Towards a method for detecting the potential genotoxicity of nanomaterials

Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis

Final report

Characterisation of MNs for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis
March, 2013

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Co-funded by the Health Programme of the European Union

Grant Agreement n° 2009 21 01

WP6: *In vivo* testing of nanomaterial genotoxicity

Deliverable 6:

Characterisation of MNs for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis

Deliverable leader: ANSES

Valérie Fessard

Laboratory of Fougères

Toxicology of contaminants

10B rue Claude Bourgelat - CS 40608

35306 Fougères cedex, FRANCE

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Workflow	
Author(s) All the WP6 partners	**Reviewer(s)** WP Leader: V. Fessard Coordinator: N. Thieriet, F. Etoré

Document status: v. Creation date: 11/ 03/ 2013

Confidentiality level of the deliverable		
PU	Public	PU
CO	Confidential, only for members of the consortium (including the Commission Services)	

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Contents

Objectives

To generate data from selected in vivo genotoxicity tests, and to assess the correlation between in vivo and in vitro results taking into account the kinetic results.

Schedule

The WP6 lasted for 16 months from October 2011 until the end of the Joint Action (February 2013). Few months at the beginning were devoted to trainings and trials before performing the experiments on Manufactured Nanomaterials (MNs). Even though, some last results are not yet available and will be collected after the end of the Joint Action, especially on the micronucleus on colon as well as on histology.

Methodology

This workpackage investigated the genotoxicity of MNs. For this purpose, 3 complementary tests were performed on rodents:

- Comet assay (early DNA damage)
- Micronucleus assay (chromosome and genome mutations)
- Mutation Lac Z assay (gene mutations).

From an ethical point of view, the genotoxicity tests were deliberately combined in the same animal in order to reduce the number of animals. Therefore, comet and micronucleus tests were performed concomitantly for all the MNs. Similarly, during the mutation LacZ assay, samples for comet and micronucleus assays were also collected.

Moreover, other endpoints, concerning inflammation and oxidative stress which have been reported to be involved in MNs toxicity, were also measured:

- Broncho alveolar cells count for intratracheal instillation
- Histology (mainly if a positive response with the comet assay was detected)
- Modified comet assay with FpG enzyme for selective detection of oxidative lesions as well as lipid peroxidation product malondialdehyde (MDA) in plasma.

Except the mutation LacZ assay which was performed on mice, all the experiments were performed on rats, this species being the main model for toxicological studies. As no indication was available on the gender sensitivity, males (Sprague Dawley or Wistar strains) were chosen as recommended by EFSA (2012) for the in vivo comet assay. Several groups of animals were distinguished with 4 to 5 animals per group: vehicle control, positive control and 3 treated groups (3 doses of MNs). Two routes were principally investigated as representing the main routes of human exposure: intratracheal instillation and gavage. The MNs were administered 3 days in a row at 24h interval and with samples collection 3 to 6 h after the last administration in order to increase the sensitivity and according to the recommendations of EFSA (2012) on the in vivo comet assay. Several organs/tissues were collected both from the site of contact and systemic organs (Figure 1).

For the comet assay, at least 5 organs were investigated among intestine, colon, blood, liver, spleen, kidney and bone marrow for gavage experiment and among bronchoalveolar fluid, lung, blood, liver, spleen, kidney and bone marrow for intratracheal exposure. In most cases, the FpG modified comet assay was also performed.

Bone marrow and colon were also used for micronucleus assay. On bone marrow, the assay was conducted according to the OECD guideline n°474.

Pieces of organs for histology could have also been collected within the same experiment.

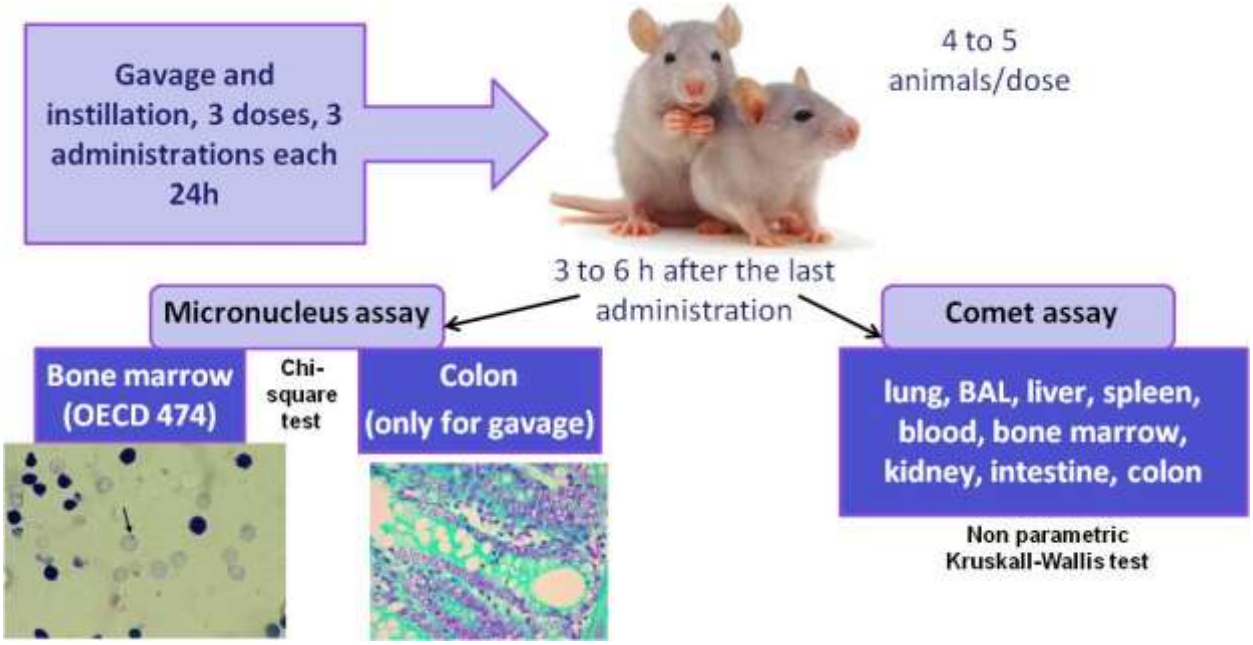


Figure 1: Schematic overview of the genotoxicity tests performed and the organs investigated

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

The Lac Z mutation assay was performed on mice (C57Bl6/J strain). Only two iv (intravenous) administrations at 24h interval were done as some toxicity on the site of injection had been observed in a preliminary trial, after the 3rd injection. Following a fixation period of 28 days after MN exposure, the mutation rate in selected organs was measured. Liver and spleen were investigated as they show some accumulation of TiO2. Complementary genotoxic assays were done concomitantly: comet assay on the selected organs and micronucleus assay in peripheral blood reticulocytes (Figure 2).

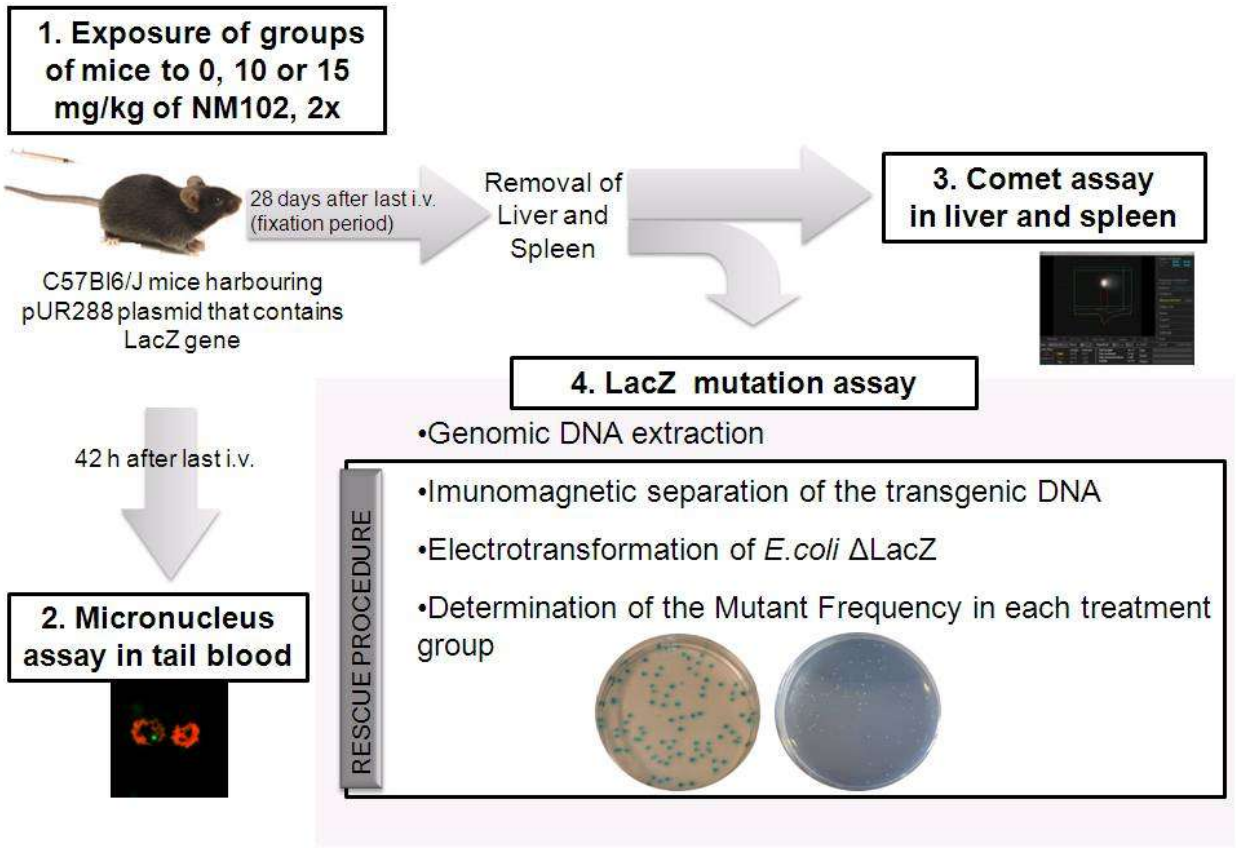


Figure 2: Schematic overview of the Lac Z assay with NM102 and the genotoxic endpoints investigated

Training

Training was necessary for the colon micronucleus assay as only one laboratory out of the three involved in this part of the workpackage had already used this assay for other projects during the last 5 years. Few days training were done in September 2011 in one of the two laboratories. However, since face to face training was not possible for the other laboratory, the most experienced laboratory provided English translation of the protocol, explanations and a video in order to promote the set up of this technique in the last laboratory.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Trials

1) Micronucleus on bone marrow

Trials for the bone marrow micronucleus assay were planned before the experiments with MNs as the work for scoring was shared between several laboratories. In most cases, the laboratory performing the comet assay scored the comet slides and sent the micronucleus bone marrow slides to another laboratory in charge of their scoring. Some slides exchanges were therefore settled between the laboratories so as the quality of the slides provided by the collecting laboratory was correct for the scoring lab.

2) Positive controls chemical and nanosized

Two chemical positive controls were tested: MethylMethaneSulfonate (MMS) and Cyclophosphamide (CPA). They were both administered by gavage according to the schedule of administration (3 administrations, 24h interval and tissue collection after 3 to 6h of the last administration). The results are summarized in the Table 1.

The Carbon Black (CB) Printex® 90 was selected for investigating its use as a nanosized positive control. Both oral and intratracheal studies were conducted and the main results are presented in the Table 1.

Table 1: Results of the comet and micronucleus assays (MNA) following gavage of various investigated compound as positive controls (MMS, CPA and CB)

		Comet assay									MNA bone marrow	MNA colon
		Lung	BAL	Blood	Liver	Spleen	Bone marrow	Intestine	Colon	Kidney		
	MMS	++	++	++	++	++	++	++	++	++	+	+
	CPA	nd	nd	-	-	-	+	-	+	-	toxic	+
CB (µg/kg)	250	nd	nd	-	-	-	-	-	+	-	-	-
	1250	nd	nd	-	-	-	-	-	+	-	-	+
	2500	nd	nd	-	-	-	-	-	+	-	-	+

MMS 100 mg/kg (x3) except for BAL and lung (25 mg/kg x3)
CPA 40 mg/kg (X3)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Set up of protocols

Rodents were bred, maintained, treated and sacrificed according to European Union directives.

An effort was made within this workpackage to reduce the number of animals used in each experiment, by integrating multiple endpoints and analyzing several organs concomitantly in the same animal.

1) Positive controls

1.1 Chemical

According to the results obtained during the trials, the MMS was selected as the chemical positive control. It was given by gavage for both routes (oral and intratracheal) but with a different dose.

For oral administration, it was recommended to give 100 mg/kg 2 days at 24h interval and then 80 mg/kg for the last administration to be sure that it will induce micronuclei formation in the colon. However, for intratracheal route, it was recommended to use 50 mg/kg 48 h before tissue collection and then 25 mg/kg 24 and 3 h before animal necropsy as this dose was enough to induce micronuclei formation in the bone marrow. CPA has been used also as positive chemical for the micronucleus assay in certain laboratories due to historical controls.

However, one laboratory has also added EthylNitrosoUrea (ENU) as a second chemical positive control for blood and bone marrow.

1.2 Nanosized

A genotoxic positive response with CB was only observed on few organs following gavage or intratracheal exposure (table 1). Moreover, some results were inconsistent from laboratory to laboratory and the addition of this compound was not compulsory for in vivo experiments.

2) Comet assay

The comet assay protocol was not harmonized due to the lack of time available for performing those experiments. Therefore, each laboratory used their own protocol both for cell isolation (Tables 2 & 3) and for the technical conditions (V/cm, % agarose, time of unwinding, electrophoresis, FpG concentration and time of incubation, etc).

Table 2. Methods used for cell preparation for the ***in vivo*** **comet assay after instillation** with Nanogenotox MNs. (NP = No Specific Preparation; ND = Not done)

Tissue	BAL fluid	Lung	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	Lavage	Freezing+ mechanical	ND	ND	Freezing+ mechanical	Freezing+ mechanical	Freezing+ mechanical
NM-103	Lavage	Freezing+ mechanical	ND	ND	Freezing+ mechanical	Freezing+ mechanical	Freezing+ mechanical
NM-104	Lavage	Freezing+ mechanical	ND	ND	Freezing+ mechanical	Freezing+ mechanical	Freezing+ mechanical
NM-105	Lavage	Freezing+ mechanical	ND	ND	Freezing+ mechanical	Freezing+ mechanical	Freezing+ mechanical
SiO_2							
NM-200	Lavage	Enzymatic	NP	Flushing	Mechanical	Enzymatic	Enzymatic
NM-201	Lavage	Enzymatic	NP	Flushing	Mechanical	Enzymatic	Enzymatic
NM-202	Lavage	Enzymatic	NP	Flushing	Mechanical	Enzymatic	Enzymatic
NM-203	Lavage	Enzymatic	NP	Flushing	Mechanical	Enzymatic	Enzymatic
MWCNT							
NM-400	ND	Mechanical	ND	ND	Open in liquid	Mechanical	Mechanical
NM-401	Lavage	Mechanical	ND	ND	Open in liquid	Open in liquid	Mechanical
NM-402	Lavage	Mechanical	ND	ND	Open in liquid	Open in liquid	Mechanical
NRCWE-006	Lavage	Mechanical	ND	ND	Open in liquid	Open in liquid	Mechanical

Table 3. Methods used for cell preparation for the ***in vivo*** **comet assay after gavage** with Nanogenotox MNs. (NP = No Specific Preparation; ND = Not done)

Tissue	Intestine	Colon	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	Scrapping + freezing	Scrapping + freezing	Lymphocytes isolation	Flushing + freezing	Freezing + Open in liquid, mechanical	Freezing +Chopping in liquid, enzymatic	Freezing +Chopping in liquid, enzymatic
NM-103	Scrapping + freezing	Scrapping + freezing	Lymphocytes isolation	Flushing + freezing	Freezing + Open in liquid, mechanical	Freezing +Chopping in liquid, enzymatic	Freezing +Chopping in liquid, enzymatic
NM-104	Scrapping + freezing	Scrapping + freezing	Lymphocytes isolation	Flushing + freezing	Freezing + Open in liquid, mechanical	Freezing +Chopping in liquid, enzymatic	Freezing +Chopping in liquid, enzymatic
NM-105	Scrapping + freezing	Scrapping + freezing	Lymphocytes isolation	Flushing + freezing	Freezing + Open in liquid, mechanical	Freezing +Chopping in liquid, enzymatic	Freezing +Chopping in liquid, enzymatic
SiO_2							
NM-200	Scrapping	Scrapping	NP	Flushing	Flushing	Mechanical	Mechanical
NM-201	Scrapping	Scrapping	NP	Flushing	Flushing	Mechanical	Mechanical
NM-202	Scrapping	Scrapping	NP	Flushing	Flushing	Mechanical	Mechanical
NM-203	Scrapping	Scrapping	NP	Flushing	Flushing	Mechanical	Mechanical
MWCNT							
NM-400	ND	Scrapping	ND	ND	Open in liquid	Mechanical	Mechanical
NM-401	ND	Scrapping + enzymatic	ND	ND	Open in liquid	Open in liquid	Mechanical
NM-402	ND	Scrapping + enzymatic	ND	ND	Open in liquid	Open in liquid	Mechanical
NRCWE-006	ND	Scrapping + enzymatic	ND	ND	Open in liquid	Open in liquid	Mechanical

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

3) Micronucleus assays

- On bone marrow, according to the OECD guideline n°474; staining was done whether by Giemsa or by acridine orange
- On paraffin sections of swiss-rolled colon; DNA staining was done with Feulgen and counterstaining with Brilliant Green.

Statistics

1) Comet assay and LacZ mutation assay

The unit for statistical analysis was the animal. For comet values, the Kruskal-Wallis test was first performed. If a positive result was obtained, the Mann-Whitney test with a Bonferroni correction was done in order to know whether data from exposed animals were significantly different from the control. For MMS and ENU, the Mann-Whitney test was used.

2) Micronucleus assays

For bone marrow, blood and colon assays, the chi-square test was done.

3) Cytological and biochemical parameters

Data were log-transformed and an ANOVA followed by a Dunnett test was performed.

Methods and results

1) TiO2

Four MNs (NM102, NM103, NM104, and NM105) were tested. NM 101 was also investigated by intratracheal instillation. The protocol of dispersion agreed on the one used within WP7.
Dispersion protocol:
For each MN, a solution of 2.56 mg/ml in EtOH+0.05% RSA was prepared in glass vials with a total volume of 22 ml and homogenized for 16 min.

1.1 Intratracheal

1.1.1 Materials and Methods

1.1.1.1 Preparation of exposure suspensions

TiO2 samples were prepared according to the dispersion protocol established by WP4. In short, a 2.56 mg/ml stock suspension was prepared by prewetting the TiO2 powder in 0.5 vol % ethanol followed by dispersion in 0.05 wt% Rat Serum Albumin (RSA) in ultrapure water. The sonication of the suspensions was performed on ice (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA, equipped with a disruptor horn Model number: 101-147-037) for 16 minutes on ice/water. The stock suspensions were diluted (9:1 vol/vol) with 10x concentrated phosphate buffer pH 7.4 (702 mg NaH2PO4 x2H2O, 4155 mg Na2HPO4 x7H2O, dissolved in 1L) and used immediately.

