronchoalveolar lavage (BAL)) and genotoxicity were determined on day 1, 28 or 92. Histopathology of the lungs was performed on day 28 and 92. All MWCNT induced similar histological changes. Lymphocytic aggregates were detected for all MWCNT on day 28 and 92. Using adjusted, multiple regression analyses, inflammation and genotoxicity were related to dose, time and physicochemical properties. The specific surface area (BET) was identified as a positive predictor of pulmonary inflammation on all post-exposure days. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (-OH and -COOH) was predictor of lowered inflammation on day 28. BET surface area, and therefore diameter, significantly predicted genotoxicity in BAL fluid cells and lung tissue such that lower BET surface area or correspondingly larger diameter was associated with increased genotoxicity. This study provides information on possible toxicity-driving physicochemical properties of MWCNT. The results may contribute to safe-by-design manufacturing of MWCNT, thereby minimizing adverse effects.

2. Inflammatory activity in the lungs after exposure to MWCNT was observed. We found lymphocytic aggregates, macrophage infiltrates and foreign body type granulomas and the incidences of the histopathological findings varied for the different MWCNT. There was however no widespread interstitial inflammations, which would seem to be most directly associated with fibrosis and malignancy. The lymphocytic aggregates seen have been described earlier by us in a study concerning of mice exposed to HARN different MWCNT for 3 months. Lymphocytic aggregates in some control mice but the exposure to HARNs clearly increased the numbers of these aggregates. The presence of lymphocytic aggregates in control mice could be seen as a feature of aging mice as we did not observe them in control mice at earlier time points (28 days or 3 month) assessed in the earlier study. (Data have been uploaded to the NANoREG database.)

Examination of visceral pleura from the diaphragm and the chest revealed no tumors or other significant histopathological changes detectable. In the lungs we observed a few tumors but the numbers of cases were too low to allow statistical evaluation. The study was really not designed for evaluation for tumor formation, as the group size was too small and follow up time was too short. Only few fibrotic lesions were observed across the MWCNT exposed groups. The numbers of fibrotic lesions were also relatively low and the fibrotic lesions were rather focal and did not suggest a generalized or multifocal interstitial fibrosis as seen in e.g. asbestosis (Knudsen et al in preparation).

Foreign body granulomas surrounding MWCNT were observed for many, but not all, of the exposure groups. Morphologically, they appeared rather inactive, with often a very thin layer of macrophage separating the material in question from the lung tissue. Granulomas protect the organism from potentially dangerous particles by insulating the particles from tissues with a layer of adherent macrophages. The most fiber-like MWCNT in the study are NM-401 and NRCWE-006 (MWNT XNRI-7) as these MWCNT appear straight and needle-like in dark field images. Neither NM-401 nor NRCWE-006 caused the formation of granulomas. In addition, it was very difficult to detect NM-401 or NRCWE-006 by light microscopy alone as the two MWCNTs appeared well dispersed in the lung interstitium. NRCWE-006 was however detected as single CNTs in the lung using dark-field imaging. Significantly, these features of NM 401 and NRCWE-006 are similar to that of asbestos.

MWCNT were observed in the lungs one year after exposure to them using only light microscopy for all MWCNTs except NM-401 and NRCWE-006 suggesting that most MWCNT are biopersistent in the lungs. We were also in most cases able to detect MWCNT in mediastinal lymph nodes suggesting that the material exit the lungs to some extent by lymphatic drainage. NM-401 and NRCWE-006 did not induce granuloma formation, lymphocyte aggregation or macrophage infiltration. Thus, these two long, straight and thick MWCNT were not observed as aggregates in the lungs 1 year after exposure. We did not observed NRCWE-006 or NM-401 in lymph nodes either, as the lymph nodes appeared normal in these mice.

In this comparison of the toxicological response to 11 MWCNT with different physico-chemical properties, we found large variation in the toxicological and histopathological response 1 year

after exposures. Thinner MWCNT and less iron content correlated with granuloma formation and formation of lymphocyte aggregates in an multivariate regression analysis. Thinner MWCNT were more associated with macrophage infiltration. The thicker, and hence more fiber-like MWCNT, induced less of the histological changes compared to their thinner counterparts. Co was a predictor of lymphocytic infiltration and granuloma formation determined in the regression analysis.The majority of the MWCNT were observed in lung tissue using light microscopy as agglomerates or aggregates one year after the exposure suggesting that these nanomaterials are biopersistent. Two of the studied MWCNTs, characterized as long and thick, were not found as agglomerates or aggregates, but were present as single fibers in the lung. We did not find significant fibrosis in lungs or pleura. However, this may be because the chosen dose, (54 µg/mouse corresponding to 3 times the estimated work-life dose at the proposed OEL for CNT) is too low. We found that the 11 different MWCNTs induced varying degrees of inflammation inflammation-related histologic findings including lymphocyte aggregation, macrophage infiltration and granuloma formation.

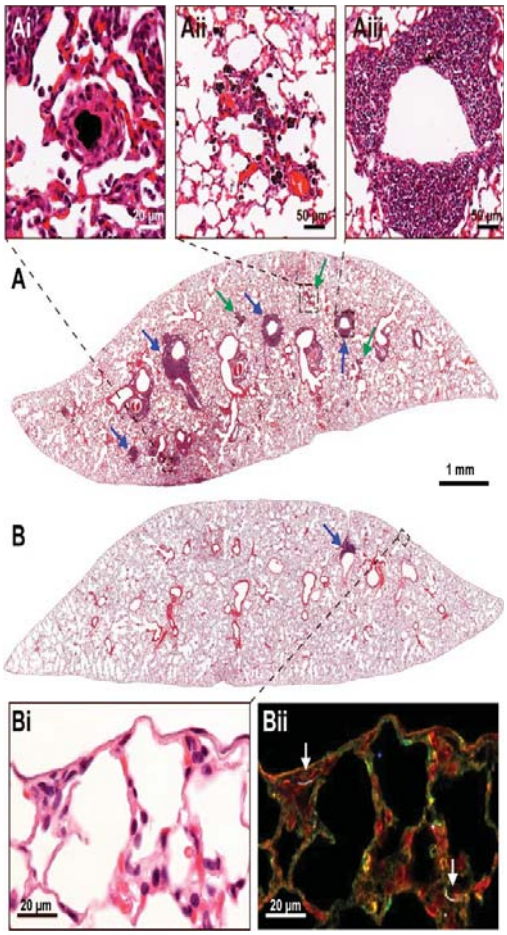

Figure 1: Representative overviews of H&E stained paraffin-embedded lung tissue from mice exposed to NM-403 (Fig 1A) and NRCWE-006 (Fig 1B) one year after exposure. MWCNT were generally observed as black aggregates in macrophages or granulomas (Fig 1Ai-ii and Fig S1-2) for all MWCNT except NM-401 and NRCWE-006. Only a few NRCWE-006 aggregates were found in lobes of a few exposed mice. With enhanced dark field microscopy, NRCWE-006 (Fig 1Bi-iiA) and NM-401 were found to be distributed as single fibers spread throughout the lung.

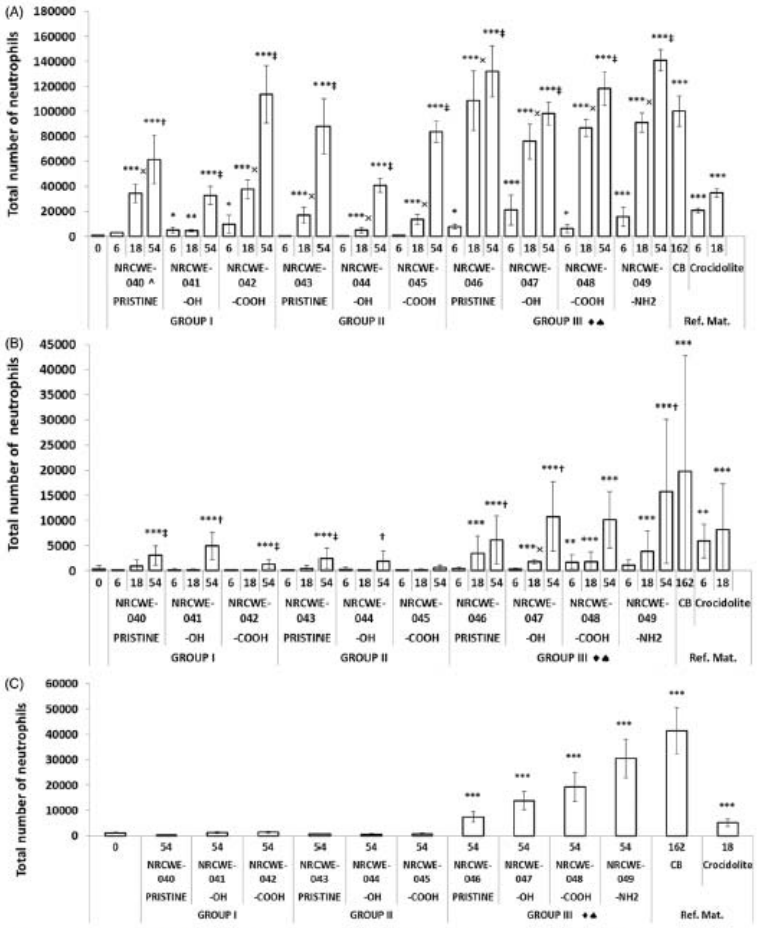

Figure 2. Total number of neutrophils in the BAL fluid after exposure to MWCNT and reference materials. Error bars indicate SD. (A) Day 1. (B) Day 28. (C) Day 92. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to vehicle controls. †: 54 > 6 μg/ml; ‡: 54 > 6 and 18 μg/ml; x: 18 > 6 μg/ml; ^: higher than the –OH form; ♦: higher than Group I; ♠: higher than Group II; ♣: higher than Group III.

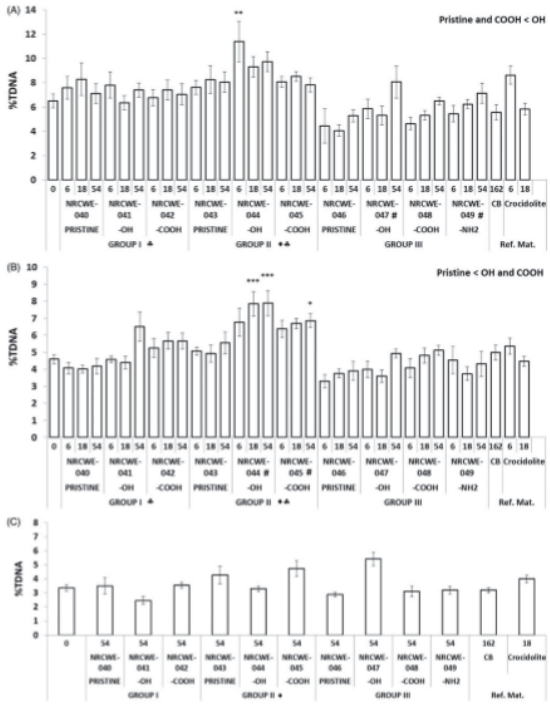

Figure 3: DNA strand breaks in the lung tissue after exposure to MWCNT and reference materials. Error bars indicate SD. (A) Day 1. (B) Day 28. (C) Day 92. *p50.05, ***p50.001 compared to vehicle controls. #: higher than the pristine form; ˙: higher than Group I; f: higher than Group III.

3. Thirteen-week old female Sprague Dawley rats were housed in individually ventilated cages in 12h/12h light/dark cycle, and when not in contention tubes had ad libitum access to food and water. They were exposed by nose only inhalation, 2x3h/day; 5 days/week for 4 weeks to filtered air or 2 concentrations (0.5 and 1.5 mg/m3) of aerosolized carbon nanotubes, NM-401 and NM-403. Three, 30, 90, and 180 days after the end of exposure in biological effects were examined.

Aerosols monitoring and in-depth characterization were ensured by 1) the use of real-time devices: an optical particle sizer (OPC FIDAS mobile, Palas), a Condensation Particle Counter (CPC) (TSI model 3007), a scanning mobility particle sizer (SMPS, Grimm) composed of a Vienna-type Differential Mobility Analyser and a CPC, an aerodynamic particle sizer (TSI APS model 3321) and an electrical low pressure impactor (ELPI, Dekati), and 2) samples taken for off-line analyses (gravimetric analysis, mass size distribution from cascade impactor (SIOUTAS, SKC), and TEM observations).

Table I: Characteristics of the carbon nanotubes (from NRCWE and WP2)

		Length (µm)	Diameter (nm)	SSA (m^2/g)	Purity (%)
NM-401	Pristine MWCNT	4.0 (±0.37)	67 (24-138)	18	98
NM-403	Pristine MWCNT	0.4 (±0.03)	12 (5-37)	189	96.9

Table II: Main characteristics of the aerosols

	Target concentration (mg/m^3)	Mass concentration (mg/m^3)	Number concentration ($particles/cm^3$)	MMAD (GSD) (nm)	NMAD (nm)
NM-401	1.5	1.59 ± 0.24	~2200	790 (1.83)	280
	0.5	0.54 ± 0.11	~815		
NM-403	1.5	1.48 ± 0.63	~540	1940 (1.48)	1440
	0.5	0.50 ± 0.14	~130		

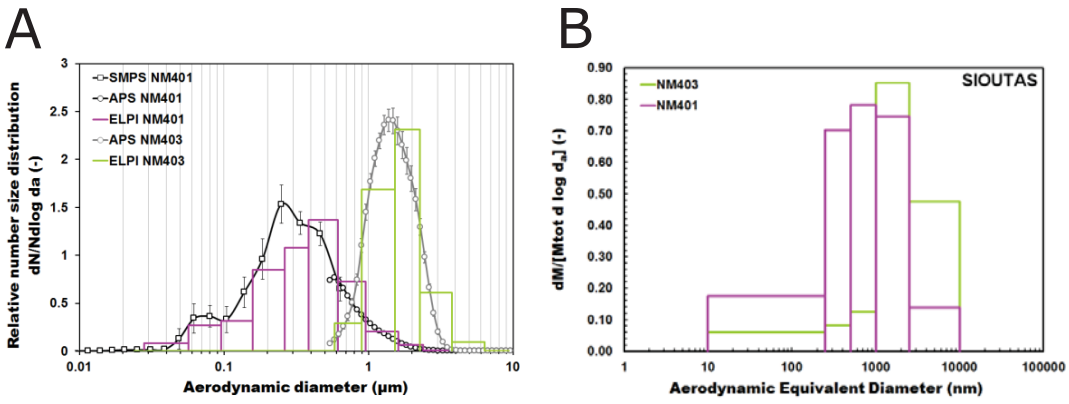

Figure 4: Number (A) and mass (B) distribution of the aerosols.