1.1.1.2 Dynamic Laser Scattering (DLS) of TiO2 suspensions

The hydrodynamic particle number/size distribution of the particles in the exposure liquids were analysed by photon correlation spectroscopy using a Dynamic laser scattering DLS Zetasizer nano ZS (Malvern Inc., UK.). The number distributions were calculated by the DTS software using the viscosity for H2O (0.6864), temperature of 25 ºC and material refractive (Ri) and absorption indices (Rs) for TiO2 (Anatase: Ri 2.49; Rs 0.10 and rutile: Ri 2.90; Rs 0.10). NM-105 contains 85% anatase and was analysed as anatase. Vehicle controls were analysed using both settings. Scans were performed on all samples to determine optimal measure parameters. A laser attenuation factor of 2 and measurement position of 0.45 mm was optimal for all TiO2 containing samples. It was not possible to find optimal parameters for samples without TiO2 particles (Vehicle control). These were analysed using a laser attenuation factor of 7 and a measurement position of 4.65 mm. All samples were analysed within 30 min after sonication, i.e. within the same time frame as the oral exposures occurred. Data quality was analysed by evaluating the intensity correllelogram, cumulants fit and the distribution fit of the laser scattering intensity data.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

1.1.1.3 Animals and caging conditions

Male Sprague Dawley rats, 4-5 weeks old were obtained from Taconic (Ry, Denmark). The rats were randomly divided into groups of 2-3 housed in polypropylene cages with pinewood sawdust bedding and aspen wood enrichment (Brogaarden, Denmark). The cages were stored in rooms with a 12 h light period from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were 21 ± 2°C and 50 ± 5%, respectively. The cages were sanitized twice weekly. All rats were given free access to tap water and standard mouse chow diet (Altromin no. 1324, Christian Petersen, Denmark). The rats were kept under pathogen-limited conditions and were allowed to acclimatize for 2-3 weeks before they entered the experimental protocol. All rats were 6-7 weeks old at exposure start. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and the Animal Experiment Inspectorate under the Ministry of Justice, approved the study (#2010/561-1779).

1.1.1.4 Experimental protocol (materials and doses)

The rats were exposed to 3 intratracheal instillations on each of 3 consecutive days. They were sedated by 4% isoflurane inhalation before (not during) the instillation procedure. A BD autoguard 16GA (1.7x45 mm, ref: 381857) and a 1 ml syringe was used for the instillation. It was filled with 400 µl of air followed by 200 µl suspension/100g body weight. Animals were dosed with a high (4.6 mg/kg), middle (2.3 mg/kg) or low dose (1.15 mg/kg) of 5 TiO_2s (NM-101, -102, -103, -104 and -105). Additionally, vehicles (Nanogenotox protocol or MilliQ) and MMS (25 mg/kg oral) were tested. The experiment was terminated 3h after last instillation, where the rats were sedated by a minimum of 60 mg/kg of Pentobarbital with lidokain.

1-5	Vehicle	(Nanogenotox Protocol)		x3 days	
6-10	Vehicle	(MilliQ)		x3 days	
11-15	NM-101	(Nanogenotox protocol)	4.6mg/kg	x3 days	
16-20	NM-101	(Nanogenotox protocol)	2.3mg/kg	x3 days	
21-25	NM-101	(Nanogenotox protocol)	1.15mg/kg	x3 days	
26-30	NM-102	(Nanogenotox protocol)	4.6mg/kg	x3 days	
31-35	NM-102	(Nanogenotox protocol)	2.3mg/kg	x3 days	
36-40	NM-102	(Nanogenotox protocol)	1.15mg/kg	x3 days	
41-45	NM-103	(Nanogenotox protocol)	4.6mg/kg	x3 days	
46-50	NM-103	(Nanogenotox protocol)	2.3mg/kg	x3 days	
51-55	NM-103	(Nanogenotox protocol)	1.15mg/kg	x3 days	
56-60	NM-104	(Nanogenotox protocol)	4.6mg/kg	x3 days	
61-65	NM-104	(Nanogenotox protocol)	2.3mg/kg	x3 days	
66-70	NM-104	(Nanogenotox protocol)	1.15mg/kg	x3 days71-75	NM-105
	(Nanogenotox protocol)		4.6mg/kg	x3 days	
76-80	NM-105	(Nanogenotox protocol)	2.3mg/kg	x3 days	
81-86	NM-105	(Nanogenotox protocol)	1.15mg/kg	x3 days	
106- 110	MMS Positive Control (Saline)		oral 25mg/kg	x3days	
111-115	Unexposed Controls				

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

1.1.1.5 Broncho Alveolar Lavage (BAL) and isolation of organs

BAL was withdrawn by flushing the lungs five times with 5 ml 0.9% sterile saline through the trachea. The BAL fluid was briefly centrifuged (400g, 10 min, 4°C) and the total number of live/dead cells was determined by NucleoCounter (ChemoCounter, Denmark). The remaining cells were used for a cytospin slide and comet preparations (with 10% DMSO, stored at -80°C). The collected organs were lung, liver, kidney, and spleen. Small tissue sections of 20-40mg of each organ were snap frozen in liquid N2 and then transferred to -80 °C.

1.1.1.6 Comet and micronucleus assay

For the comet analysis, the tissue sections were in frozen state placed in a metal stapler (diameter 0.5 cm, mesh size 0.4 mm) and quickly homogenised into ice cold Merchant's media for a single cell preparation and protection. BAL or tissue cells were mixed with low melting point agarose (10 µl:90 µl) and 30 ul gels were cast on Trevigen 20 well slides. Final agarose concentration was 0.7%. After overnight lysis the slides were subjected to electrophoresis (38V (1.15V/cm), 292-300 mA; 40 min for BAL cells and 30 min for tissues). The slides were dried and stained (SYBR Green) before being imaged on a fluorescence microscope (Olympus BX41; 100x magnification) equipped with an Allied Dolphin F-145B camera (Vision Technologies) and the automated Imstar Pathfinder scoring system (Imstar, France). Both femurs were collected for micronucleus scoring. Slides were prepared, dried and shipped to the scoring laboratory.

The FpG-Comet assay has not been performed. Several tests were unsuccessful, and FpG results will therefore not be included in this report for intracheal instillation of TiO_2.

The bone marrow from both femurs (separate) was flushed out using 5 ml impuls cytofotometer solution (tri-sodiumcitrate-dihydrate 3.22 g, sodium dihydrogen phosphate dihydrate 3.40 g, disodium hydrogenphosphate dehydrate 3.87 g, citricacid monohydrate 1.17 g, glucose 3.65 g, sodiumchloride 4.96 g, dest.H2O ad 1000 ml). Fifty µl of the well mixed cell suspension was used for a cytospin slide. All slides were May-Grünwald – Giemsa stained before being sent to the scoring laboratory for analysis.

1.1.1.7 Statistical analysis

All comet assay results are shown as mean of five medians ± SD. Statistics was performed by non-parametric Kruskal-Wallis one-way test. Identical analyses were also performed with non-parametric Mann-Whitney U-test yielding very similar results. Statistical analysis was performed using Minitab 15 (Minitab Ltd. UK). The statistical significance was set at $p < 0.05$.

1.1.2 RESULTS

1.1.2.1 Broncho alveolar fluid cytology

Viability of the BAL cells was between -9% and +11% (NM-101 and NM-105 High Dose, respectively) compared to unexposed rats. Nanogenotox vehicle exposed rats had a 3% reduced viability compared to unexposed rats. Figure 3 illustrates the particle induced changes in total cell number as well as in composition of cells in the BAL fluid.

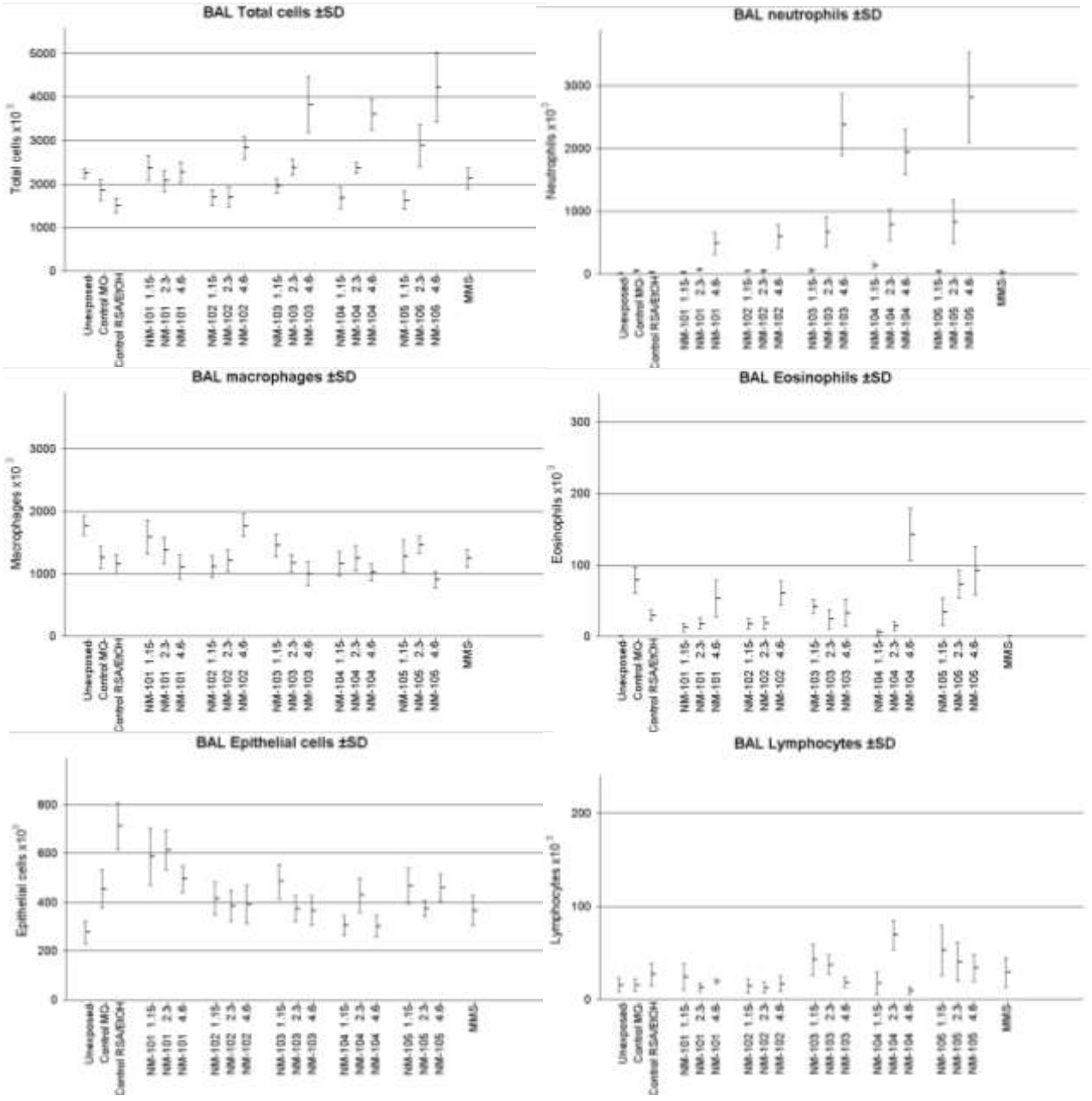


Figure 3. Particle induced changes in the numbers and composition of BAL cells. Visual identification of neutrophils, macrophages, eosinophils, epithelial cells and lymphocytes from control and exposed rats. Results represent mean ± SD.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

All materials showed a dose dependent increase in neutrophils. However, the influx of neutrophils was much greater with materials NM-103, NM-104 and NM-105 (hydrophobic rutile, hydrophilic rutile and 15% rutile) compared to NM-101 and NM-102 (anatase).

1.1.2.2 Comet assay

Imaging/scoring of the comet assay was performed on BAL cells, lung, liver, spleen and kidney tissue.

In BAL and lung, the level of genotoxicity was not increased for any of the TiO2 exposed groups (Figure 4).

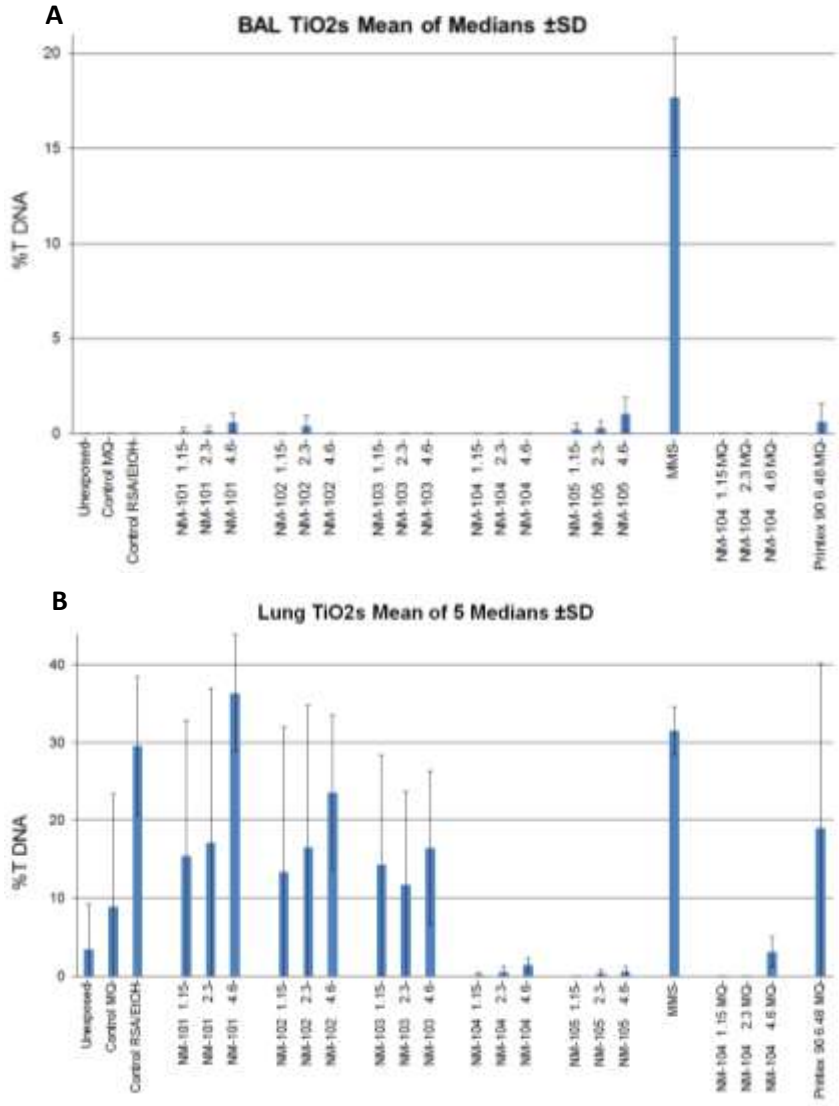
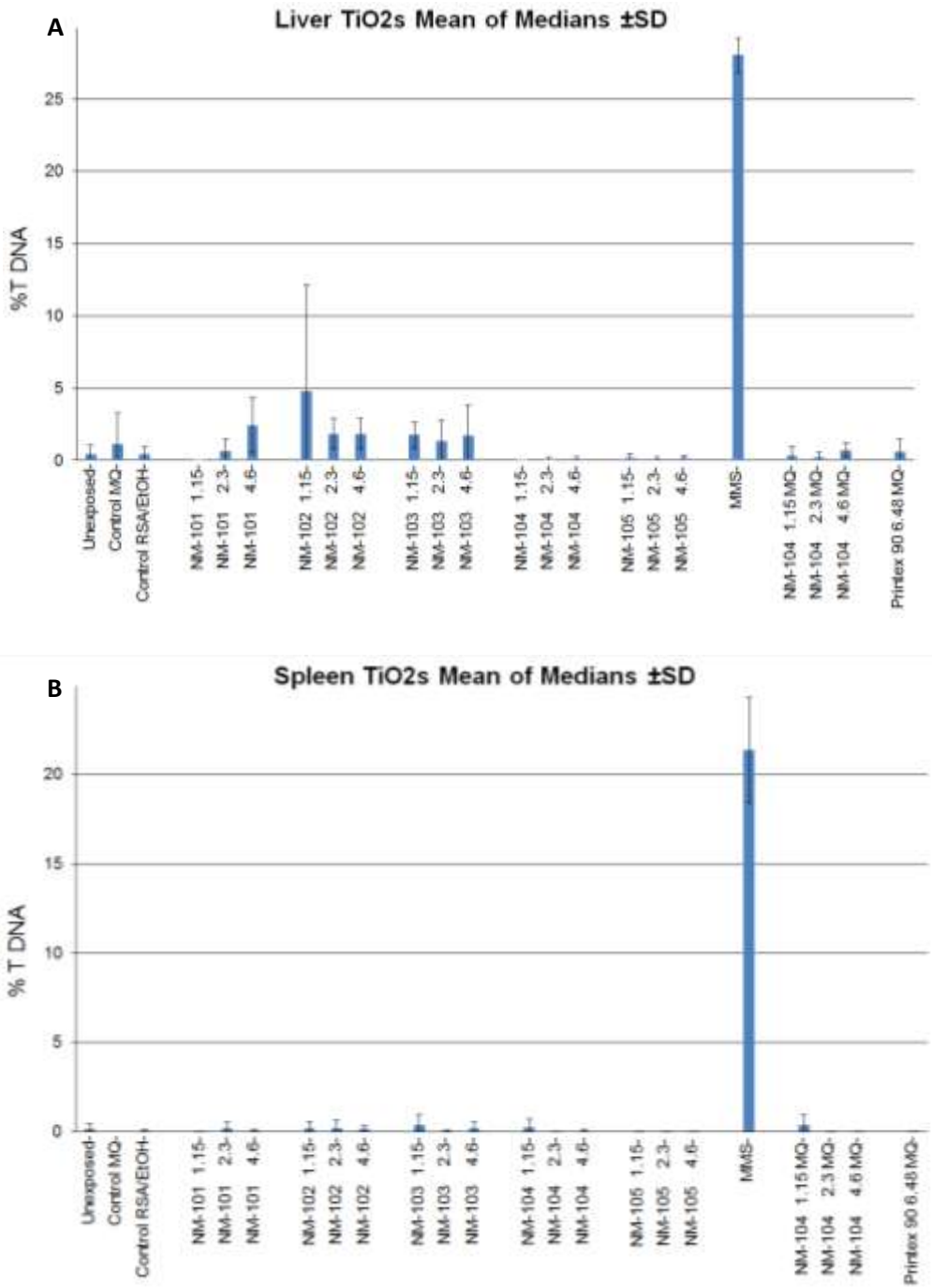


Figure 4. Genotoxicity measured as median %DNA in the tail. The mean of five medians ± SD are shown for BAL cells (A) and lung (B) after TiO_2 intratracheal instillation to rats. MMS was used as a chemical positive control. MQ= in MilliQ water.

In liver, spleen and kidney, the level of genotoxicity was not increased for any of the TiO_2 exposed groups (Figure 5).