Animals were anesthetized with pentobarbital, tail vein blood was then collected on Na_2EDTA tubes for hematology and comet assay. Rats were finally exsanguinated through the abdominal aorta. Animal chest was then opened; the heart and thymus were removed. The trachea was cut as high as possible and the lung removed. The right primary bronchus was clipped and

bronchoalveolar lavage was performed on the left lung. Lobes of the right lungs were isolated for different analyses. DNA damage was determined by the comet assay and paraffin embedded tissue blocks of lung and lung associated lymph nodes (LALN) from animals exposed to the highest concentrations of CNTs and their matched controls were analyzed for histopathology. Cytokines in BAL fluid were determined.

The highest concentration we could achieve was 1.5 mg/m^3. The two selected concentrations were 0.5 and 1.5 mg/m^3. Even though the assessment of the deposited dose by a quantitative method such as thermogravimetric needs to be performed, an estimated deposited dose has been determined using the Multiple-Path Particle Deposition model (MPPD v3.04) (https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304): 362 to 377 µg and 128 to 192 µg of NM-401 and NM-403 were deposited into the pulmonary region respectively (Table III) which are within the range of the doses tested by intratracheal instillation (180 and 540 µg) by the NRCWE.

Table III: CNT deposition in the respiratory tract

		Total deposition fraction*	Total TB deposition fraction*	Total pulmonary deposition fraction*	Estimated pulmonary deposited mass (µg) #
NM-401	NMAD	0.582	0.419	0.099	362
	MMAD	0.577	0.407	0.103	377
NM-403	NMAD	0.386	0.226	0.035	128
	MMAD	0.406	0.251	0.053	192

* estimated from symmetric Sprague-Dawley model using NMAD or MMAD (MPPD v 3.04)
# after 1 month exposure at 1.5 mg/m^3

Two carbon nanotubes were tested: the "long and thick" NM-401 and the "short and thin" NM-403 (see Table I for their main characteristics). Both were pristine without any surface modification. Based on TEM analysis (Figure 5), NM-401 nanotubes appeared to have the shape of individualized fibers with different lengths which were sometimes entangled. NM-403 nanotubes were highly entangled in such a way that at low magnification, it did not look like carbon nanotubes but rather like particles with irregular shapes. At high magnification, individual nanotubes could be seen protruding from the entanglement. NM-403 aerosol had a low particle number because of high entanglement while NM-401 nanotubes were almost not entangled.

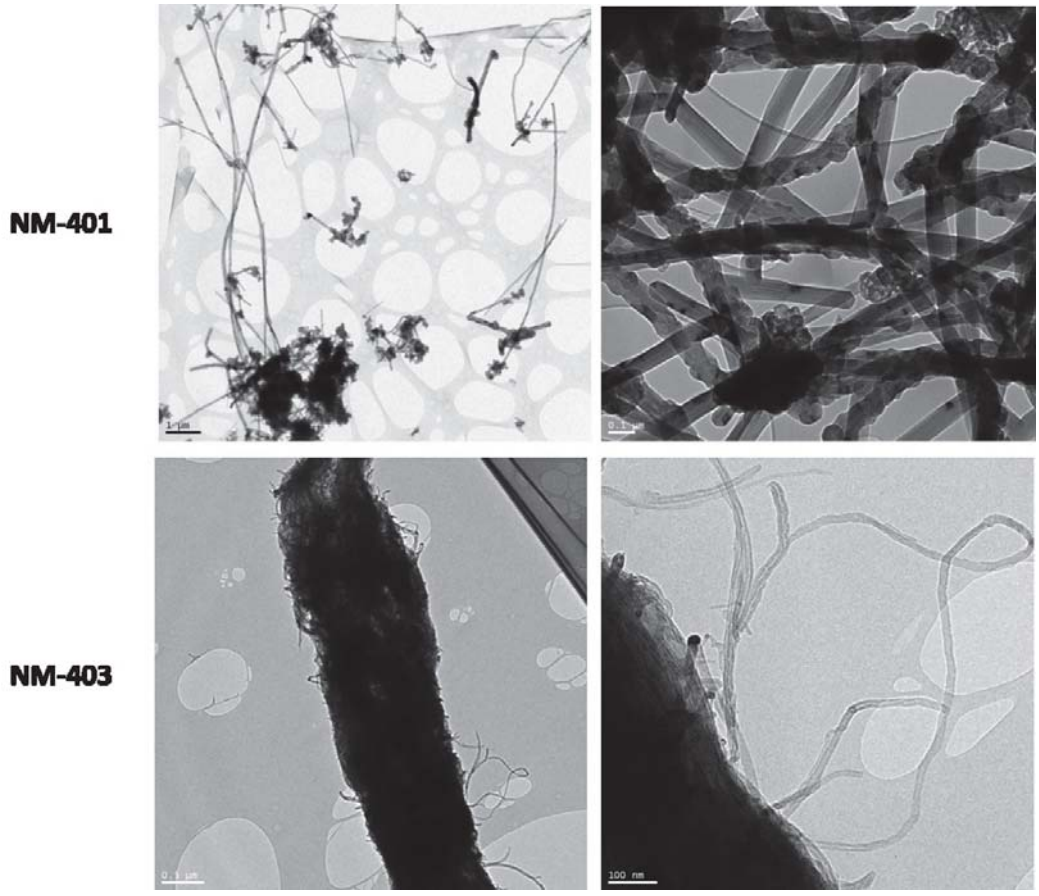

Figure 5: Representative transmission electron microscopy images of NM-401 and NM-403 aerosols. Carbon nanotubes aerosols were collected on TEM grids and observed with a Philips Transmission electron microscope equipped with a Gatan digital camera.

The sub-acute inhalation of the highest concentration of NM-401 (1.5 mg/m^3) led to a massive influx of granulocytic neutrophils 3 days after the end of the exposure. The number of neutrophils decreased overtime but was still visible and significant 180 days post-exposure (Figure 6). Also there were more lymphocytes 3 days post-exposure and the number decreased over time. The sub-acute exposure to the lowest concentration of NM-401 (0.5 mg/m^3) led to a small but significant decrease of macrophages in the BALF 3 and 30 days post-exposure but no change in neutrophils or lymphocytes was observed (Figure 7).

The exposure to NM-403 induced increased neutrophils for both concentrations 3 days after the end of exposure. This influx was still observed 30 days post-exposure but was lower in animals exposed to 0.5 mg/m^3 of this carbon nanotube samples. 90 days post-exposure, while no increase in neutrophils was noticed in the BALF from animals exposed to 0.5 mg/m^3 of NM-403, it was still significant with the highest dose and associated with a small increase of lymphocytes (Figures 8 and 9). Surprisingly, a decrease of macrophages was noticed 90 days post-exposure in animals exposed to the lowest dose of NM-403. In addition, 180 days after the end of exposure, while more granulocytes were counted at the highest dose of NM-403, an increase was also seen at the lowest dose. The morphology the nanotubes within BALF macrophages from animals exposed to NM-401 or NM-403 was similar to that observed on TEM grids (Figures 5 and 10).

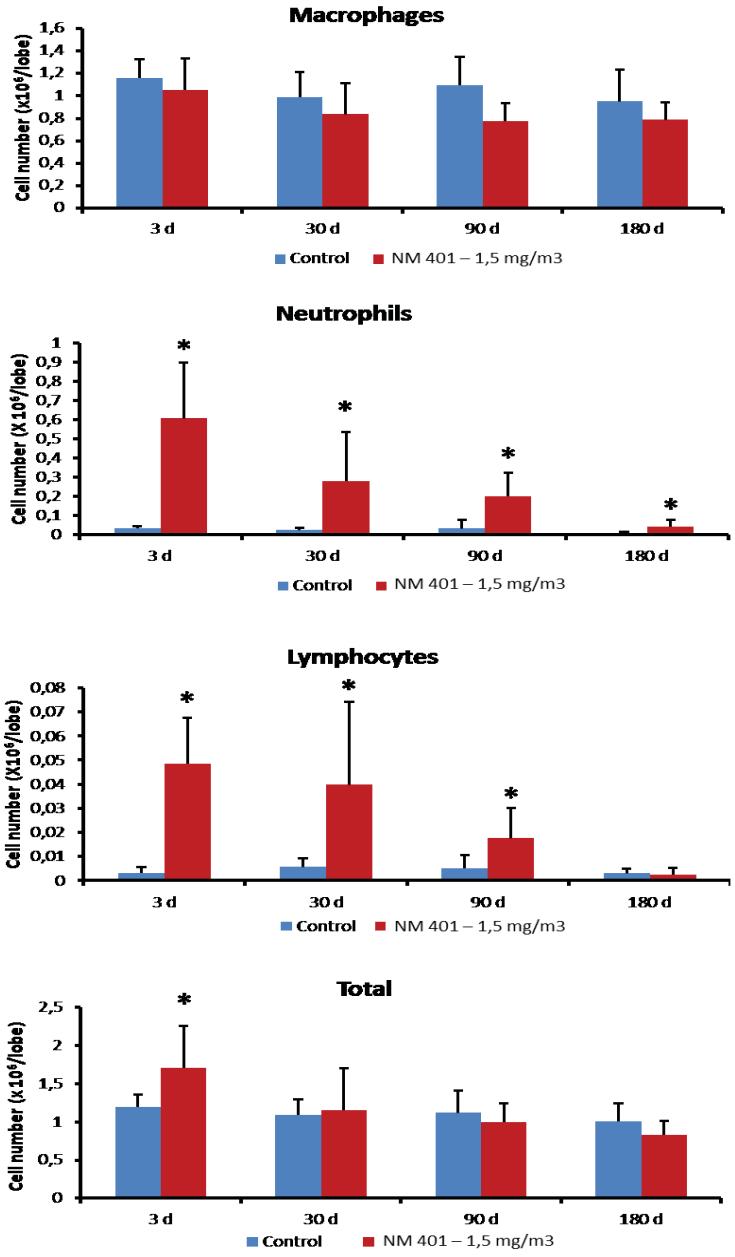

Figure 6: Cytology of bronchoalveolar lavage fluid from control and animals exposed by inhalation to NM-401 (1.5 mg/m^3). * Significantly different from the control (ANOVA and post hoc Tukey HSD test, $p<0.05$).

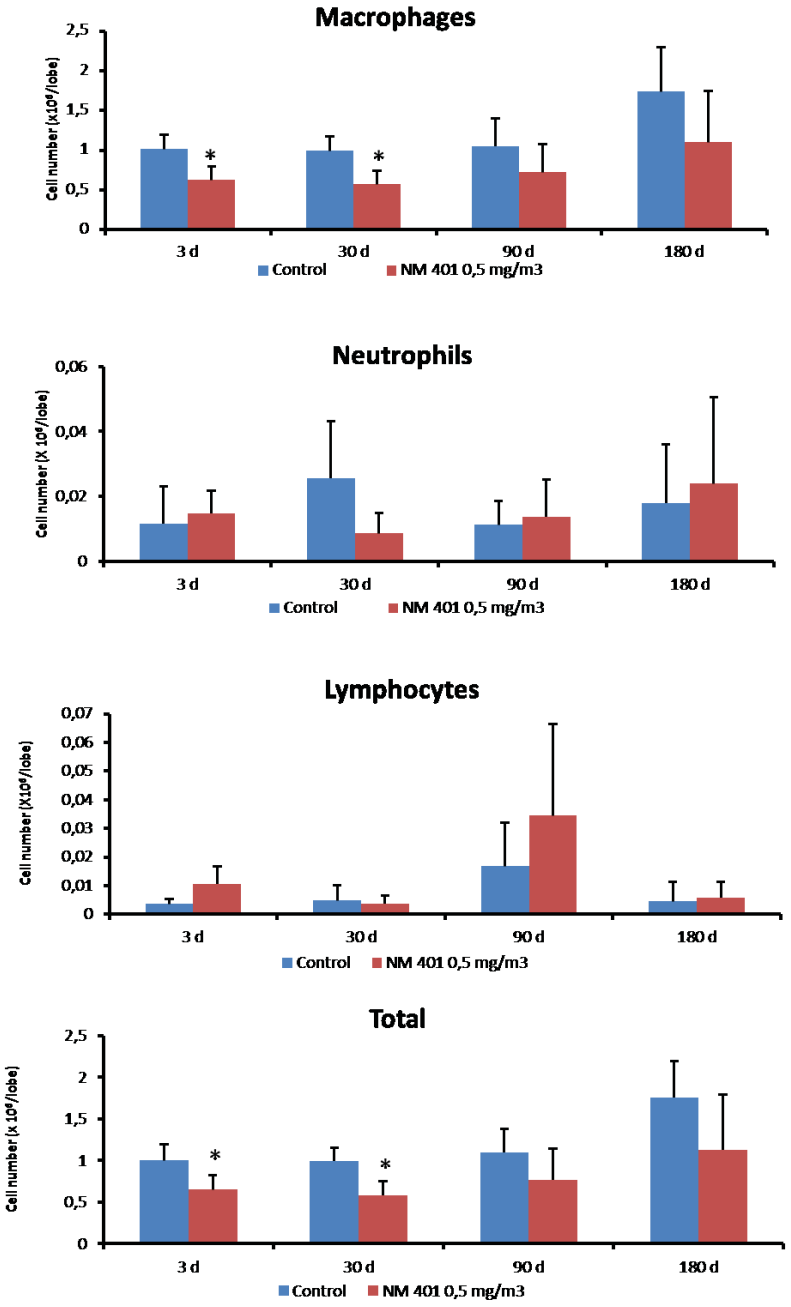

Figure 7: Cytology of bronchoalveolar lavage fluid from control and animals exposed by inhalation to NM-401 (0.5 mg/m^3). * Significantly different from the control (ANOVA and post hoc Tukey HSD test, $p<0.05$).