The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

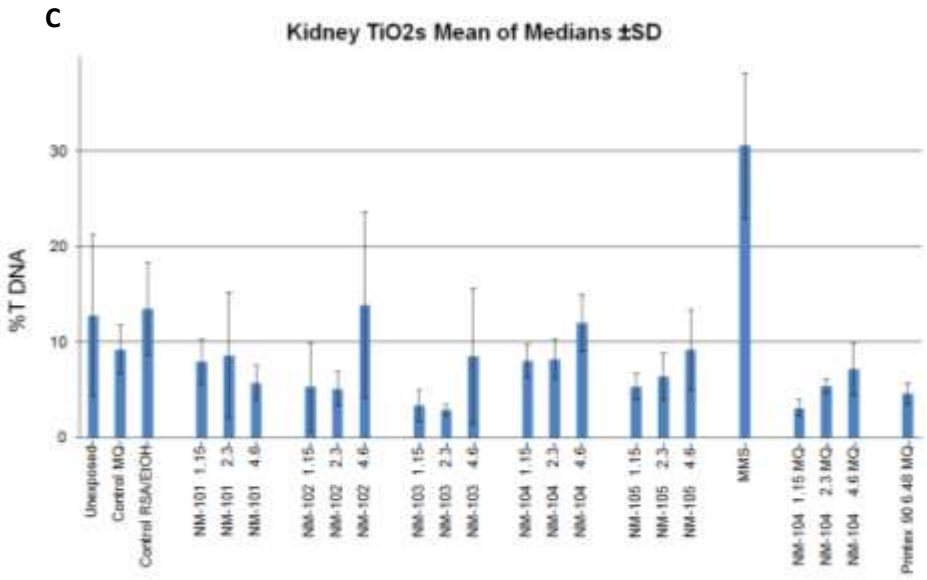


Figure 5. Genotoxicity measured as median %DNA in the tail. The mean of five medians ± SD are shown for Liver (A), spleen (B) and kidney (C) after TiO_2 intratracheal instillation to rats. MMS was used as a chemical positive control. MQ= in MilliQ water.

All TiO_2 materials caused pulmonary inflammation determined as influx of neutrophils. Largest inflammation was caused by the rutile containing NM-103, NM-104 and NM-105. However, no statistical effect was noted on the genotoxicity in any of the tested organs. These results are in line with one of our previous studies were instillation of TiO2 caused pulmonary inflammation but no genotoxicity (Saber et al., 2012).

Very low median values were observed for some organs (BAL cells, spleen and partly liver). All of these results show much higher values when viewed as tail-length and mean % DNA in tail. It therefore appears that for some organs a majority of cells are less damaged than a minority. However, for all mentioned measures the conclusion would be the same; no statistically significant genotoxicity observed.

Our median values may also be slightly lower than others due to the corrections made by the Imstar Pathfinder system. This system corrects for the halo of DNA diffused in all directions. This amount of DNA is subtracted from the tail.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

1.1.2.3 Micronucleus assay

A total of 230 slides were received in duplicate Bone marrow slides of 40 animals out of 115 could not be read due to the abundance of cells in the slide preparation. Both slides from the right and the left femurs were scored.

No increase in micronucleus frequency was induced in all the TiO_2 exposed groups (Table 4).

Table 4: Genotoxicity (frequency of micronucleated Immature Erythrocytes) and toxicity (PCE/NCE ratio) induced in bone marrow by TiO_2 following intratracheal instillation. Positive control MMS was administered by gavage as described in the Material & Methods. PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes

		Genotoxicity				Toxicity			
	[mg/kg]	Micronucleated Cells Mean	SD	n	SE	PCE/NCE ratio Mean	SD	n	SE
NM 101	0	0,323	0,150	10	0,047	1,203	0,228	10	0,072
	4,6	0,350	0,235	6	0,096	1,662	0,313	6	0,128
	2,3	0,386	0,090	7	0,034	1,675	0,443	7	0,167
	1,15	0,313	0,164	8	0,058	2,044	0,647	8	0,229
MMS	25	1,320	0,239	5	0,107	1,334	0,107	5	0,048
NM 102	0	0,323	0,150	10	0,047	1,203	0,228	10	0,072
	4,6	0,430	0,116	10	0,037	1,180	0,210	10	0,066
	2,3	0,525	0,249	8	0,088	1,593	0,391	8	0,138
	1,15	0,388	0,136	8	0,048	1,485	0,447	8	0,158
MMS	25	1,320	0,239	5	0,107	1,334	0,107	5	0,048
NM 103	0	0,323	0,150	10	0,047	1,203	0,228	10	0,072
	4,6	0,343	0,190	7	0,072	1,307	0,431	7	0,163
	2,3	0,214	0,135	7	0,051	1,840	0,521	7	0,197
	1,15	0,250	0,122	6	0,050	1,893	0,404	6	0,165
MMS	25	1,320	0,239	5	0,107	1,334	0,107	5	0,048
NM 104	0	0,323	0,150	10	0,047	1,203	0,228	10	0,072
	4,6	0,125	0,126	4	0,063	1,744	0,266	4	0,133
	2,3	0,217	0,117	6	0,048	1,890	0,471	6	0,192
	1,15	0,150	0,100	4	0,050	1,732	0,198	4	0,099
MMS	25	1,320	0,239	5	0,107	1,334	0,107	5	0,048
NM 105	0	0,323	0,150	10	0,047	1,203	0,228	10	0,072
	4,6	0,200	0,089	6	0,037	2,059	0,709	6	0,290
	2,3	0,433	0,266	6	0,109	1,631	0,389	6	0,159
	1,15	0,371	0,125	7	0,047	2,427	0,725	7	0,274
MMS	25	1,320	0,239	5	0,107	1,334	0,107	5	0,048

1.1.2.4 DLS of TiO2 suspensions

The number/size distribution of the suspended TiO2 particle preparations used for oral gavage was characterised by dynamic light scattering (DLS). Analysis of the vehicle solution was challenging, and the DTS software could not establish optimal measuring parameters. The count rate and the results (Z-average, intensity) varied highly between each of the 12 analysis, leading to a high Polydisperty Index and unreliable DLS data. Analysing the vehicle for rutile or anatase TiO2 did not influence the results.

All TiO2 containing suspensions yielded reliable results according to the DTS software (Table 5). The majority of particles occurred as agglomerates between 80–150 nm (peak number distributions, Figure 6). Suspensions of NM-103, NM-104 and NM-105 contained the smallest agglomerates (80-90 nm) whereas the largest agglomerates were observed with NM-101 and NM-102 (140-150 nm). NM-101 and NM-102 additionally showed a bi-modal size-distribution with another much less frequent mode of ~1 µm. No agglomerates were observed between 3 µm and 10 µm (the upper limit of the Zetasizer nano ZS) for any of the materials.

Table 5. Z-average (intensity distribution peak) and Polydispersity Index (PdI) of the TiO2 suspensions used for oral gavage. Results are the mean of 12 analyses conducted on two separate days. *A high Polydispersity Index means unreliable data.

Material	Conc.	Vehicle	Z-Average (int)	PdI
NM-101	2.56	EtOH/RSA/PBS	278 nm	0.323
NM-102	2.56	EtOH/RSA/PBS	367 nm	0.290
NM-103	2.56	EtOH/RSA/PBS	127 nm	0.384
NM-104	2.56	EtOH/RSA/PBS	108 nm	0.274
NM-105	2.56	EtOH/RSA/PBS	93 nm	0.151
Control	0.00	EtOH/RSA/PBS	477 nm	0.817*

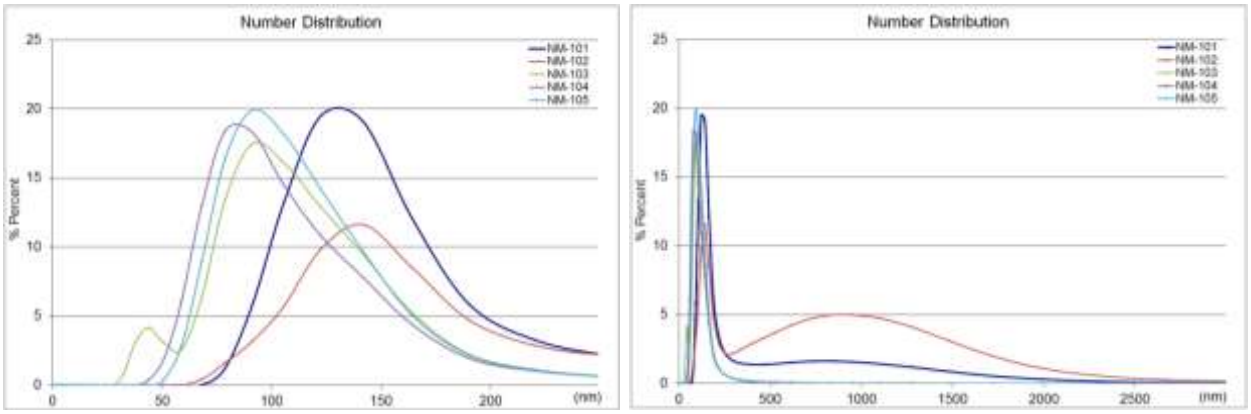


Figure 6. Hydrodynamic number/size distribution of NM-101, -102, -103, -104 and -105 (left and right). The majority of particles were observed as agglomerates between 50-200 nm. Suspensions of NM-101 and NM-102 contained an additional mode between 250 and 2500 nm. No agglomerates were observed above 3 µm. Results are the mean of 12 analyses conducted on two separate days.

1.2 Gavage

1.2.1 MATERIALS AND METHODS

1.2.1.1 Animals

Male, 8- 10 weeks old Wistar rats were obtained from the animal facility of the Bulgarian Academy of Sciences (EBBA-BAS, Slivnitsa, Bulgaria). They were randomly assigned to 4 experimental groups, with two controls (placebo and positive (MMS) control) with five animals per group. Following 5-10 days of adaptation, one millilitre of TiO2 (NM102, -103,-104, and -105) dispersion, vehicle and MMS were given by gavage once daily for 3 consecutive days. Three hours following the last gavage, the animals in different groups were anaesthetised with ketamine (100 mg/kg b.w) and sacrificed. Tissue and blood collections are described below. During all housing period the animals were kept in Tecniplast cages at a room temperature of 21 ± 2°C, a relative humidity of 50 ± 5% and a 12-h light/dark cycle. The drinking water and conventional feed (Complete lab chow, Topmix Ltd, Kaloyanovo, Bulgaria) were provided ad libitum. These experiments were approved by the Animal Research Committee of the Institute of Neuroscience – BAS (Protocol N 27/02.06.2011) prior to the study.

1.2.1.2 MN Sample preparation

MNs samples were prepared according to the dispersion protocol developed within WP4. In short, a 2.56 mg/ml stock suspension was prepared by pre-wetting the TiO2 powder in 0.5 vol % ethanol followed by dispersion in 0.05 wt% Rat Serum Albumin (RSA) in ultrapure water. The sonication of the suspensions was performed on ice (Cell Disruptor Sonicator W-375 (Ultrasonic Inc.)) for 16 minutes at 40% power output. The stock suspensions were used for the gavage within 30 min.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Methyl methanesulfonate (MMS) was purchased from Sigma Aldrich (ref# 129925), diluted in 0.9% NaCl and administered by gavage.

1.2.1.3 Tissue collection and cells' isolations

Blood samples from treated animals were collected together with liver, spleen, kidney, jejunum, colon, tibia and feces. Immediately after collection, the bone marrow cells and epithelial cell from the colon and jejunum were isolated in Epithelium growth media (EBM) or Bone marrow one, and were frozen at -80 °C (in cell protected media) until further analysis.

- Bone marrow isolation

Following the dissection and cleaning of tibia, both ends of the bone were opened and flushed with 6 ml serum into a 50 ml tube. The suspensions were mixed well with a sterile pipette and load on cell strainers (70 and 50 µm mesh), centrifuged at 170 g for 10 min at 23 °C (Eppendorff 5430R) and wash two times with EBM media. The supernatant was removed and the remaining cell pellet was suspended in 2 ml EBM. The cells for comet assay were frozen in EBM media containing 20%FBS and 10% DMSO until further analysis.

- Lymphocytes isolation

Blood sample (+anticoagulant): 1 ml PBS (or 1ml RPMI medium (w/o PhenolRed)+10% FCS) was added to the 30 µl blood in a 1.5 ml Eppendorf tube. Mixed and kept on ice for 30 min. Then underlay with 100 µl Histopaque 1077 (Sigma), using a pipette. Span at 200 x g, 3 min, 4°C. Lymphocytes were retrieved in 100µl in the layer just above the boundary between PBS (RPMI) and Histopaque. They were washed with 1 ml PBS and centrifuged again. The supernatant was removed as much as possible and kept for comet analysis.

- Liver, kidney and spleen cells isolation

Cells from liver, kidney and spleen were obtained by enzymatic tissue dissociation using a mixture of Collagenase type IA and papain (Sigma Aldrich) at 37 °C for 1h. In case of incomplete dissociation the samples were further destructed by pipetting or with Xenox motorized hand tool. The cell suspensions were filtered through cell strainers (70 and 50 µm, Fisherbrand)) and frozen at -80 °C in cell protected media until further analysis.

- Colon and Jejunum

Following the washing of the colon and the intestine with PBS they were open and the internal side was scratched with a scrapper and mixed with EBM media. The mixtures were pippeted up-down several times and were filtered through cell strainer (50 µm, Fisherbrand). The cell suspension was centrifuged at 800 x g for 5 min and the resulting pellet was washed twice with PBS. Finally the cells were used immediately for comet assay or stored at -80 °C in cell protected media until further analysis.

The counting of the cells was performed using an automated cell counter Countess (Invitrogen) following staining with Trypan blue.

1.2.1.4 Comet assay

The alkaline version of the comet assay was performed following the recommendations of Tice et al. (Tice et al. 2000), Burlinson (Burlinson et al. 2007), and Guidance on a Strategy for Testing of Chemicals for Mutagenicity (2000). The formamidopyrimidine DNA glycosylase (FpG) was used to detect oxidative DNA damage according to Collins et al (Collins et al. 2008).

Briefly, cells were embedded in 0.8 % of low melting agarose (Invitrogen) and spread on microscope slides pre-coated with 1 % agarose or GelBond films (Lonza). After lysis for 1h at 4°C (buffer: 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris-HCl, pH10, 10 % DMSO and 1 % Triton X100) and equilibration, the electrophoresis was performed in buffer containing 0.3 M NaOH and 1 mM EDTA (25V, ~300 mA, 30 min at 4°C). Following electrophoresis the slides or films were neutralized by 3 x 5 min washes with neutralizing buffer (0.4 M Tris, pH 7.5) in horizontal staining jars at 4°C and the excess of the neutralizing buffer was carefully blotted with filter paper. The slides were washed twice with ultrapure water for 5 min and dehydrated for 10 min into 96% ethanol before being dried at 45°C and stored in the dark at room temperature.

For FpG comet assay, the films were supported by slides, washed three times in enzyme buffer (50 mM Na3PO4, 10 mM EDTA, 100 mM NaCl, pH 7.5), drained and incubated with 50 µl of either buffer or FpG (Sigma Aldrich, USA) (1 µg/ml in enzyme buffer) placed onto gel and cover with cover slip, and then transferred into moist box (prevents desiccation), and incubate at 37ºC for 30 min. The slides were then placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 40 min at 4°C to allow denaturing and unwinding of the DNA and the expression of alkaline-labile sites. The running conditions were the same as above.

For scoring, the slides were stained with 4'6-diamidine-2-phenylindol dihydrochloride (DAPI, 1µg/ml). Pictures were taken with a fluorescence microscope (Axiovert 200M, Zeiss) and the analyses were performed with TriTek CometScore™ software (TriTek Corporation). A minimum of 100 nucleoids per slide were scored and the percentage of DNA in the tail was recorded.

1.2.1.5 Titanium content analysis

Atomic emission spectrometer with inductively coupled plasma (ICP-OES, ULTIMA 2, HORIBA Jobin Yvon) was used for the determination of titanium concentration in samples solutions (optimal instrumental parameters are presented in details in Deliverable #7 for NM-105). Results obtained are calculated and expressed as micrograms Ti or TiO2 per gram fresh tissue or faeces.

- ❖ Tissue analysis

Titanium content analysis in the tissue samples were performed according to the protocols developed in WP7 for NM-105.

- ❖ Feces analysis

The collection of feces began following the first gavage and ended at the time of euthanizing. All collected feces were kept frozen until the analysis. Frozen feces were homogenized and two parallel samples were digested according to the procedure: About 1 g sample was weighed in a high pressure Teflon vessels for microwave digestion, 5 ml c.HNO_3 (ultrapure grade, Merck) were added and

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

samples left to stay overnight (8 hours). Than 0.5 ml HF (ultrapure grade, Merck) were additionally added and samples were digested in a microwave assisted digestion system (CEM MARS, USA) according to the program: step 1: 5 min at 300W; step 2: 5 min at 600W. After cooling the solutions were quantitatively transferred to plastic tubes and diluted to 12 ml with Milli Q water.

- Food analysis

Procedure for food analysis: About 0.5 g complete lab chow (Topmix Ltd, Kaloyanovo, Bulgaria) was weighed in a high pressure Teflon vessels for microwave digestion, 5 ml c. HNO_3 (ultrapure grade, Merck) were added and samples left to stay overnight (8 hours). Than 0.3 ml HF (ultrapure grade, Merck) were additionally added and samples were digested in a microwave assisted digestion system (CEM MARS, USA) according to the program: step 1: 5 min at 300W; step 2: 5 min at 600W. After cooling the solutions were quantitatively transferred to plastic tubes and diluted to 10 ml with Milli Q water.

1.2.1.6 Statistical analysis

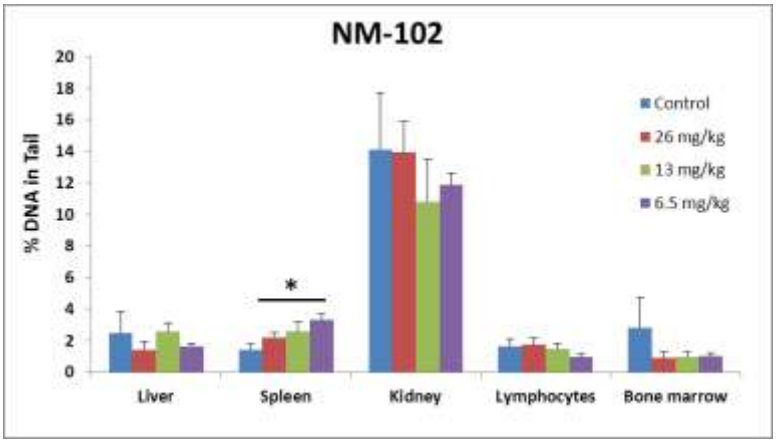
Results were expressed as mean ± standard deviation. For Ti concentrations multigroup comparisons of the means were carried out by one-way analysis of variance (ANOVA) followed by Bonferroni's test to compare the differences between the groups.
The results from comet assay were analysed by multi-group comparisons of the means were carried out by Mann–Whitney U test followed by Jonckheere-Terpstra trend test (PASW Statistics 18.0, IBM, USA). The statistical significance for all tests was set at $p < 0.05$.

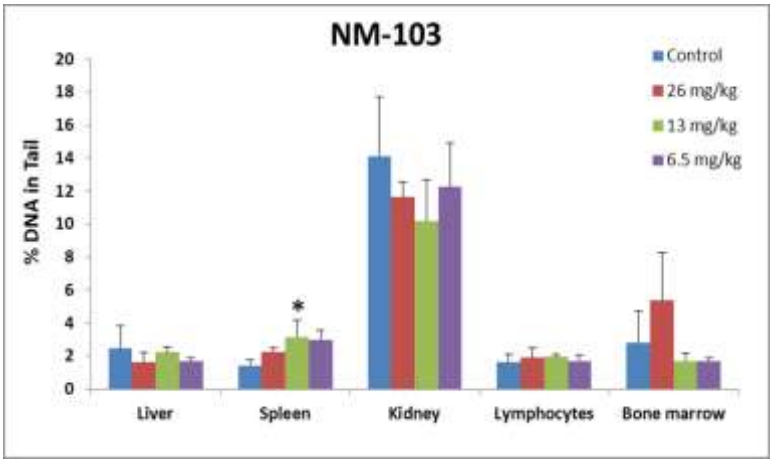
1.2.2 RESULTS

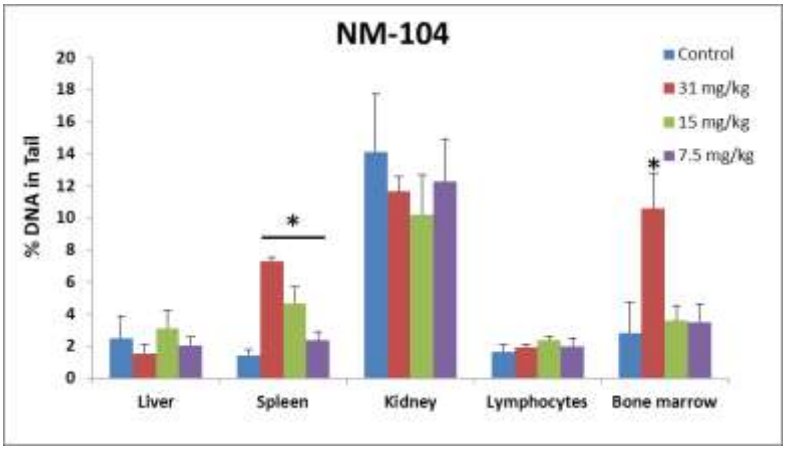
1.2.2.1 Toxicity

The gavage performed with NM 102, 103, and 104 was well tolerated in all animals studied, with exception of NM 105 (26 mg/kg b.w) where diarrhea was registered in 3 animals on day 3. In all other animals no adverse effects were observed during the experiment.

1.2.2.2 Comet assay







The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

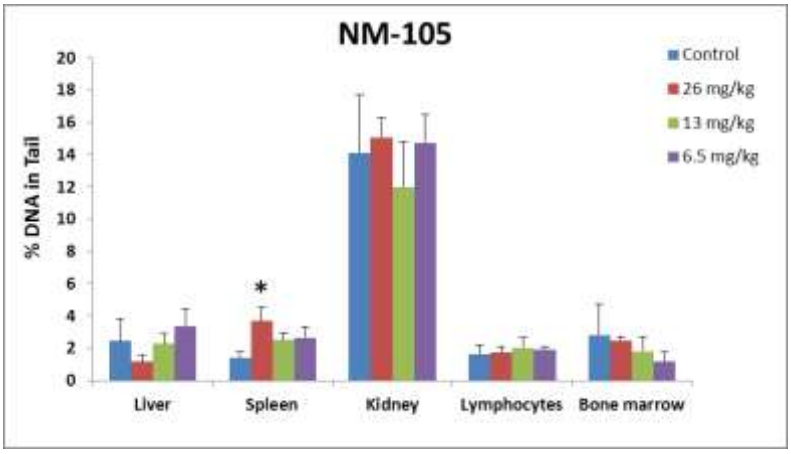
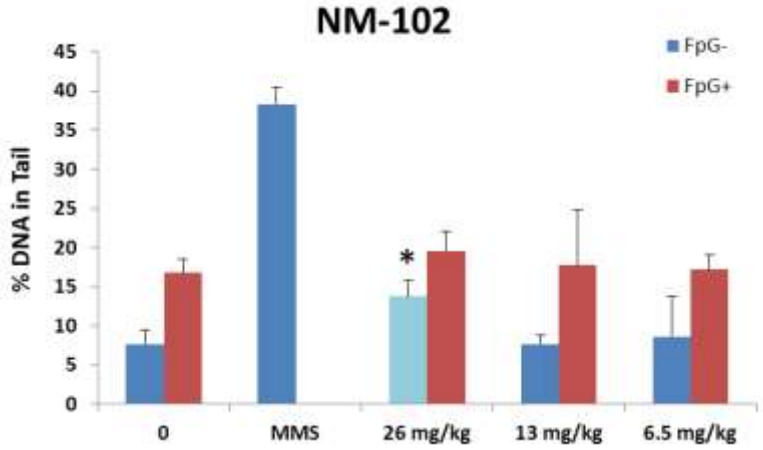


Figure 7. DNA fragmentation assessed by the comet assay without FpG in the organs and tissues from the rats treated by gavage with TiO_2 nanomaterials for 3 consecutive days. All data are presented as mean of the median of the present DNA in the tail (Mann-Whitney test, * $p<0.05$).

The data following assessment of oxidative DNA fragmentation in the colon detected by comet assay using FpG are shown in Figure 8.



The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

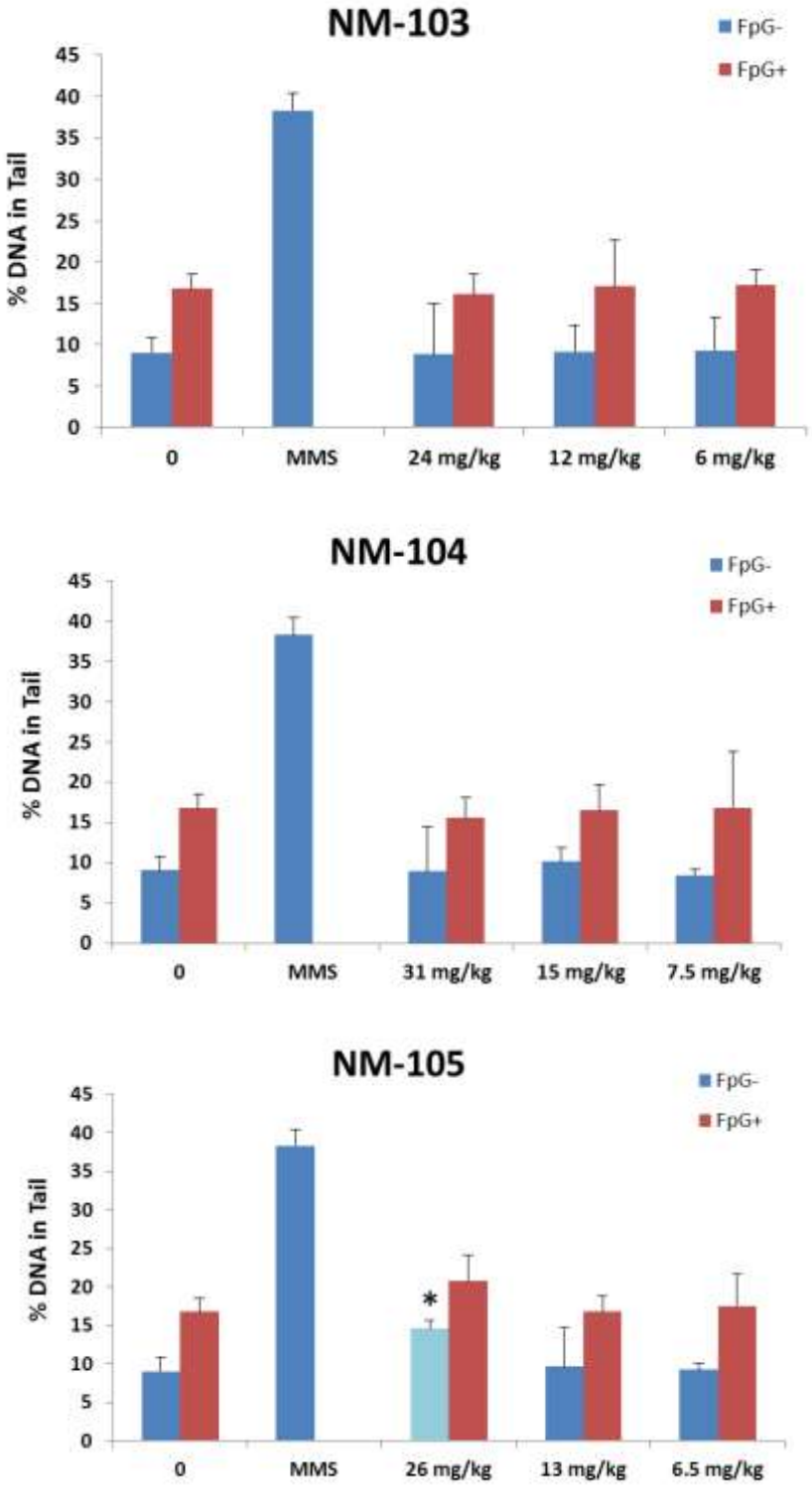
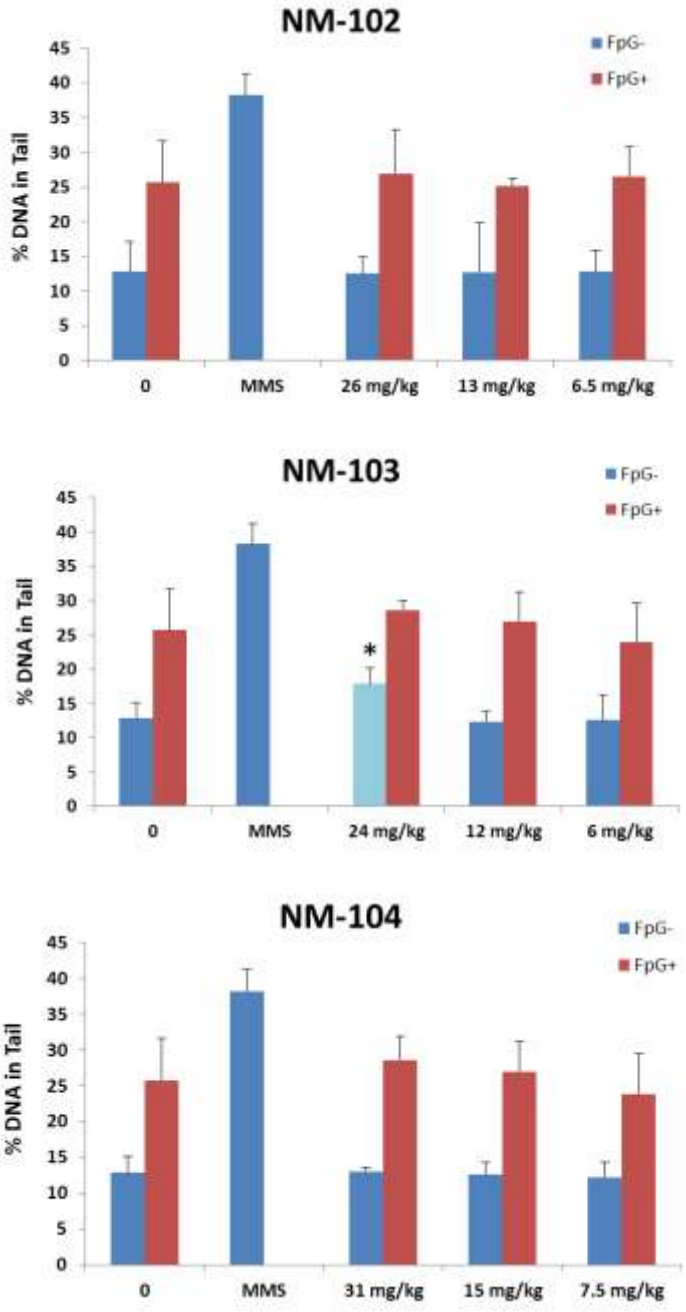


Figure 8. Oxidative DNA damage in the colon of rats exposed to TiO_2 at the indicated doses using a comet assay without formamidopyrimidine DNA glycosylase (FpG-) and with FpG+. 0: controls animals; MMS: Methylmethanesulfonate (positive control). Results are expressed as % DNA in Tail. Mean ± SD are shown, from five different animals with duplicate cell samples. (Mann-Whitney test, * $p<0.05$)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

The data following assessment of oxidative DNA fragmentation in the jejunum detected by comet assay using FpG are shown in Figure 9.



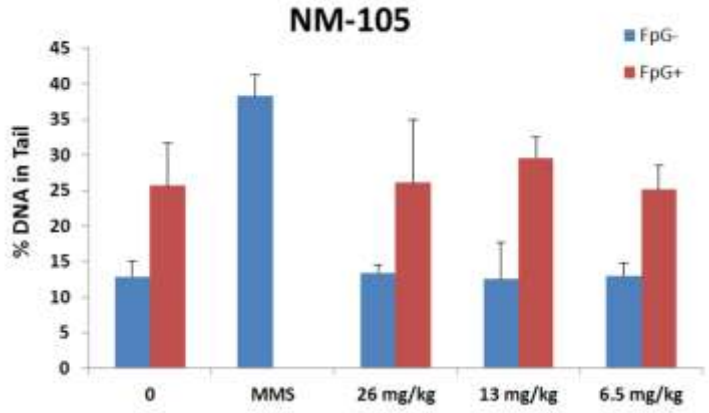


Figure 9. Oxidative DNA damage in the jejunum of rats exposed to TiO_2 at the indicated doses using a comet assay without formamidopyrimidine DNA glycosylase (FpG-) and with FpG+. 0: controls animals; MMS: Methylmethanesulfonate (positive control). Results are expressed as % DNA in Tail. Mean ± SD are shown, from five different animals with duplicate cell samples. (Mann-Whitney test, * $p<0.05$)

1.2.2.3 Micronucleus assay

- Bone marrow

Table 6. Percentage of polychromatic erythrocytes (PCE) micronucleous (PCEMN) in the micronucleus test in vivo following gavage with TiO_2 nanomaterials for 3 consecutive days

	Dose (mg/kg. b.wt)	PCEMN/1000 PCE (%)	Proportion of PCE(%)	PCEMN/1000 PCE (%)	Proportion of PCE (%)
Control	0	0.48	42	1.27	41
MMS	280	nd	nd	12.02	25
NM-102	26	0.43	61	1.70	87
NM-103	24	0.19	50	1.14	66
NM-104	31	0.27	nd	2.90	66
NM-105	26	0.38	48	1.93	53

- Colon

The slides from the colon micronucleus assays are still under scoring and the results cannot be presented in this deliverable.

1.2.2.4 Tissue distribution

All six organs investigated (liver, kidney, spleen, stomach, jejunum, and colon) following gavage administration of MNs for 3 consecutive days, showed a small detectable Ti content only for the highest dose used (24-31 mg/kg b. w). The highest Ti concentrations detected in the liver and spleen were for NM-105 – 270±0.06 ng/g tissue and 243±140 ng/g tissue respectively (Figure 10). For all other MNs tested Ti concentration in the spleen was about 170±50 ng/ g tissue. The presence of Ti in the GI tract was also detected and the results are shown in (Figure 11).

The Ti concentration within the organs of Wistar rats treated with NM-105 were measured by ICP-OES following the methods developed in WP7.

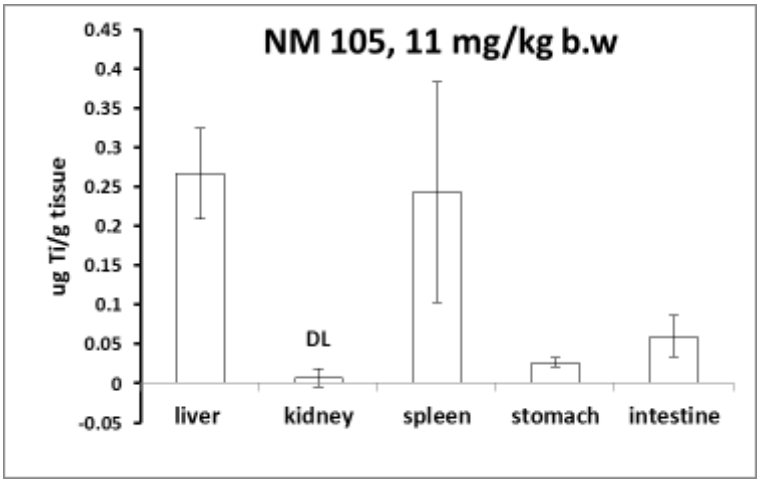


Figure 10. Distribution of Ti in the organs of rats treated by gavage with 2.56 mg/ml NM105. Each bar repre sents the mean of 5 test animals (mean ± SD). DL: below the detection limit.

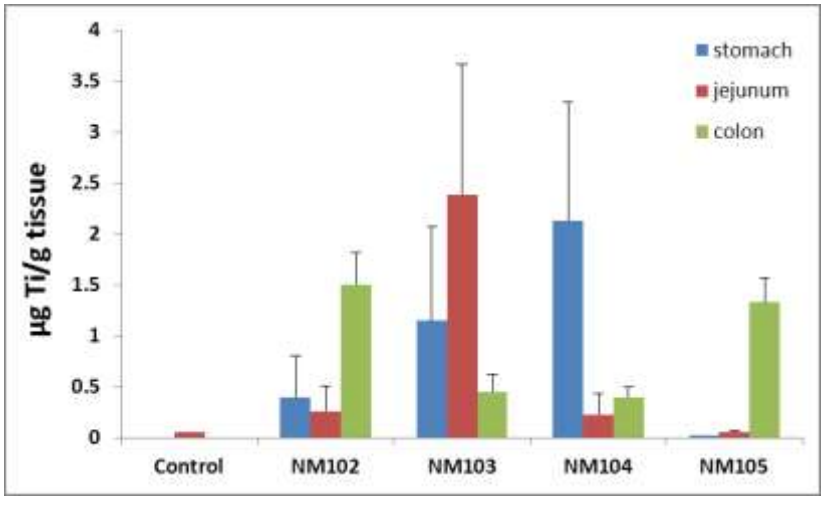


Figure 11. Distribution of Ti in the organs of rats treated by gavage with 2.56 mg/ml TiO_2 for 3 consecutive days. Each bar represents the mean of 5 test animals (mean ± SD).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

After the repeated TiO_2 nanomaterial administration very high Ti levels were detected in the faeces (Table 7), as an indication of MNs extraction vie GI tract.