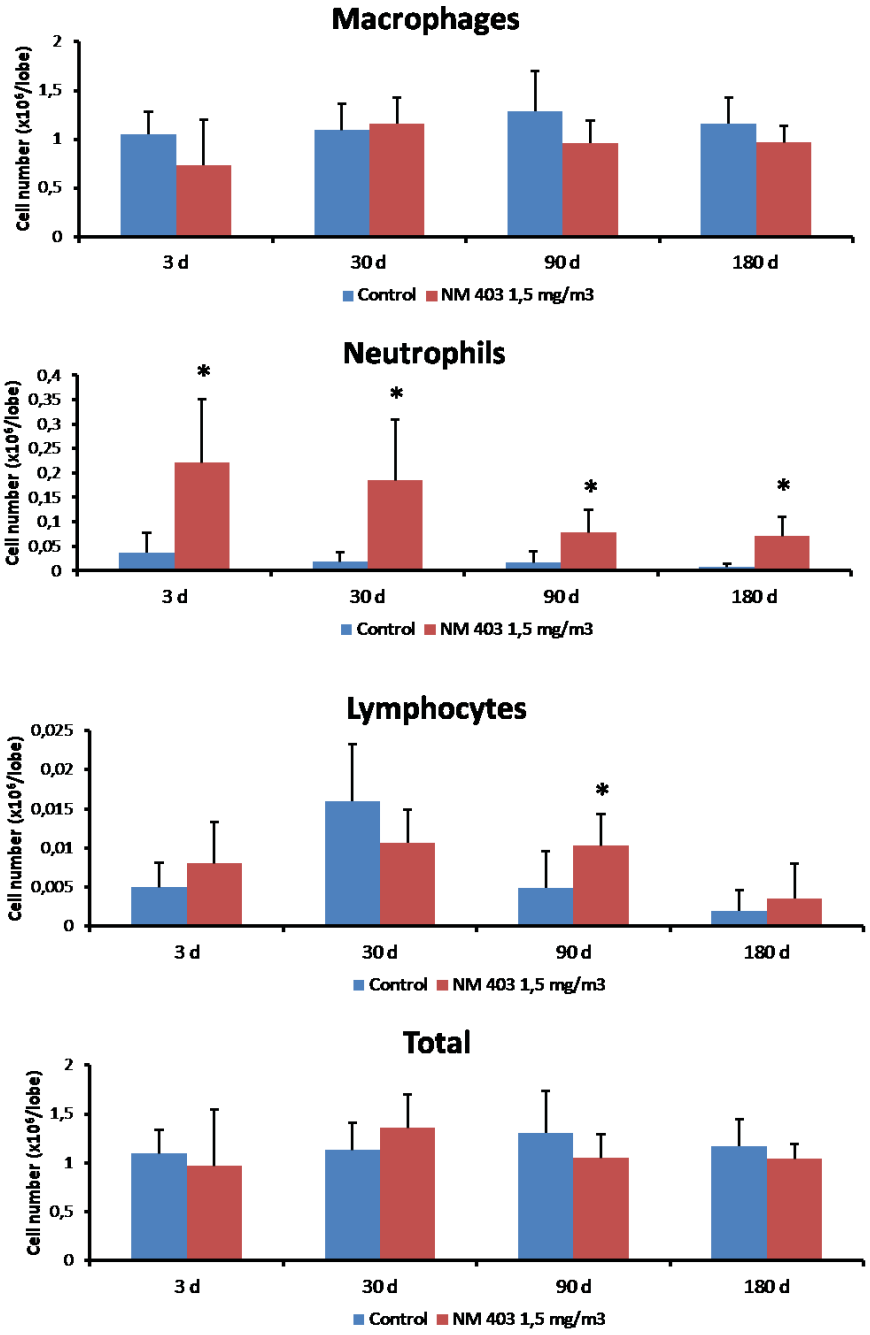

Figure 8: Cytology of bronchoalvolar lavage fluid from control and animals exposed by inhalation to NM-403 (1.5 mg/m^3). * Significantly different from the control (ANOVA and post hoc Tukey HSD test, $p<0.05$).