Table 7 Presence of Ti (µg) in faeces of TiO_2 treated animals

	Dose (mg/kg. b.wt.)	Dose (mg/rat)	Excreted feces (average number)	Excreted TiO_2/ feces (µg)
Control	0	0	103	89 ± 26
NM102	26	6.9	95	4122 ± 310
NM103	24	6.9	117	3894 ± 427
NM104	31	6.9	93	3166 ± 866
NM105	26	6.9	90	4180 ± 370
	TiO_2 in the food chow			0.6 ± 0.1 µg/g

1.3 Intravenous administration in rats

1.3.1 Materials and Methods

During an experiment performed in the toxicokinetics workpackage for the tissue distribution of NM-103 and NM-104, extra bone marrow samples were obtained for evaluation of the presence of micronuclei. In this experiment animals received a single (day 1) or repeated (five times on five consecutive days, day 1 to day 5) intravenous administration of TiO_2 NM-103 and NM-104.

1.3.1.1 Animals

Animals were bred under specific pathogen-free (SPF) conditions and barrier maintained during the entire experiment in Macrolon cages at a room temperature of 23 ± 1°C, a relative humidity of 50 ± 5% and a 12-h light/dark cycle. Standard feeding chow diet and water were provided *ad libitum*. Rats were randomly divided into groups of 1-3/cage. The rats were kept under pathogen-limited conditions and were allowed to acclimatize for 1-2 weeks before they entered the experimental protocol. All rats were 9-10 weeks at exposure start.

1.3.1.2 MN Sample preparation

TiO_2 samples were prepared according to the dispersion protocol by WP4. In short, a 2.56 mg/ml stock suspension was prepared by pre-wetting the TiO_2 powder in 0.5 vol % ethanol followed by dispersion in 0.05 wt% Rat Serum Albumin (RSA) in ultrapure water. The sonication of the suspensions was performed on ice (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA, equipped with a disruptor horn Model number: 101-147-037) for 16 minutes at 10% power. The stock suspensions were diluted (9:1 v/v) with 10x concentrated phosphate buffer (2mM) pH 7.4 (702 mg NaH_2PO_4 x$2H_2O$, 4155 mg Na_2HPO_4 x$7H_2O$, dissolved in 1L) and used immediately.

1.3.1.3 IV administration of NM-103, and NM-104

The TiO_2 doses for the IV route were administered using a TiO_2 dispersion containing 2.30 mg TiO_2 per ml. The single dose groups received a dose per animal of 2.3 mg per animal resulting in a dose between 8.7 – 9.7 mg/kg body weight (b.w.) for male animals, and 12.4 – 13.7 mg/kg b.w. for female animals depending on the actual weight of the animal (weight range males 238 g – 265 g, females 168 g – 186 g at the start of the experiment).
The repeated dose groups received total cumulative dose per rat between 43.5 – 48.5 mg/kg b.w. for male rats, and 62 – 68.5 mg/kg b.w. for female rats depending on the actual weight of the animal. All experiments were approved by an independent Ethical Committee on Animal Experimentation and conducted in compliance with all applicable provisions of the national laws of the Netherlands i.e., the Experiments on Animal Decree and the Experiments on Animal Act. Study numbers were: DPA registration 201000255, 201000287, and 201100309.
As positive control for the induction of micronuclei in the bone marrow, methyl methanesulfonate (MMS) was used. Animals received either a single an IV administration of 30 µg/kg, or a repeated IV administration for 5 days (days 1 to 5) of 30 mg/kg resulting in a cumulative dose of 150 mg/kg.

1.3.1.4 Bone marrow isolation, staining and scoring

After IV administration of NM-103, NM-104, and MMS the rats were anaesthetized by inhalation of isoflurane (Isoflu®, AST Pharma, Oudewater, The Netherlands) in oxygen and subsequently euthanized by drawing blood from the abdominal aorta. For evaluation of micronuclei in the bone marrow, bone marrow cells were isolated by flushing the right femur with 4 ml of ICD (Impuls Cytophotometer) solution consisting of tri-sodiumcitrate-dihydrate (3.22 g), sodium dihydrogen phosphate-dihydrate (3.40 g), disodium hydrogenphosphate- dehydrate (3.87 g), citric acid-monohydrate (1.17 g), glucose (3.65 g), sodium chloride (4.96 g), and aqua destillata ad 1000 ml. The solution has a pH 7.4 at 20 °C, and was stored for maximally one month at 4 °C.
Bone marrow cells were isolated at 24 h after a single IV administration, and at 24 h after the last of five repeated IV administrations on day 1 to 5. A cytospin preparation was prepared in a cytospin centrifuge (Shandon Cytospin 2, Shandon, Thermo Shandon, Pittsburg, USA), and cells were fixed with methanol abs. and stained by May-Grünwald–Giemsa stain [May-Grünwald solution: 50% May-Grünwalds eosine-methyleneblue solution (Merck KGaA, Darmstadt, Germany) and 50% water. Giemsa solution: Giemsa stain (Fluka, Sigma-Aldrich, St Louis, USA), 8% v/v solution in water].

Evaluation

Using light microscopy approximately 500 cells were evaluated for determining the frequency of immature erythrocytes. The number of immature erythrocytes was between 50% and 65% of the number of erythrocytes present in the bone marrow. At least 1000 immature erythrocytes were scored for the presence of a micronucleus.

1.3.2 RESULTS

No increase in the micronucleus frequency was induced in all the TiO_2 exposed animals, irrespective of the gender (Table 8).

Table 8: Genotoxicity (frequency of micronucleated Immature Erythrocytes) and toxicity (PCE/NCE ratio) induced in bone marrow by TiO_2 following intravenous administration. Positive control MMS was administered by gavage as described in the Material & Methods. PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes

	[mg/kg]	Genotoxicity				Toxicity			
		Micronucleated Cells Mean	SD	n	SE	PCE/NCE ratio Mean	SD	n	SE
NM 103 (once a day)	0	0,050	0,058	4	0,029	1,347	0,155	4	0,077
	M	0,050	0,071	2	0,050	1,320	0,023	2	0,016
	F	0,050	0,071	2	0,050	0,950	0,038	2	0,027
MMS	25	2,150	0,423	6	0,173	1,512	0,199	6	0,081
NM 104 (once a day)	0	0,050	0,058	4	0,029	1,347	0,155	4	0,077
	M	0,050	0,058	4	0,029	1,216	0,107	4	0,053
	F	0,125	0,096	4	0,048	1,460	0,176	4	0,088
MMS	25	2,150	0,423	6	0,173	1,512	0,199	6	0,081
NM 103 (5x once a day)	0	0,125	0,096	4	0,048	1,594	0,421	4	0,210
	M	0,183	0,098	6	0,040	1,664	0,209	6	0,085
	F	0,217	0,117	6	0,048	1,123	0,313	6	0,128
MMS	25	3,400	0,716	6	0,292	0,672	0,155	6	0,063
NM 104 (5x once a day)	0	0,125	0,096	4	0,048	1,594	0,421	4	0,210
	M	0,217	0,172	6	0,070	1,211	0,183	6	0,075
	F	0,050	0,084	6	0,034	1,257	0,135	6	0,055
MMS	25	3,400	0,716	6	0,292	0,672	0,155	6	0,063

1.4 Intravenous administration in mice

1.4.1 MATERIAL & METHODS

The lac Z assay was performed on mice only with NM-102 which was selected according to its genotoxic response within the WP5 and its accumulation potency detected within the WP7.

1.4.1.1 Animals and treatments

C57Bl/6 pUR288 transgenic mice were bred, maintained, treated and sacrificed at Instituto Nacional de Saúde Dr. Ricardo Jorge, according to European Union directives. In these mice, the pUR288 plasmid is inserted in head-to-tail sequences in homozygosity on both chromosomes 3 and 4 and contains the lacZ reporter gene.

A dispersion of the titanium dioxide nanomaterial NM-102 was prepared according to agreed protocol. A solution of 2.56 mg/ml in EtOH+0.05% BSA was prepared in glass vials, homogenized by sonication for 16 minutes and 10x concentrated saline was added before injection in mice. Groups of 5-6 male mice, 3 months-old, were intravenously injected twice, in consecutive days, with 0 (vehicle only), 10 or 15 mg/kg of body weight. The positive control, N-ethyl-N-Nitrosurea (ENU; Sigma-Aldrich, St. Louis, MO, CAS# 759-73-9) was dissolved in dimethylsuphoxide and a dilution in saline (25 mg/ml) was prepared for injection. One group of lacZ mice mice was intraperitoneally injected with a single dose of ENU, 120 mg/kg body weight.

Mice were sacrificed 28 days later and livers and spleens were removed, flash frozen, and stored in liquid nitrogen until DNA extraction for lacZ mutation analysis. Part of liver and spleen were collected for immediate processing for the comet assay.

1.4.1.2 Micronucleus assay

Five microlitres of peripheral blood were obtained from a tail blood vessel 42 hr after the last injection. The blood was placed on acridine orange-coated slides, covered with a coverslip, and stored at 4ºC until analysis (Hayashi et al, 1990). For each animal, 2000 RET were blind scored under fluorescence microscopy. The frequency of micronucleus per 1000 RET and the percentage of RET were calculated for each animal and for each treatment group.

1.4.1.3 Comet Assay

The alkaline comet assay was performed in freshly-isolated liver and spleen samples that were placed in D-PBS with 20 mM EDTA and 10% DMSO, pH 7.0. Tissues were minced into fine pieces, and 5 - 10 µL of the cell suspension was embedded in low melting point agarose 1% at 38ºC. The assay was performed after overnight lysis at 4ºC. DNA was then allowed to unwind for 30 min in alkaline electrophoresis buffer (300mM NaOH, 1mM Na_2EDTA, pH>13) and electrophoresis was run for 25 min at 0.8V/cm and 300mA, at 4ºC. Following neutralization (0.4M Tris, 4M HCl, pH 7.5), slides were stained with ethidium bromide (0.125 µg/µL). One hundred randomly selected nucleoids were analysed per mice under a fluorescence microscope (Zeiss, Axioplan 2) using the Comet Imager software (MetaSystems, GmbH).

1.4.1.4 4. LacZ mutation assay

Genomic DNA extraction from homogenized livers and spleen and lacZ-plasmid rescue were done using a previously established protocol (Louro et al, 2010). Briefly, 50 µg of genomic DNA were

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

incubated with 40 U of HindIII and with magnetic beads (Dynabeads M450 sheep antimouse IgG) precoated with lacZ/lacI fusion protein.The supernatant fluid was discarded and the plasmid DNA was eluted using isopropyl-b-D-galactopyranoside, followed by heat inactivation of HindIII at 65ºC. Plasmids were circularized with T4 DNA at room temperature and ethanol precipitated. The purified plasmid DNA was electroporated into highly competent Escherichia coli C (DlacZ and galE2) host cells. To determine the number of mutant colonies, the majority (99.9%) of the transformed cells were plated on a selective top agar plate containing the lactose analogue phenyl-b-D-galactopyranoside. The remainder was plated on nonselective medium containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) to determine the total number of colonies.

Mutant frequency (MF) was calculated as the ratio between the number of mutant colonies and the total number of colonies multiplied by the dilution factor.

1.4.2 RESULTS

1.4.2.1 Comet assay

No increase in the level of DNA damage was detected, by the alkaline comet assay, in liver and spleen of NM-102 exposed mice, under the tested conditions; the positive control induced a significant increase in the level of DNA damage in liver, even 28days after injection (Figure 12).

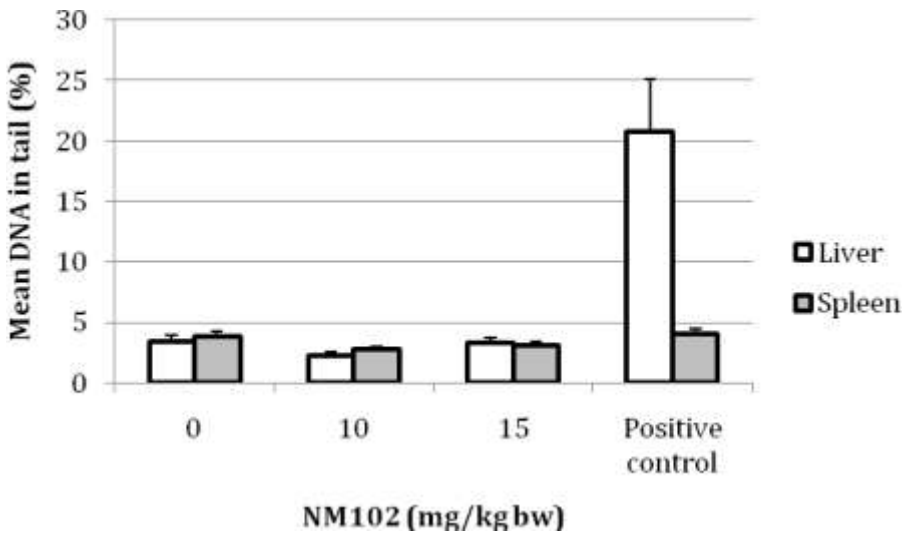


Figure 12: Results from comet assay in liver and spleen 28 days after exposure of mice by i.v. to 0, 10, or 15 mg/kg of body weight, in two consecutive days. Positive control: ethyl-nitrosourea, single i.p. of 120 mg/kg**.**

1.4.2.2 Micronucleus assay

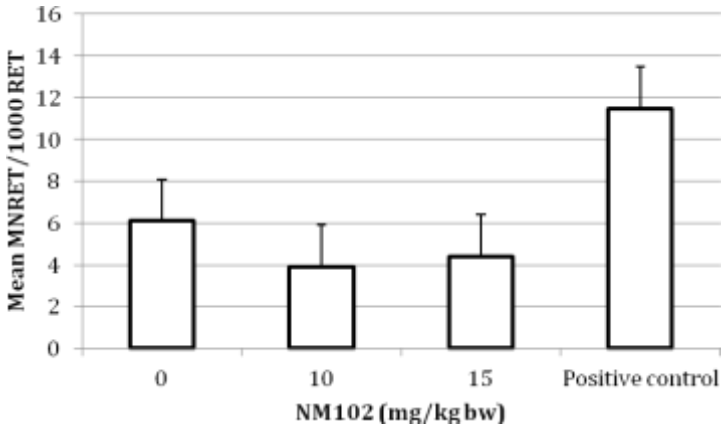


Figure 13: Results from micronucleus assay in blood from mice 42 h after exposure of mice by i.v. to 0, 10, or 15 mg/kg of body weight, in two consecutive days. Positive control: ethyl-nitrosourea, single i.p. of 120 mg/kg.

1.4.2.3 LacZ mutation assay

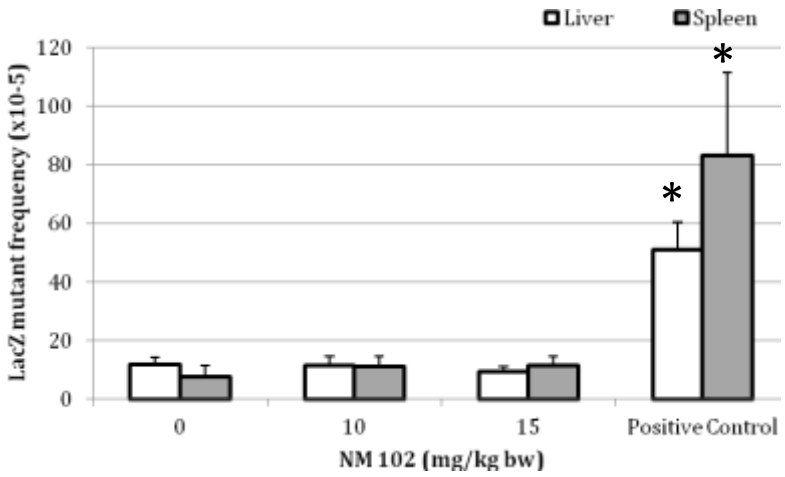


Figure 14: Results from LacZ mutation assay, 28 days after exposure of mice by i.v. to 0, 10, or 15 mg/kg of body weight, in two consecutive days. Positive control: ethyl-nitrosourea, single i.p. of 120 mg/kg (*significantly different from negative controls).

In brief for TiO2

- **Dose ranges:**

Instillation: 4.6, 2.3 and 1.15 mg/kg (x3)

Gavage: 26, 13.5, 6.5 mg/kg (x3)

Intravenous for NM-103 and NM-104 (WP7 experiments): 2.3 mg/animal (X5)

Intravenous for NM-102 (LacZ): 10 and 15 mg/kg (x2)

- **Distribution:**

Low Ti uptake from GI was recorded

Ti excretion detected via GI tract

- **Comet assay:**

Most MNs induced **no DNA damage** irrespective of the organ

except after instillation NM-105 in BAL

and after gavage in spleen, intestine (NM-103), colon (NM-102 and -105) and bone marrow (NM-104)

Genotoxic effect observed in organs **depending on the route** (BAL for instillation; spleen and GI tract for gavage)

- **Micronucleus assays:**

No mutagenicity in bone marrow after instillation, gavage or iv administration

- **Lac Z (iv administration with NM102):**

No genotoxicity in spleen and liver (comet)

No clastogenicity in blood (micronucleus)

No mutagenicity in liver and spleen (lac Z mutation)

2) SAS

4 MNs (NM-200, NM-201, NM-202 and NM-203) were tested. The protocol of dispersion agreed on the one used within WP7.

For each MN, a particle suspension solution at 6.0 mg/mL in 30 mL normal saline (NaCl 0.90% w/v) was sonicated 16 min with 10% amplitude in 50 mL polypropylene Falcon® tube. This protocol was provided by ISS scientists (Italy) who performed SAS toxicokinetics in WP7.

2.1 Intratracheal instillation

2.1.1 Material and Methods

2.1.1.1 Animals

Seven-week old Sprague Dawley rats were purchased from Janvier and were exposed by intratracheal instillation to particle suspensions or vehicle 48, 24 and 3 hours before tissue collection. Three concentrations were tested 12; 6 and 3 mg/kg b.w.

2.1.1.2 Treatment

For intratracheal instillation, animals were anesthetized by an intraperitoneal injection of a mixture of Medetomidine (Domitor, 0.25 mg/kg) and Ketamine (Clorketam 37.5 mg/kg) and 200 µL of particle suspensions or vehicles were then administered per 100 g of b.w using a 16 G I.V. catheter placed into the rat's trachea through the mouth. The sedative effect of medetomidine was reversed by subcutaneous injection of Atipamezole (Antisedan 1.25 mg/kg).

2.1.1.3 Positive controls

Methyl methanesulfonate (MMS) was purchased from Sigma Aldrich (ref# 129925), diluted in 0.9% NaCl and administered by gavage at a final concentration of 50 mg/kg b.w. 48 hours before tissue collection and then at 25 mg/kg b.w. 24 and 3 hours before tissue collection.
N-ethyl-N-nitrosourea (ENU) was also purchased from Sigma Aldrich (ref# N3385), diluted in 0.9% NaCl and administered by gavage at a final concentration of 25 mg/kg b.w. 48, 24 and 3 hours before tissue collection.

MMS was used as positive control for:

- ➔ comet assays in BAL cells, lung, liver, spleen, bone marrow and kidney
- ➔ micronucleus assay in bone marrow

ENU was used as positive control for:

- ➔ comet assays in blood and bone marrow
- ➔ micronucleus assay in bone marrow

2.1.1.4 Tissue collection

For tissue collection, animals were anesthetized with pentobarbital (60 mg/kg) and exsanguinated through the abdominal aorta. Blood was collected in K_2 EDTA tubes.
Bone marrow from the two femoral bones were collected by flushing them with a mixture of Sorensen buffer and Fetal Calf Serum (FCS) (1:1) using a 1 mL syringe connected to a 18G x 1 1/2'' needle. Bone marrow cells were centrifuged 5 min at 400 g at 4°C. Pellets were resuspended in the flushing buffer and smears were made (3 for each femur). Slides were air dried, fixed with methanol and stored until staining.
Remaining cells were kept on ice prior to comet assay.
Animal chest was 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 using 18G canula: 4 mL of ice-cold PBS was introduced into the left lung and collected into a 15 mL conical tube. 4 additional lavages were performed with 4 mL of ice cold PBS each and were collected in second 15 mL conical tube. Both tubes were centrifuged 5 min at 4°C at 400 x g. The supernatant from the first tube was collected for biochemical analysis using a Randox analyser and kept at 4°C for no more than 24 hours. The supernatant from the second tube was discarded and the two cell pellets were resuspended in 1 mL of ice cold PBS for cell count, cytospin preparation and comet assay.

Cell counts were performed using a Cellometer (Nexcelom) and after Acridine Orange/Propidium Iodide cell staining. Differential cell counts were performed on cytospin preparation stained with May Grunwald Giemsa technique, 500 cells/animal were then analyzed.

The left lung was kept in ice cold PBS before enzymatic tissue dissociation using Collagenase type IA (Sigma Aldrich ref# C9891) and Miltenyi Biotec Gentle Max dissociator.

The right caudal lung lobe was fixed with 10% neutral buffered formalin (Sigma Aldrich) and paraffin block was made.

Cells from liver, kidney were obtained by enzymatic tissue dissociation using Collagenase type IA (Sigma Aldrich ref# C9891) and Miltenyi Biotec Gentle Max dissociator. Cells from spleen were obtained by mechanical dissociation using the Miltenyi Biotec Gentle Max dissociator.

One of the kidneys and pieces of liver and spleen were fixed with 10% neutral buffered formalin (Sigma Aldrich) and paraffin blocks were made.

2.1.1.5 Comet assay

Cells were mixed with 1% low-gelling agarose (Sigma Aldrich ref# A9414) and poured onto microscope glass slides pre-coated with 1% general routine agarose (Sigma Aldrich ref# A9539). Microscope slides were laid onto an ice bed to let the agarose solidified. The slides were then immersed into the ice-cold lysis buffer (2.5 mM NaCl; 100 mM Na_2EDTA; 10 mM Tris base; 10% DMSO and 1% Triton; pH 10) overnight at 4°C. For each animal 2 slides were made: one for the regular single gel electrophoresis assay and one for the formamidopyrimidine DNA glycosylase (FpG)-modified comet assay. For the FpG-modified comet assay, following lysis, slides were washed three times 5 min with the FpG incubation buffer (Hepes 40 mM, KCl 0.1 M, EDTA 0.5 mM, bovine serum albumin 0.2 mg/mL; pH 8) at 4°C and then incubated for 30 min at 37°C with 5 U/mL of FpG (Sigma Aldrich ref#F3174) in the FpG incubation buffer. For the regular comet assay, slides were not treated the same way as for FpG-modified assay but without incubation with FpG enzyme. The slides were then immersed into the ice-cold alkaline electrophoresis buffer (300 mM NaOH; 1 mM Na_2EDTA; pH>13) for 20 min and then submitted to electrophoresis for 40 min at 0,9 V/cm. At the end of the electrophoresis, slides were immersed into the neutralization buffer (Tris base 0.4 M; pH 7.5) for 15 min at 4°C. Slides were then washed twice with ultrapure water for 5 min and dehydrated for 10 min into 96% ethanol before being dried at 45°C and stored in the dark at room temperature. For fluorescent microscopy analysis, slides were rehydrated 10 min with ultrapure water and then stained with propidium iodide 2.5 µg/mL in PBS. For each sample, 100 cells were analyzed using the Comet Assay IV software (Perceptive Instruments, Suffolk, UK) and the percentage of DNA in the tail of each comet was measured.

2.1.1.6 Micronucleus assay

Once fixed, the bone marrow slides were sent to the collaborating lab for staining and scoring.
For staining, fixed bone marrow slides were sequentially incubated with:
- filtered non diluted May-Grünwald stain (QUA-Med Polska) (3 min.)
- gently wash with Sörensen's Buffer (pH 7.2)
- Giemsa (QUA-Med Polska) stain diluted with Sörensen's Buffer (1:6, v/v; pH 7.2) (10 min.)
- gently wash with Sörensen's Buffer (pH 7.2)

After washing and leaving for few minutes on absorbent paper, the slides are soaked in 95% for 5 min.

Only one scorer had scored all the slides from a whole study. The slides were first checked with low magnification for verification of cell morphology, spacing and staining. Then the slides were examined in the chosen area, systematically, in continuous fields counting each of the following cell types:

- polychromatic erythrocytes (PCEs) distinguished by their bluish colour
- mature erythrocytes (NCEs) distinguished by their pink colour
- micronucleated polychromatic erythrocytes.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

The proportion of PCEs among total (PCEs + NCEs) erythrocytes was determined for each animal by counting a total of at least 200 erythrocytes for bone marrow. 2000 PCEs/animal were scored for the presence of micronuclei.

2.1.1.7 Histology

Paraffin embedded tissue blocks from relevant SAS concentrations were analysed for histopathology by a subcontractor (Ricerca Biosciences SAS, France).

2.1.2 RESULTS

2.1.2.1 Broncho alveolar fluid cytology and biochemistry

Changes in BAL fluid cell number and composition may be associated with a toxicological process and an inflammatory response (especially granulocytes influx). For all SAS, whatever the dose considered, a significant increase of influx of neutrophilic granulocytes was observed in BAL fluid from exposed animals with a dose-dependent trend (Figure 15). An increase of the total cell number was also noticed for all the SAS, but this change was not significant for the lowest dose of NM-200 and NM-202. Main cell types observed in BAL fluid were macrophages and neutrophilic granulocytes, some lymphocytes were also observed but their frequency and number in SAS exposed animals were not significantly different from the control group (data not shown). In addition, in this study, the percentage of neutrophilic granulocytes in control (vehicle) group appears higher than that is usually obtained following a single intratracheal instillation and may be considered as an experimental artifact due to repeated animal exposure.

Increase in lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and N-acetyl-β-D-glucosaminidase (NAG) activities and protein content are often signs of pulmonary toxicity following pulmonary exposure to particulate matter.
For all SAS, a significant increase in the broncho-alveolar fluid of LDH and NAG activities was observed (Figure 16). A significant increase in the BAL fluid of protein content was only observed for NM-202 and NM-203 (highest dose) (Figure 17). No change in alkaline phosphatase (ALP) was however observed (Figure 17).

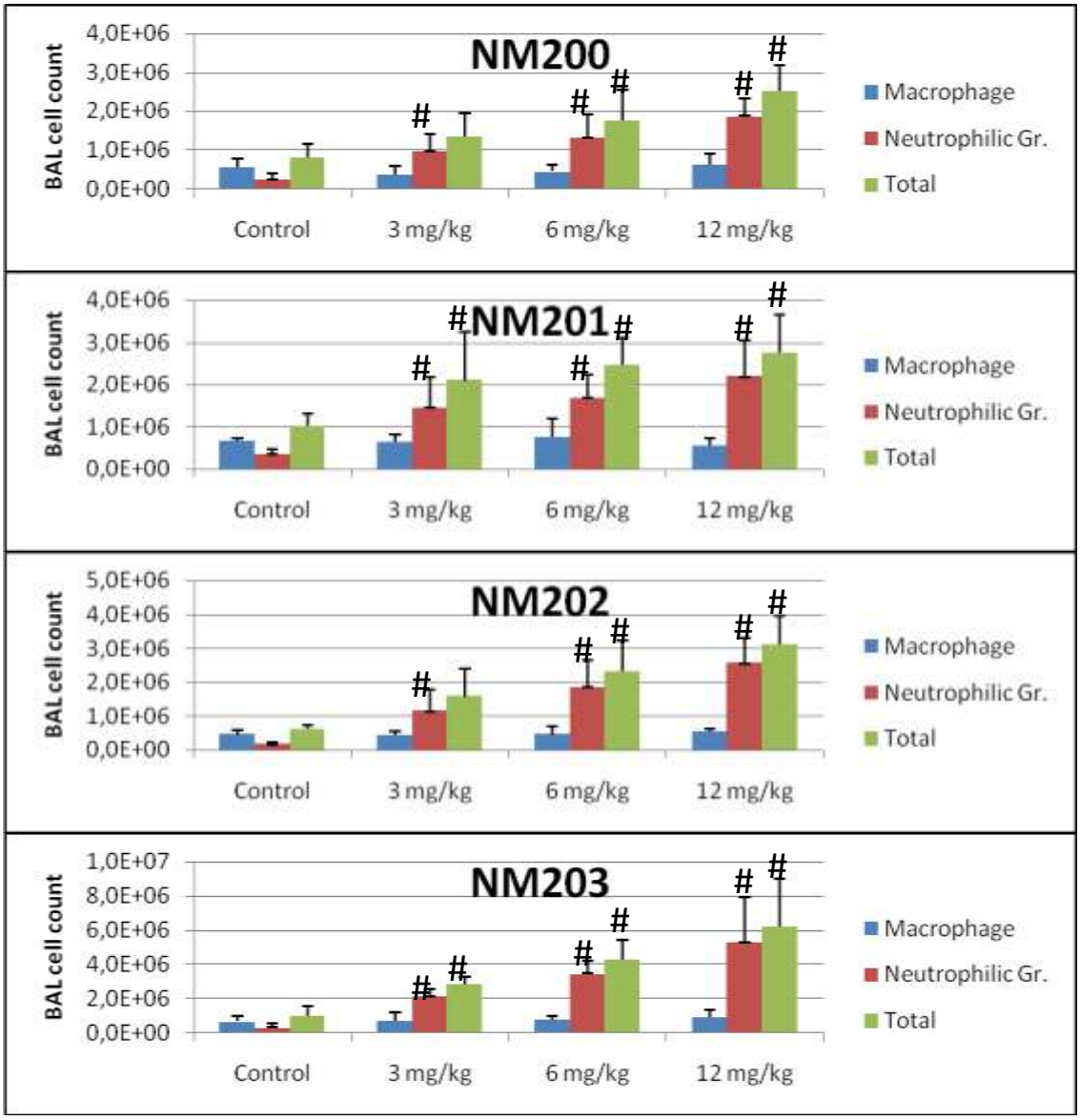


Figure 15: Cytology of bronchoalvolar lavage fluid from control and animals exposed by intratracheal instillation to SAS nanoparticles. . # Significantly different from the control (Dunnett's test, $p<0.05$).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

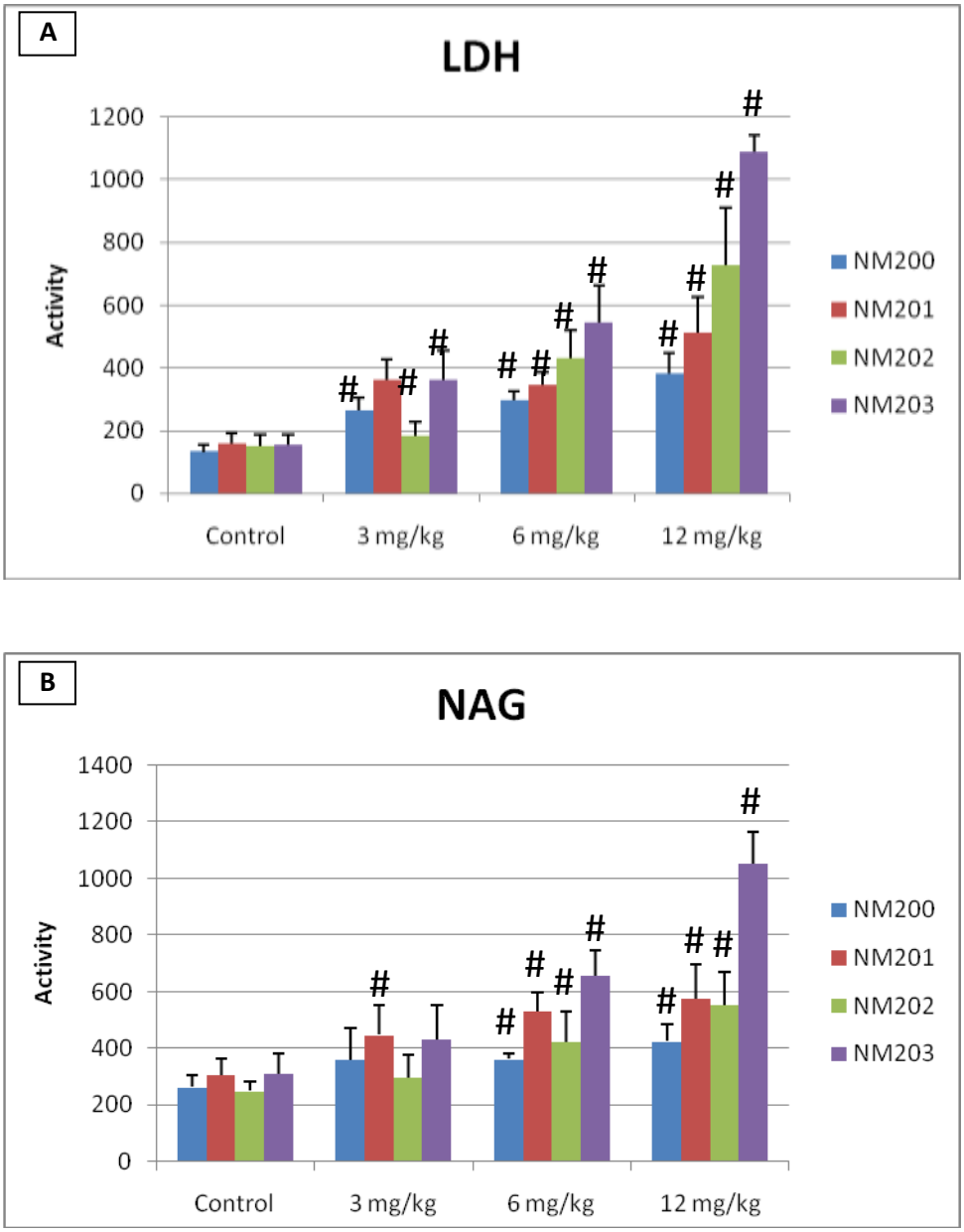


Figure 16: BAL fluid LDH (A) and NAG (B) activities in controls and rats exposed by intratracheal instillation to SAS. # Significantly different from the control (Dunnett's test, $p<0.05$).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