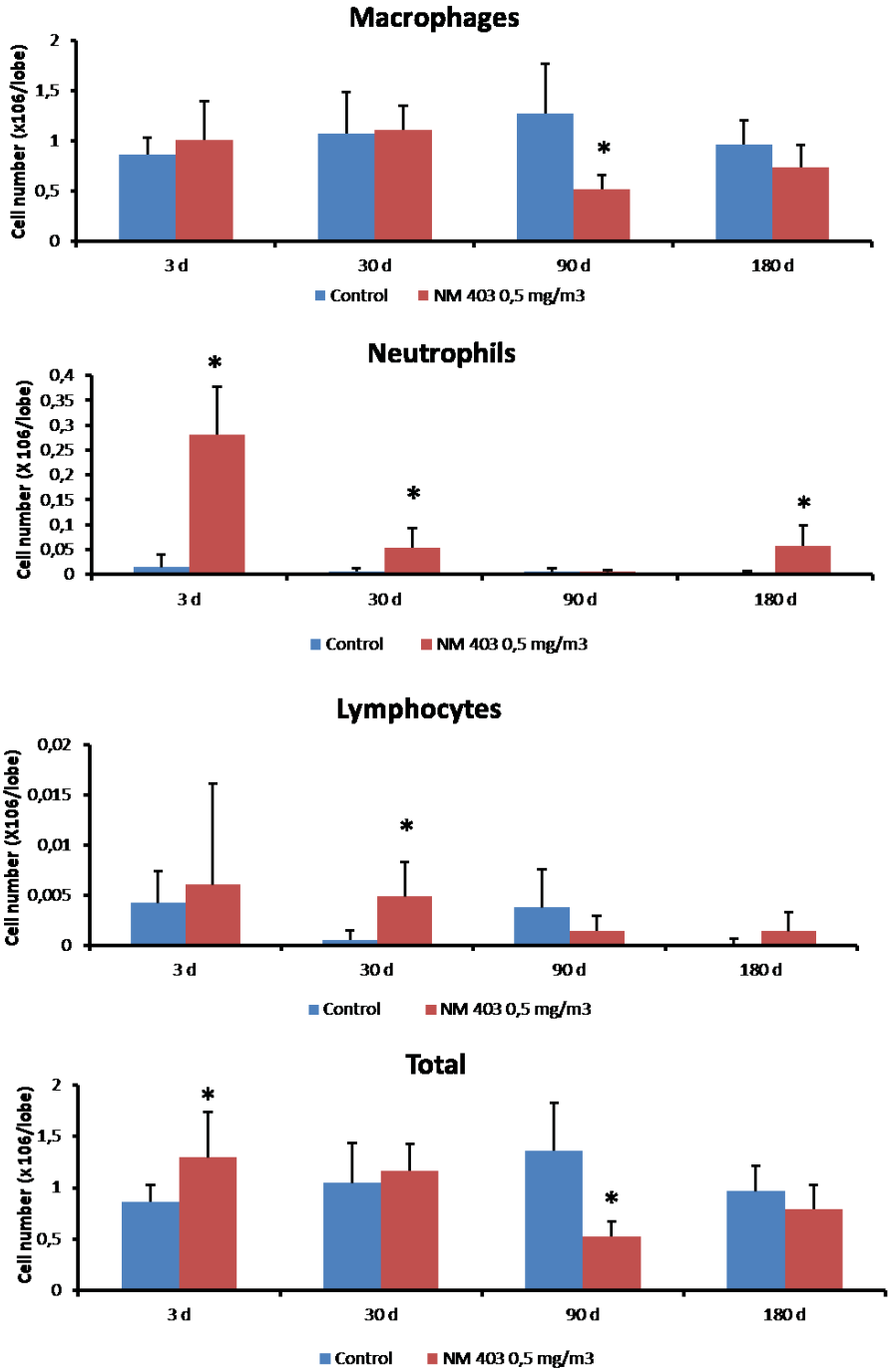

Figure 9: Cytology of bronchoalveolar lavage fluid from control and animals exposed by inhalation to NM-403 (0.5 mg/m^3). * Significantly different from the control (ANOVA and post hoc Tukey HSD test, $p<0.05$).

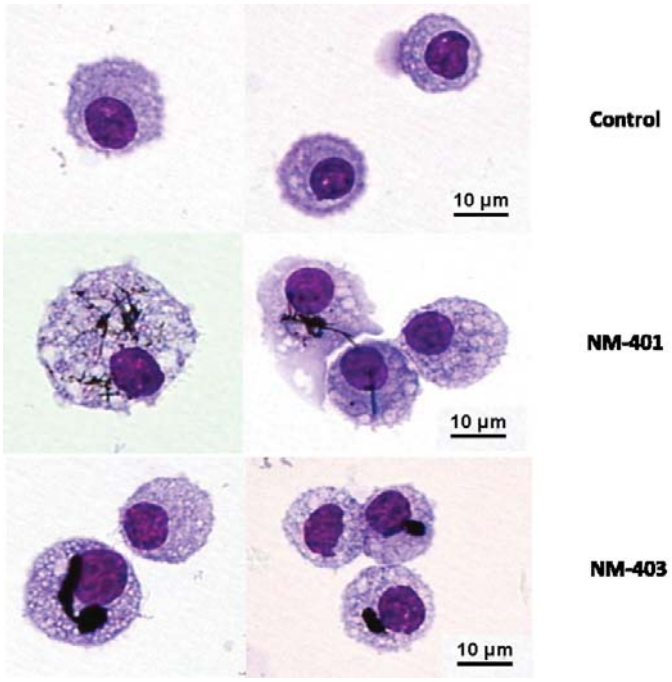

Figure 10: Representative optical microscope images of BALF macrophages from controls and animals exposed to NM-401 or NM-403 aerosol. BALF cells were cytospun on glass slides and stained with the May-Gunwald Giemsa method.

Biochemical markers and cytokines in BALF were changed sometimes until 180 days post-exposure. Lung weight was affected shortly post exposure and there were small differences in the expression profiles of cytokines or proteins

Histopathological examinations were only performed with the highest concentrations of NM-401 and NM-403 in lungs and lung associated lymph nodes.

Significant lung changes in NM-401 treated rats consisted of minimal to mild, number of macrophages containing black particles consistent with carbon nanotubes from 3 to 90 days post-exposure. There were a minimal number of carbon-laden macrophages in NM-401 exposed animals 180 days after the end of treatment. While the number of CNT-positive macrophages in exposed animals 3, 30 and 90 days post-exposure were similar, their distribution in the lung sections differed. Singular, positive macrophages in the 3-day group were randomly disseminated throughout the lung section, mainly in alveoli. In the 30-day exposed group, positive macrophages were present in alveoli, but, also, as discrete aggregates associated with terminal bronchioles. In the 30-day exposed group, fewer positive cells were noted in alveoli and more prominent number of aggregates associated with bronchioles. At the 180 days post-exposure time point, no significant number of positive macrophages was present in alveoli, and the number of macrophage aggregates associated with bronchioles was reduced. Treatment-related changes in lymph nodes consisted of minimal number of carbon, containing macrophages, In the 90 and 180-day exposed groups. Remaining lymph node changes (hemosiderin and erythrophagocytosis) were irregularly present in both control and exposed groups.

No significant difference in amount or distribution of collagen in lung was noted with the Masson's Trichrome stain between control and exposed animals.

For NM-403, at sacrifices on day 3, 30, 90 and 180, all treated rats had a minimal accumulation of dark material consistent with carbon nanotubes in the cytoplasm of alveolar macrophages. This was considered likely to reflect accumulation of carbon nanotubes in alveolar macrophages and was not accompanied by any treatment-related inflammatory change or other alterations of the lung parenchyma. No carbon nanotubes-related changes (such as interstitial fibrosis) were

observed in the Masson's trichrome stained sections of the lung from control and treated rats sacrificed on day 90 and 180 post-exposure.

In the "lung-associated" lymph nodes, a minimal accumulation of dark material, similar to that observed in pulmonary macrophages, was observed in macrophages in the sinuses of the lymph node in one rat, only, which was sacrificed on 180 post-exposure. This was considered to reflect the physiological migration of the pulmonary macrophages into the draining lymph nodes. There were no treatment-related changes in the lymphoid tissue of the lymph node.

DNA damage by the comet assay was determined in several organs. From our data, it was difficult to assess whether the carbon nanotubes NM-401 or NM-403 induced DNA damage in primary target organs (lung and BALF) (Figures 11 and 12) or in secondary tissues (liver, spleen and blood leukocytes). However, with Fpg, NM-403 seemed to induce more DNA damage compared to the control group but the genotoxic effect was not detected for all time-points (Figure 12). NM-401 at the dose of 1.5 mg/m^3 only induced significant DNA damages in lung cells at the 30 days post-exposure time (Figure 11), while at the dose of 0.5 mg/m^3 significant DNA strand breaks were seen at 30 days only with Fpg and at 180 days (with and without Fpg).