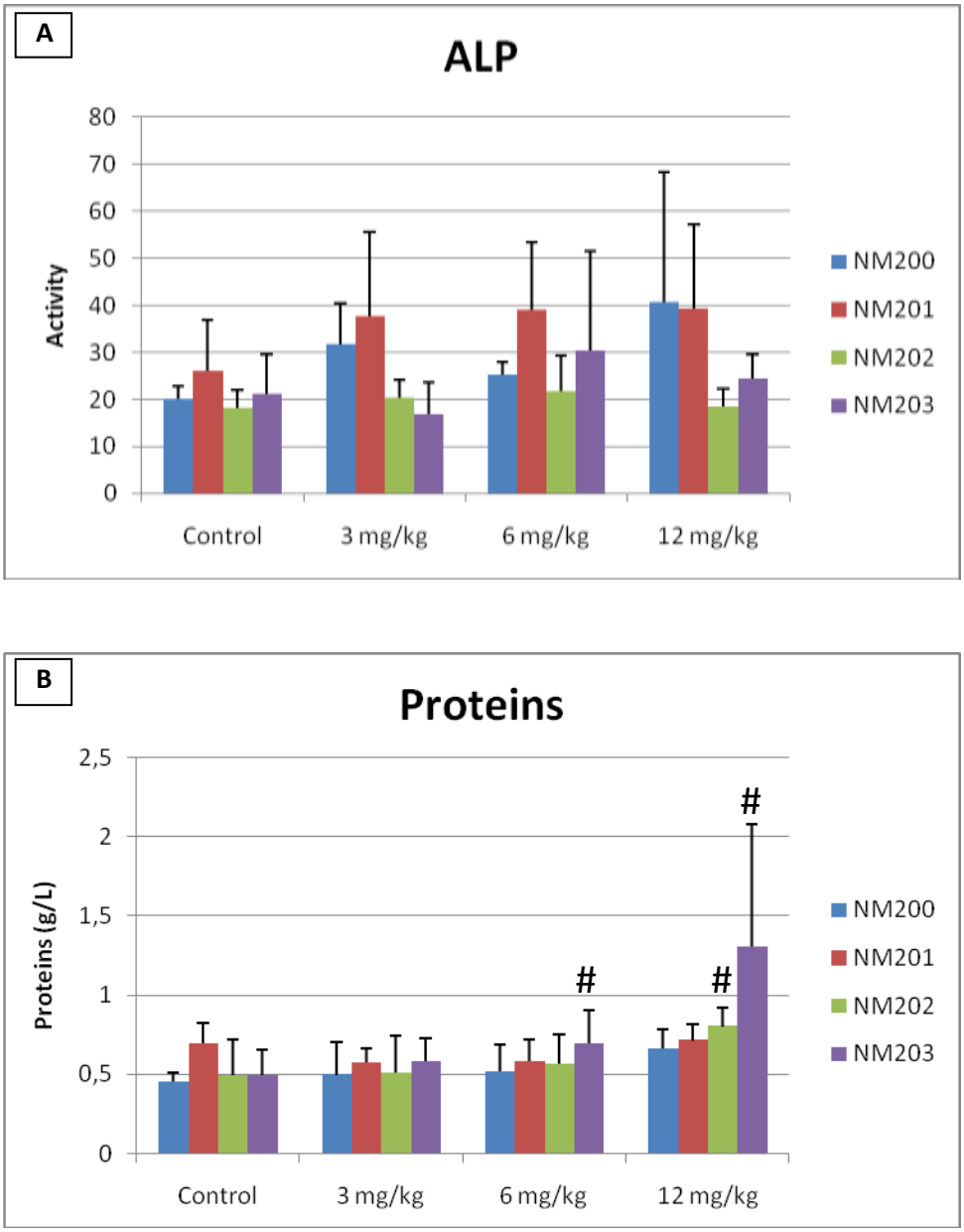
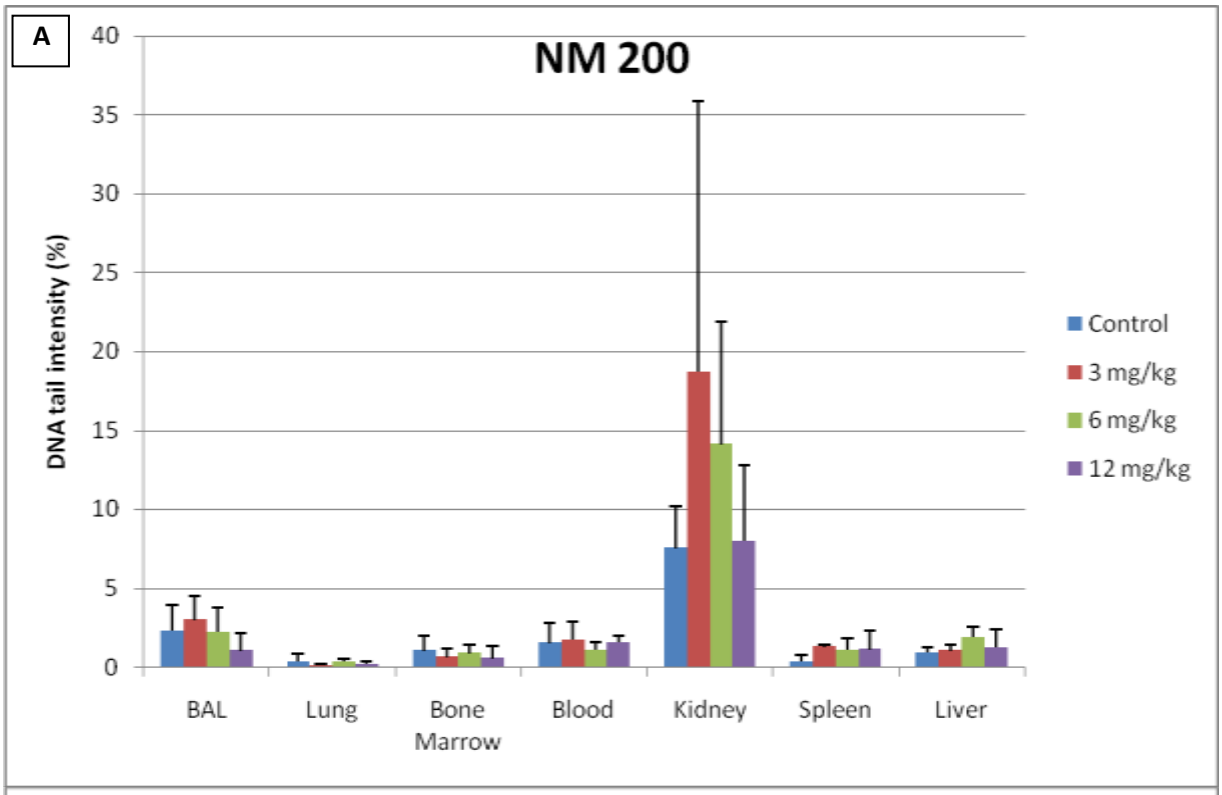
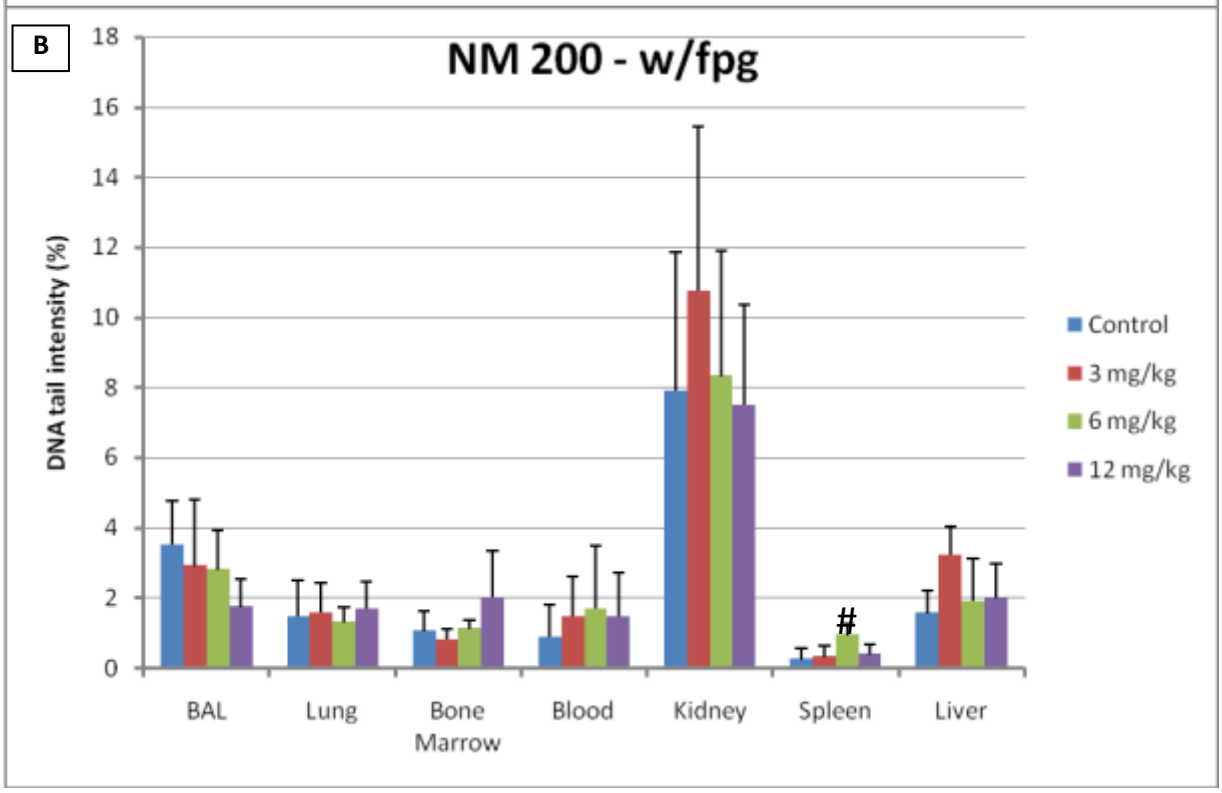


Figure 17: BAL fluid ALP activity (A) and protein content (B) in controls and rats exposed by intratracheal instillation to SAS. # Significantly different from the control (Dunnett's test, $p<0.05$).

2.1.2.2 Comet assay

❖ **NM-200**





The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

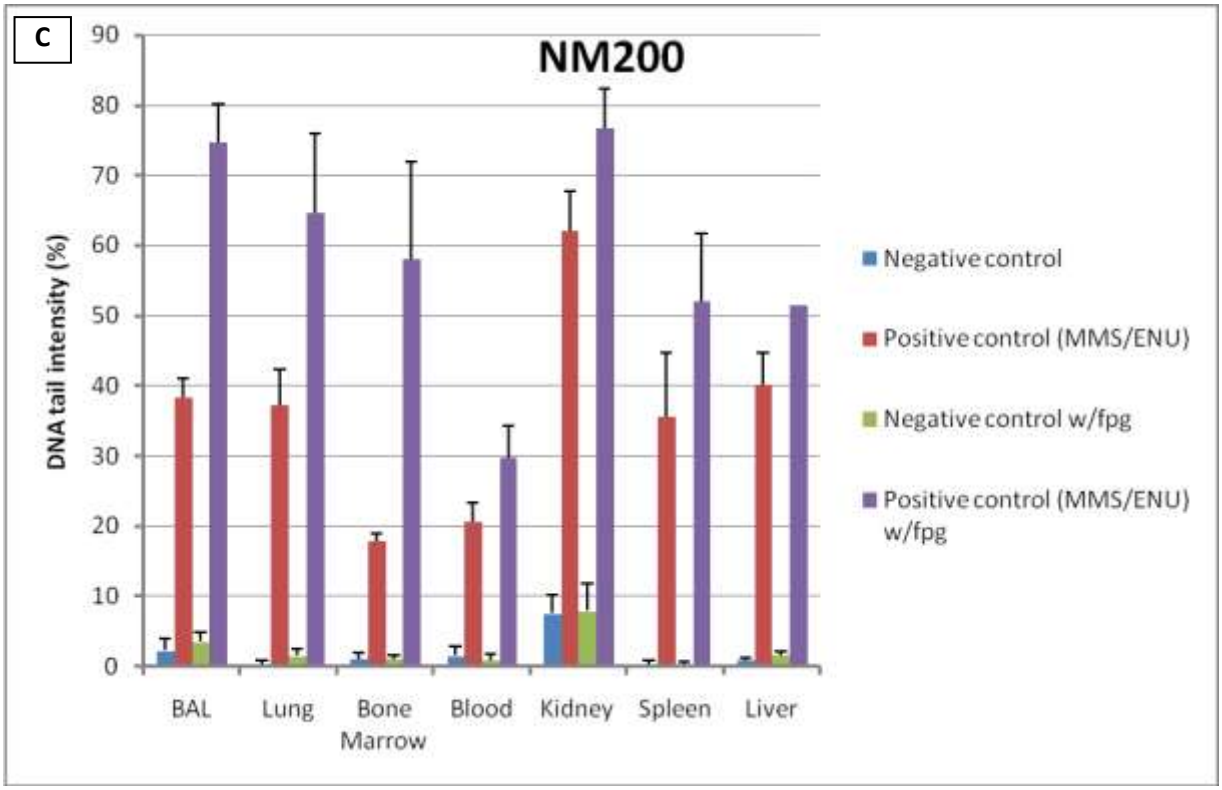
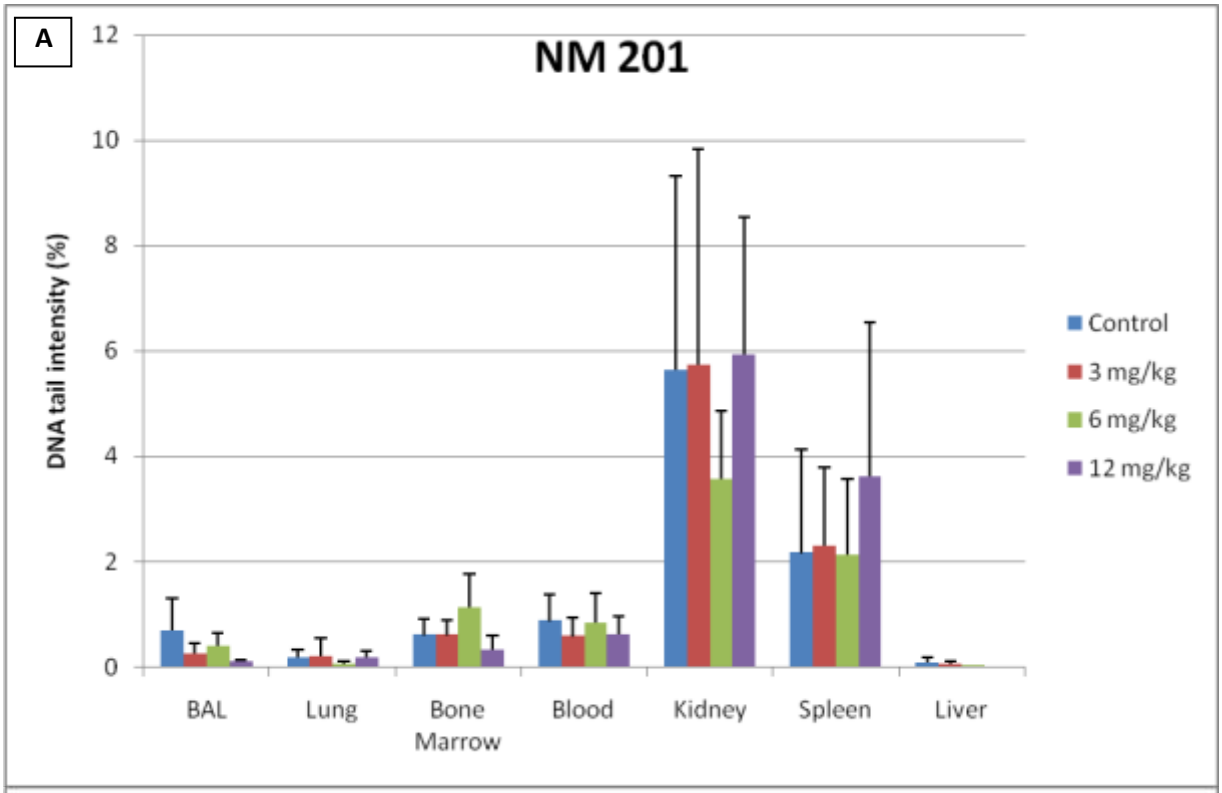


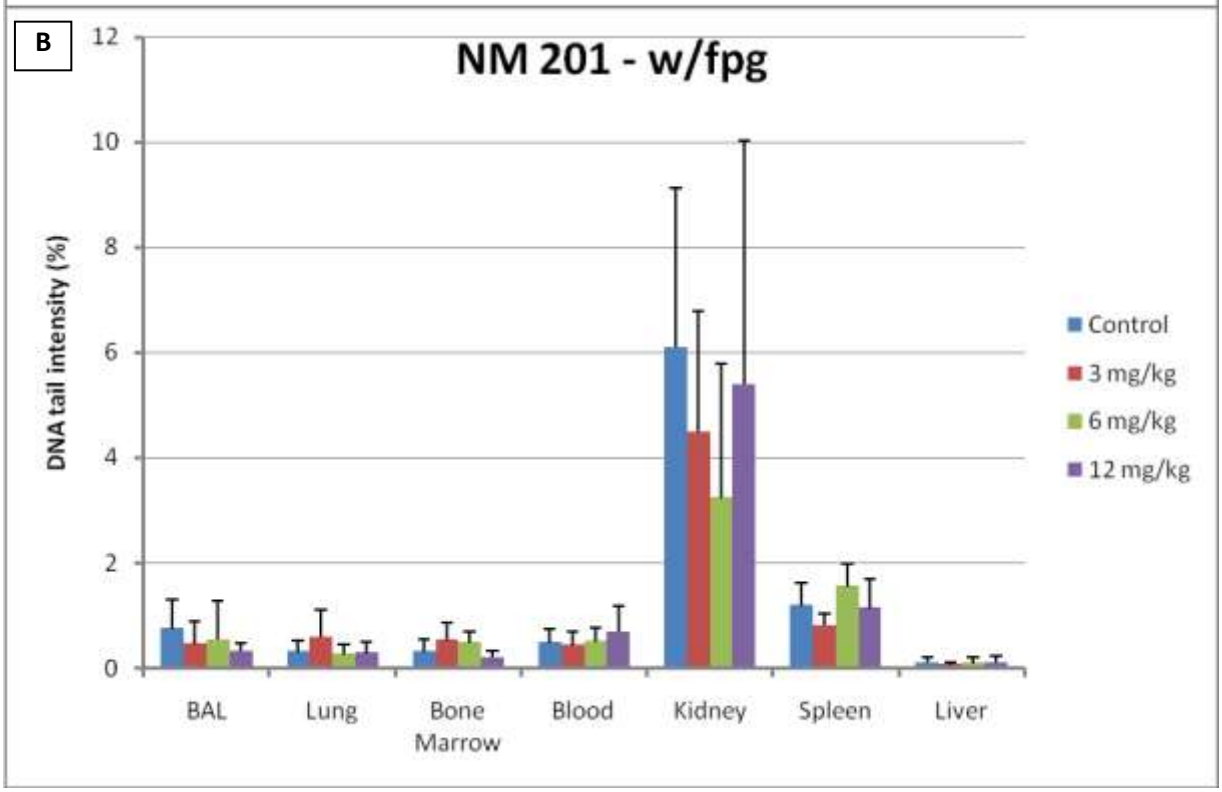
Figure 18: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by intratracheal instillation to NM-200 or by gavage to MMS or ENU (only for blood) (C). # Significantly different from the control (Mann-Whitney test, $p<0.05$). All data from MMS and ENU were significantly different from the control (Mann-Whitney test, $p<0.05$).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-201**





The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

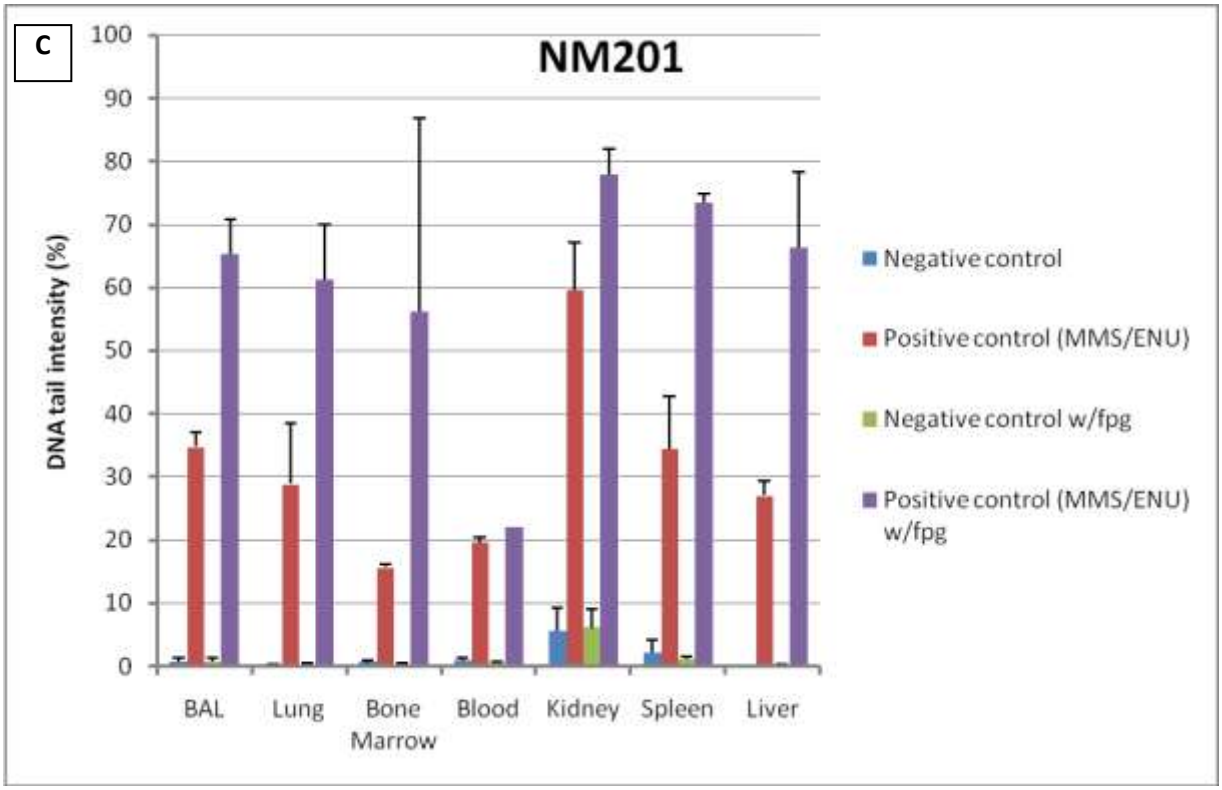
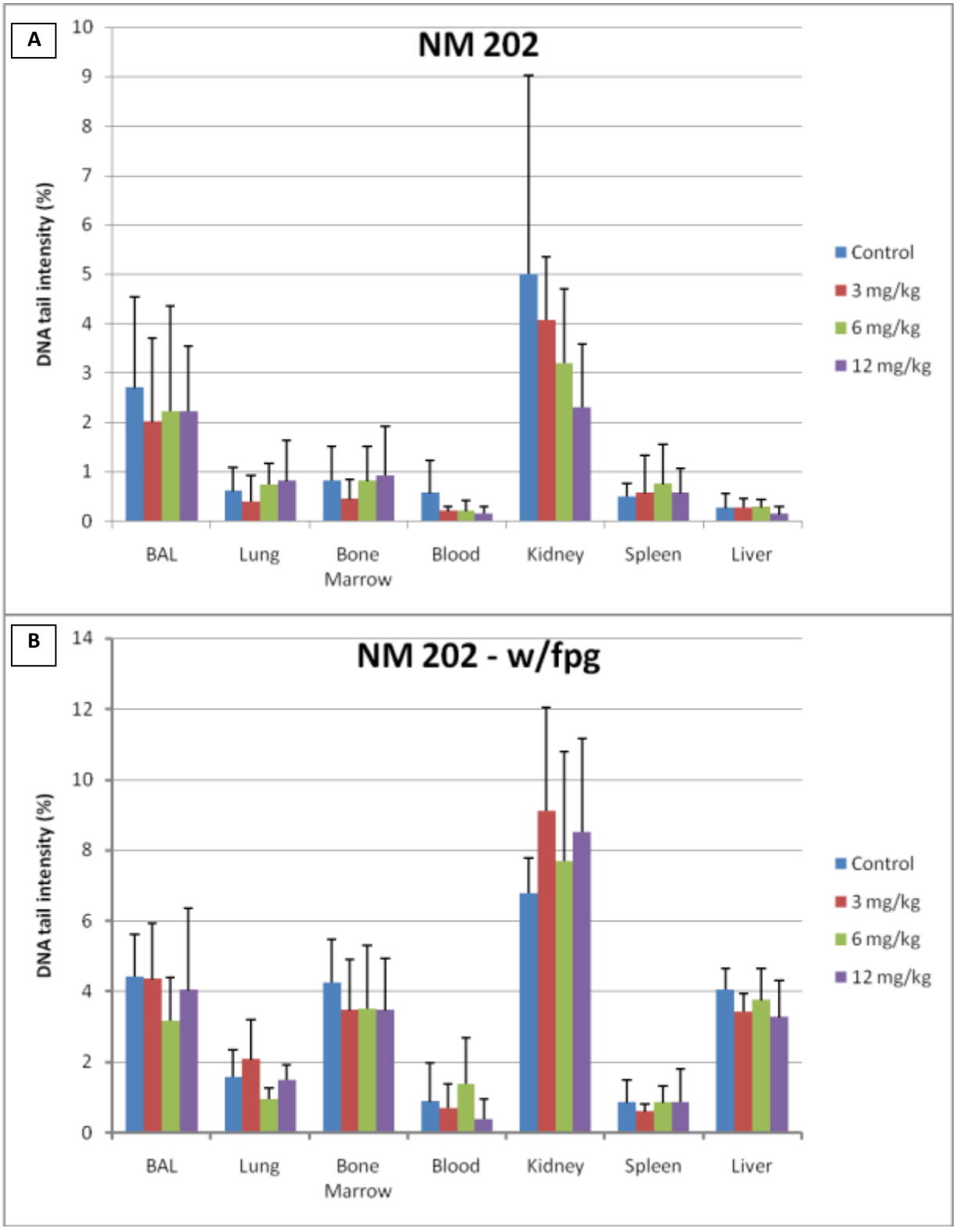