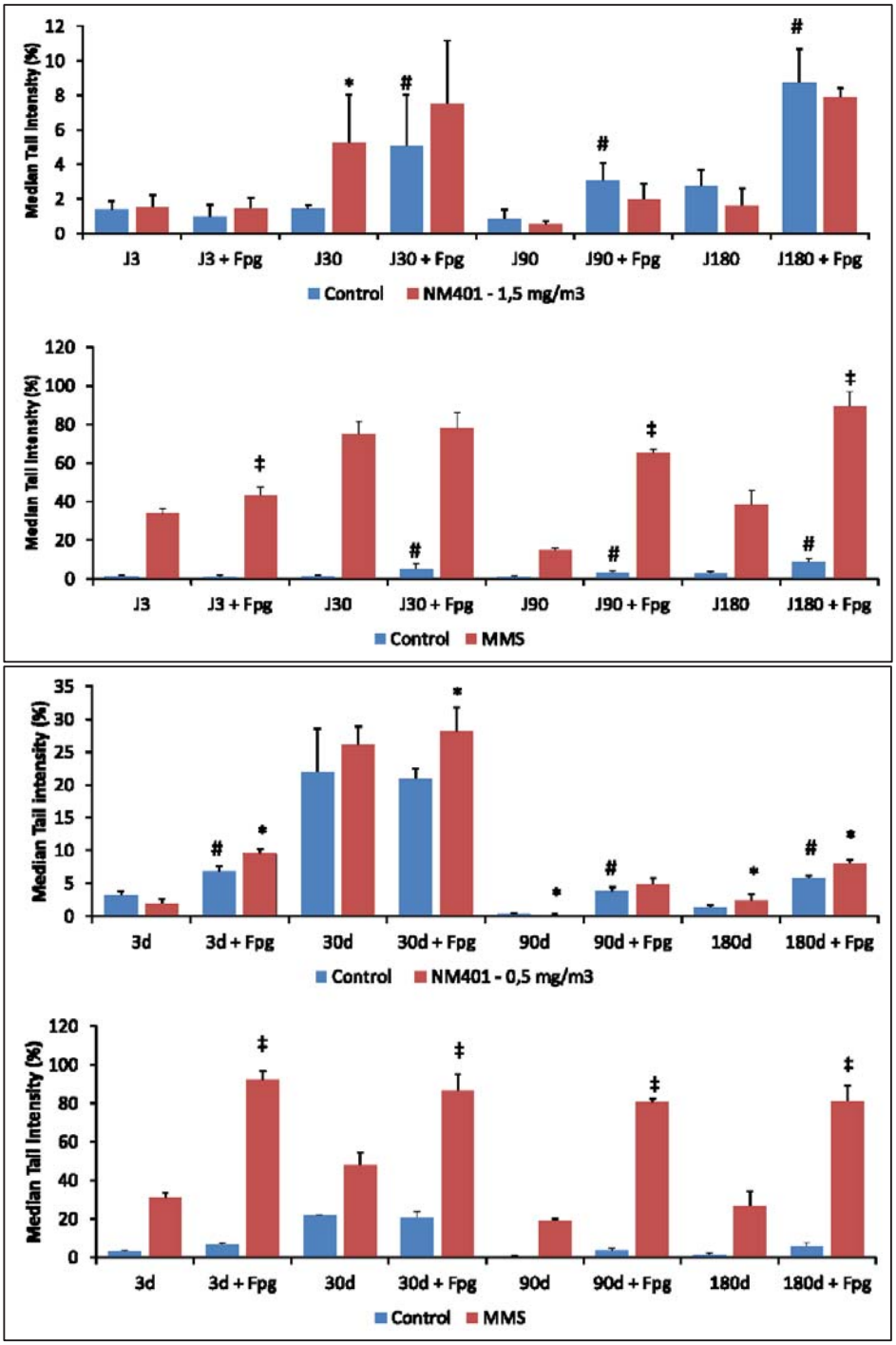

Figure 11: DNA strand breaks assessed by the comet assay in the lung from rats exposed by inhalation to filtered air or NM-401 or by gavage to MMS. All data from MMS were significantly different from the control; * significantly different from the control; ‡ significantly different from MMS alone; # significantly different from the control alone (Mann-Whitney test, $p<0.05$).

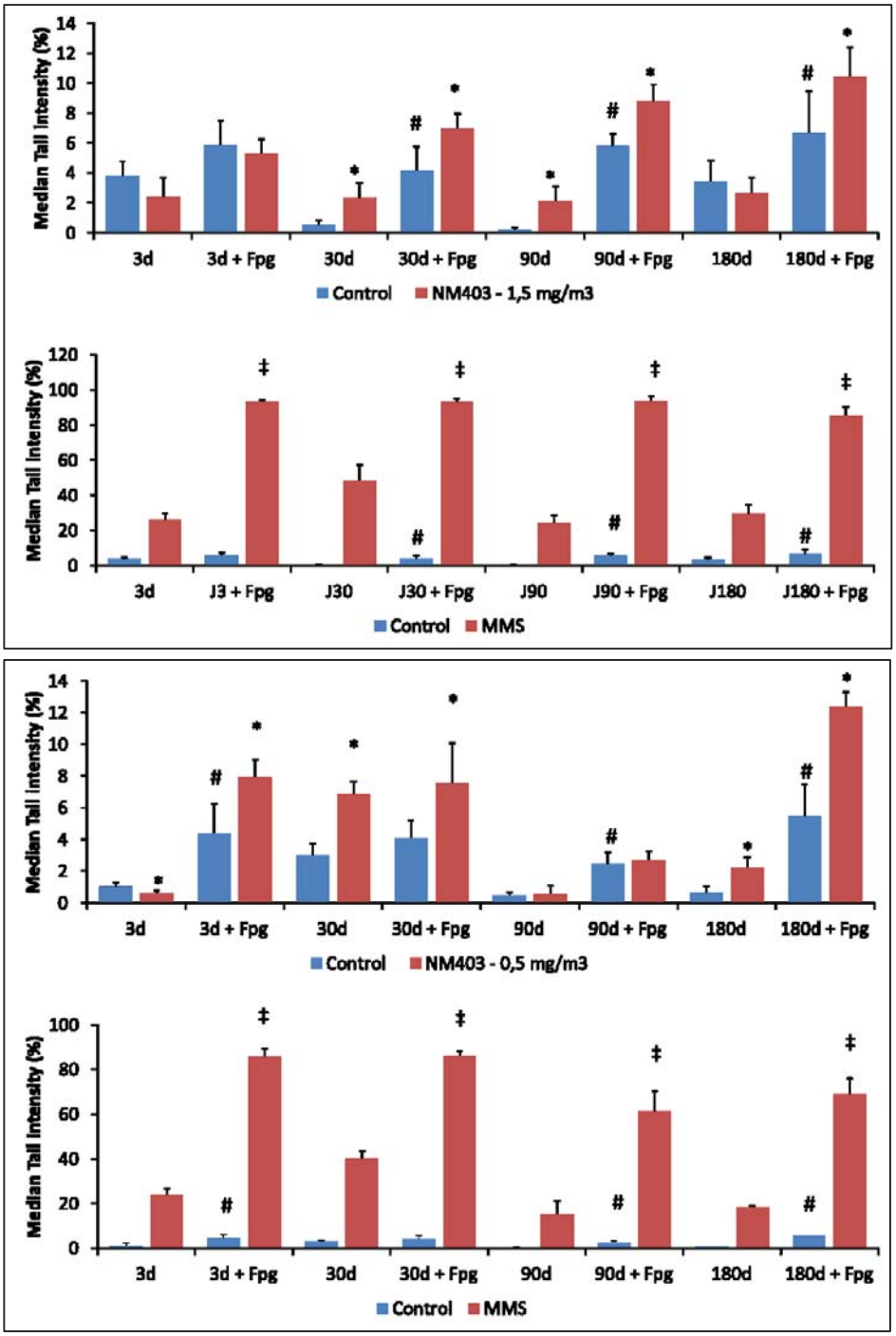

Figure 12: DNA strand breaks assessed by the comet assay in the lung from rats exposed by inhalation to filtered air or NM-403 or by gavage to MMS. All data from MMS were significantly different from the control; * significantly different from the control; ‡ significantly different from MMS alone; # significantly different from the control alone (Mann-Whitney test, $p<0.05$).

For the whole-body inhalation exposure experiments thirteen-week old female Sprague Dawley rats were housed individually in 12h/12h light/dark cycle, and had ad libitum access to food and water both during and post-exposure. They were exposed 6h/day; 5 days/week for 4 weeks to filtered air or 1.5 mg/m^3 of carbon nanotubes, NRWCE-64. The next inhalation exposure will involve a higher concentration (3 mg/m^3) and this will be followed by experiments using NM-401. Biological effects will be examined after 3, 30, 90, and 180 days of the end of exposure. To date limited results only are available for the 1.5 mg/m^3 concentration at 3 and 30 days.

The aerosols were monitored during experiments by real-time devices (Condensation Particle Counter, Aerodynamic Particle Sizer). Samples were taken for off-line analyses (gravimetric analysis, mass size distribution from NanoMOUDI, and TEM imaging). The aerosol particles were typically between 1 – 2 microns in diameter, isometric highly tangled particles with protruding individual MWCNT. The actual mass concentration was 1.56 ± 0.23 mg/m3 and the CMD 1.35 ± 0.01 µm.

Results for BALF cells analysis indicate no significant change in total cells numbers at 3 and 30 days.

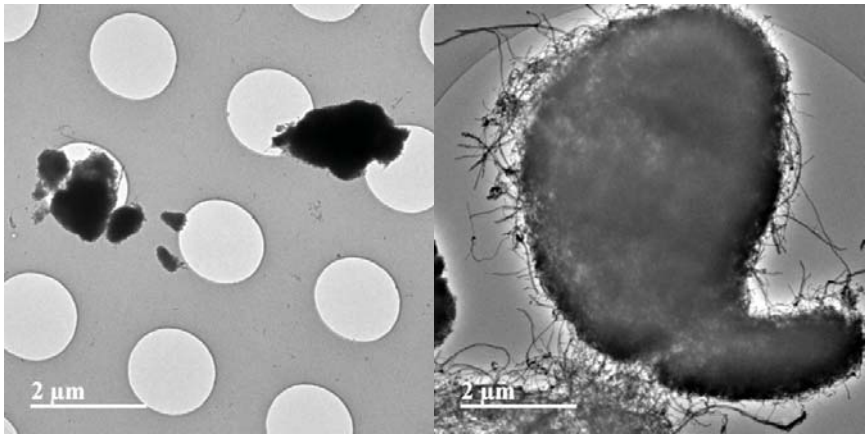

Figure 13: Representative transmission electron microscopy images of NRWCE-64, collected from aerosol.

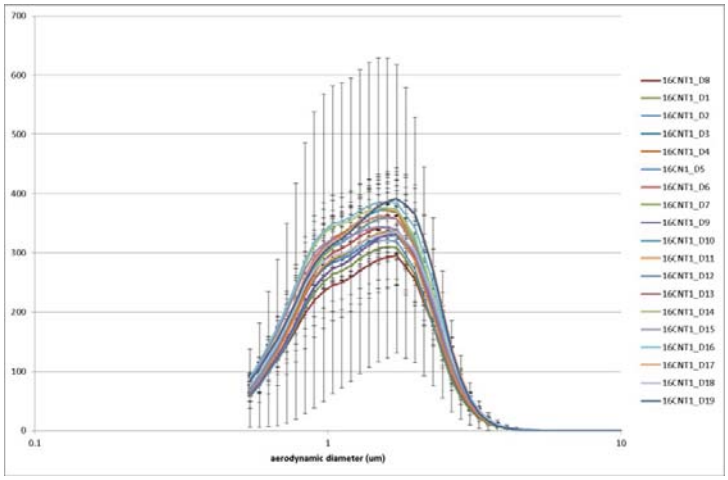

Figure 14: Particle size distribution for the aerosol on different exposure days (Aerodynamic Particle Sizer).

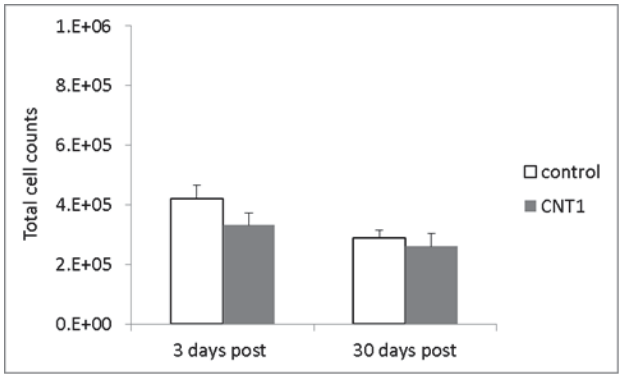

Figure 15: Cytology of bronchoalveolar lavage fluid from control and animals exposed by inhalation to NRWCE-64 (1.5 mg/m3).

4. Data from whole body inhalation study will be included during 2017-2018.

2.5 Evaluation and conclusions

The data indicate that short/curved/highly agglomerated CNT are very differently distributed in the lung than straight longer thicker ones. Over time the latter seem to disperse and diffuse out into the lung tissue whereas the fate of the shorter agglomerated ones is determined by engulfment in macrophages and granulomas. The surface properties affect the infammogenicity of CNT. Nanocelluloses were unexpectedly rather inflammogenic compared to the CNT

From the nose-only inhalation experiments, because of the differences in the shape and the size distribution of the aerosol, the pulmonary deposition of these two nanotubes was different (128-192 µg/lung vs. 362-377 µg/lung for NM-403 and NM-401 respectively). However NM-403 was more inflammogenic than NM-401. Since NM-403 had a specific surface area 10 times greater than NM-401, the use of different metrics may be useful for the interpretation of the data. The genotoxicity of these CNTs has been assessed using the comet assay, even though a thorough analysis of the data is required, it is unclear whether NM-401 and NM-403 are able to induce significant DNA strand breaks in the lung or other organs such as spleen or liver. Nonetheless, in the lung, NM-403 may be more genotoxic than NM-401 and perhaps this may occur through the production of reactive oxygen species.

Similar effects were seen after inhalation and instillation in rats as well as after instillation in mice.

Despite the persistent presence of carbon nanotubes in lung tissues, no significant histopathological changes were observed. The use of more sensitive and high throughput methods like transcriptomics or proteomics would help understanding the molecular mechanisms triggered after the exposure to such nanomaterials.

The interactions of HARN materials the biological effects and the distribution in the lung is dependent on size and agglomerate size. Surface properties were less important but had an effect on the biological effects.

2.6 Data management

Full data from the INRS and the NRCWE have been uploaded into the NANoREG data base and some preliminary data from PHE. Data will be publically available,

3 Deviations from the work plan

There have been some delays with animal experiments at the PHE partner because the final inhalation exposure system components were received late from the manufacturer and the system was commissioned in preparation for inhalation studies in the autumn of 2016. Unfortunately, the delays in receiving the whole-body exposure system and staff availability issues have led to significant delays at PHE. Initial whole-body studies have been carried out using the carboxylated MWCNT, NRWCE-064 at 1.5 mg/m^3 and some very preliminary results are available. A further study will be undertaken using a higher concentration in February. These experiments will then be repeated using NM-401 to allow comparison with the nose-only exposures. Results will be obtained beyond the NANoREG termination.