Figure 19: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by intratracheal instillation to NM-201 (A) or by gavage to MMS or ENU (only for blood) (C). All data from MMS and ENU were significantly different from the control (Mann-Whitney test, $p<0.05$).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-202**



The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

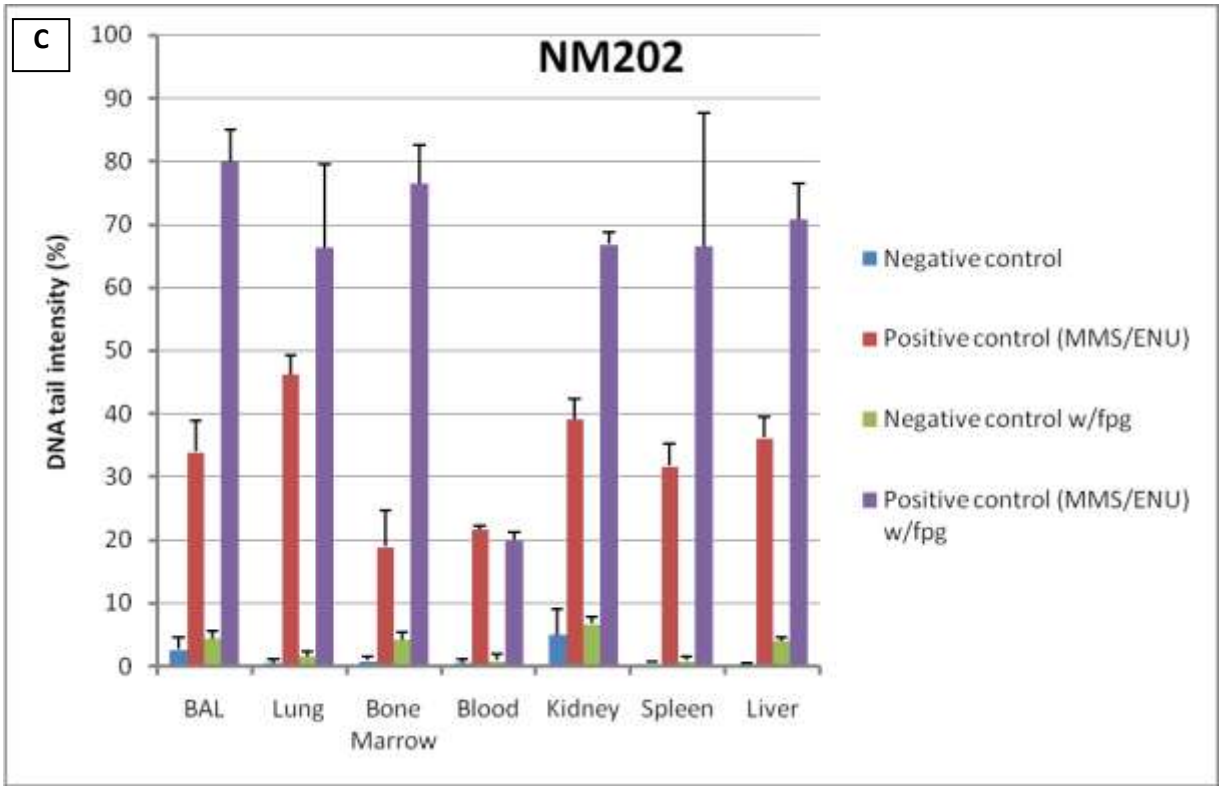
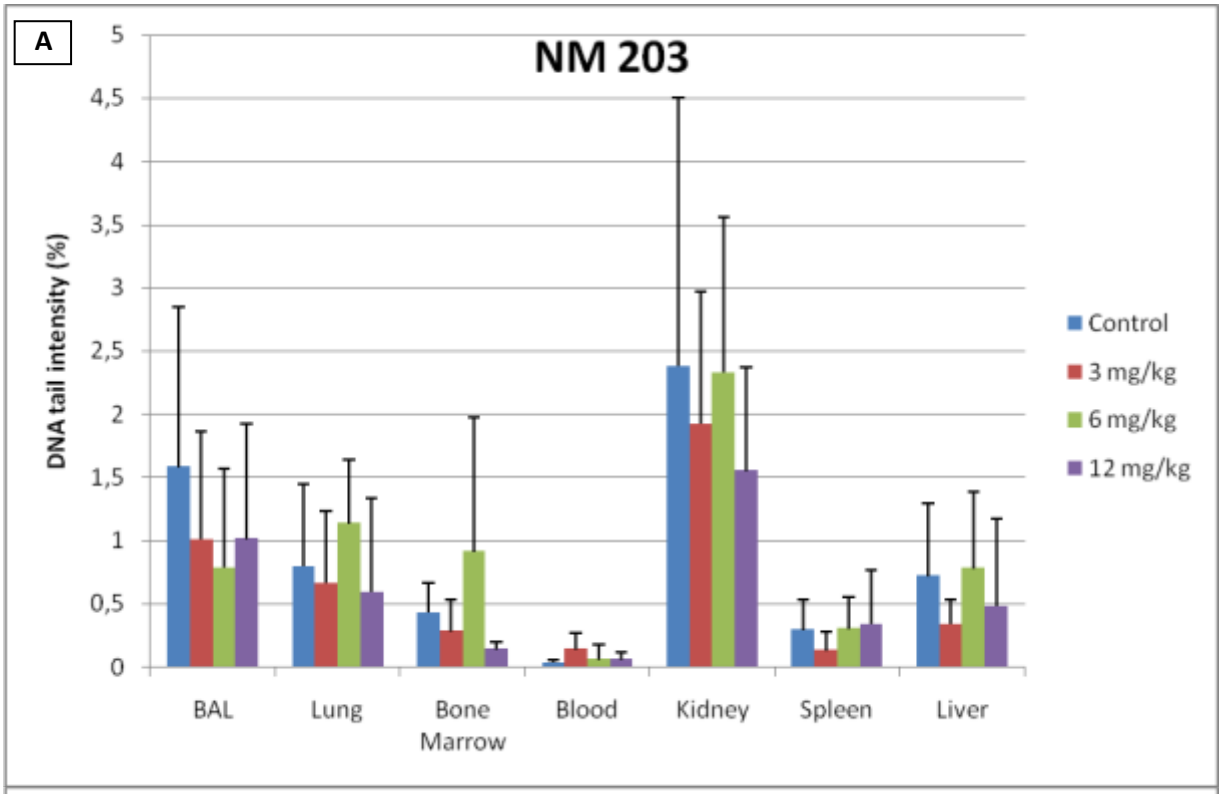


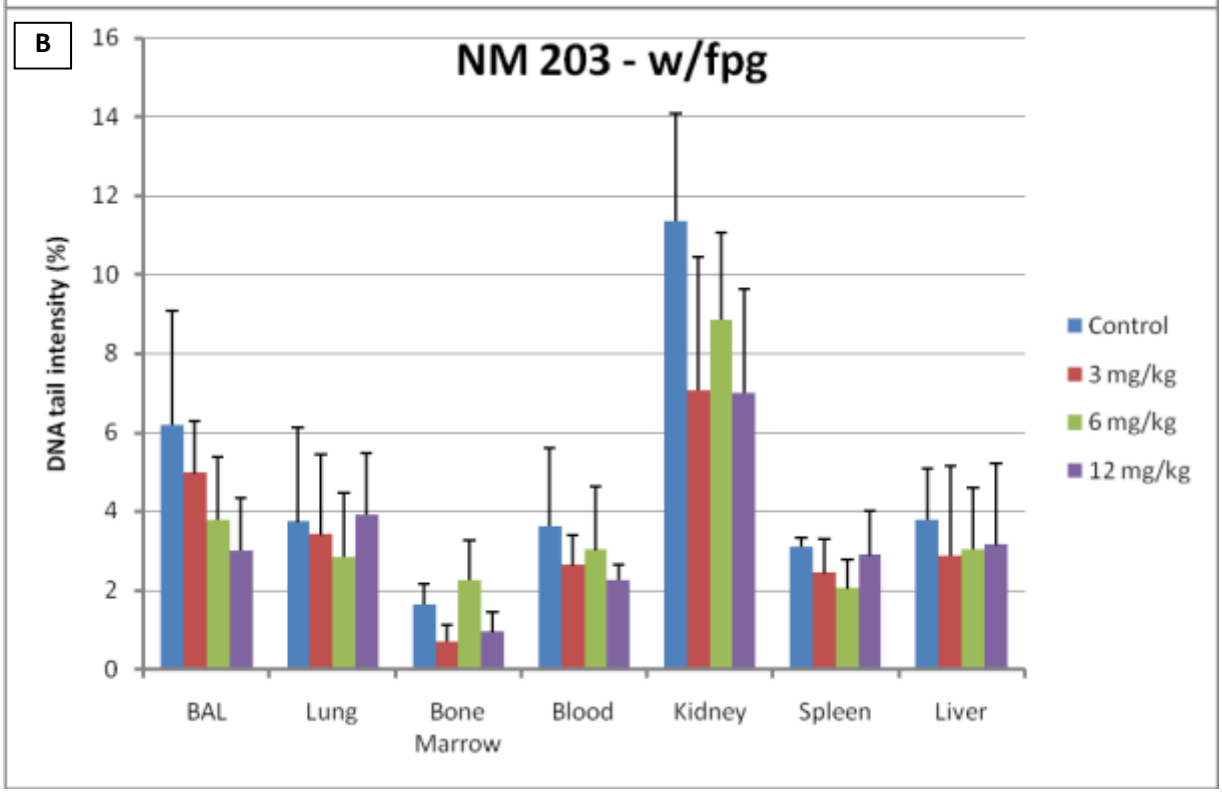
Figure 20: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by intratracheal instillation to NM-202 (A) or by gavage to MMS or ENU (only for blood) (C). All data from MMS and ENU were significantly different from the control (Mann-Whitney test, $p<0.05$).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-203**





The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

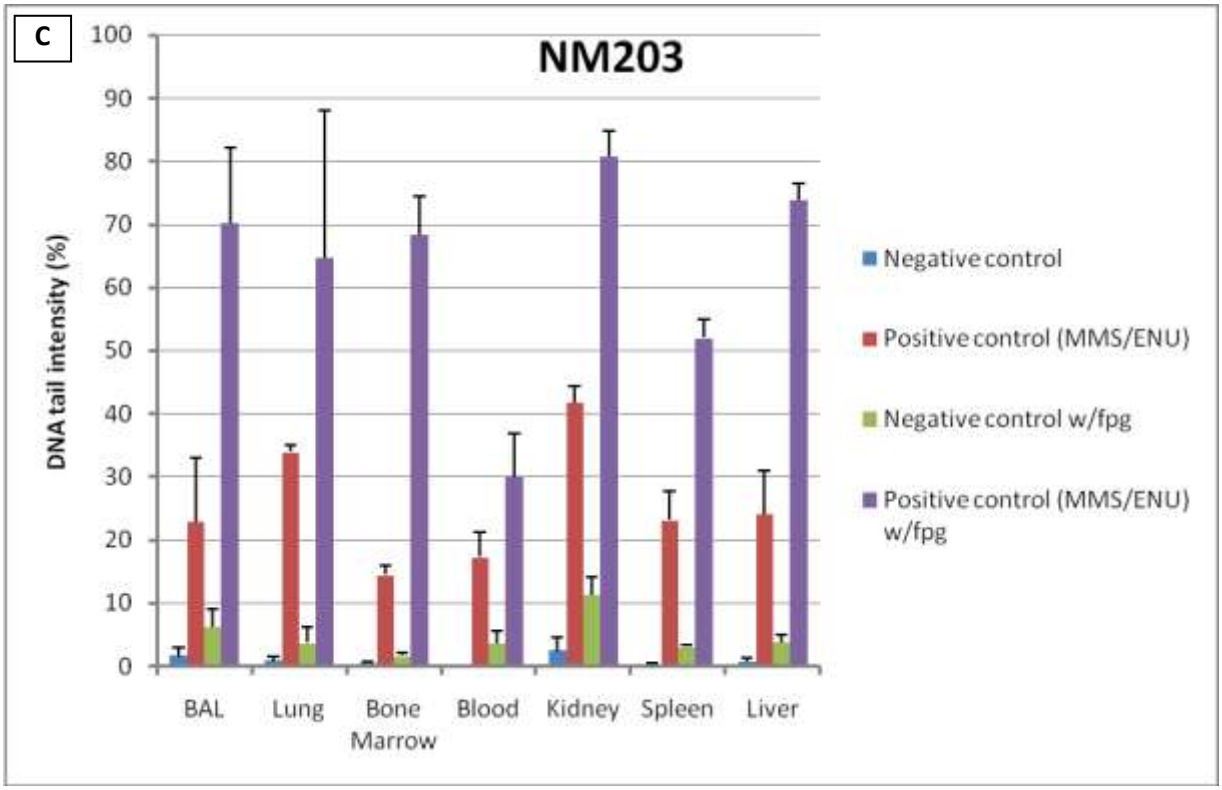


Figure 21: DNA strand breaks assessed by the comet assay in the organs and tissues investigated from rats exposed by intratracheal instillation to NM-203 or by gavage to MMS or ENU (only for blood) (B). All data from MMS and ENU were significantly different from the control (Mann-Whitney test, $p<0.05$).

In conclusion, intratracheal instillation of SAS did not induce any significant increase of DNA damages in all the organs tested. In all the experiments performed, positive controls (MMS or ENU) always induced significant DNA strand breaks in the tissues analysed.

2.1.2.3 Micronucleus assay

No mutagenicity was observed in the bone marrow micronucleus assay irrespective of the SAS (Table 9).

Table 9: Genotoxicity (frequency of micronucleated Immature Erythrocytes) and toxicity (PCE/NCE ratio) induced in bone marrow by SAS following intratracheal instillation. Positive controls MMS and ENU were administered by gavage as described in the Material & Methods. PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes

		Genotoxicity				**Toxicity**			
	[mg/kg]	**Micronucleated Cells Mean**	SD	n	**SE**	**PCE/NCE ratio Mean**	SD	n	**SE**
NM 200	**0**	**0,00070**	0,00045	5	**0,00020**	**0,64**	0,05	5	**0,02**
	3	**0,00090**	0,00042	5	**0,00019**	**0,65**	0,02	5	**0,01**
	6	**0,00150**	0,00050	5	**0,00022**	**0,71**	0,03	5	**0,01**
	12	**0,00160**	0,00042	5	**0,00019**	**0,75**	0,03	5	**0,01**
MMS	**25**	**0,00233**	0,00029	3	**0,00017**	**0,69**	0,01	3	**0,01**
ENU	**25**	**0,00283**	0,00029	3	**0,00017**	**0,66**	0,02	3	**0,01**
NM 201	**0**	**0,00100**	0,00041	4	**0,00020**	**0,61**	0,02	4	**0,01**
	3	**0,00180**	0,00027	5	**0,00012**	**0,63**	0,02	5	**0,01**
	6	**0,00110**	0,00042	5	**0,00019**	**0,65**	0,03	5	**0,01**
	12	**0,00100**	0,00061	5	**0,00027**	**0,61**	0,01	5	**0,01**
MMS	**25**	**0,00183**	0,00058	3	**0,00033**	**0,65**	0,07	3	**0,04**
ENU	**25**	**0,00250**	0,00050	3	**0,00029**	**0,65**	0,05	3	**0,03**
NM 202	**0**	**0,00070**	0,00057	5	**0,00025**	**0,67**	0,02	5	**0,01**
	3	**0,00100**	0,00050	5	**0,00022**	**0,67**	0,04	5	**0,02**
	6	**0,00100**	0,00035	5	**0,00016**	**0,69**	0,01	5	**0,00**
	12	**0,00120**	0,00045	5	**0,00020**	**0,64**	0,03	5	**0,01**
MMS	**25**	**0,00233**	0,00076	3	**0,00044**	**0,65**	0,04	3	**0,02**
ENU	**25**	**0,00250**	0,00050	3	**0,00029**	**0,61**	0,01	3	**0,01**
NM 203	**0**	**0,00050**	0,00035	5	**0,00016**	**0,65**	0,02	5	**0,01**
	3	**0,00070**	0,00045	5	**0,00020**	**0,64**	0,02	5	**0,01**
	6	**0,00050**	0,00050	5	**0,00022**	**0,63**	0,03	5	**0,01**
	12	**0,00080**	0,00045	5	**0,00020**	**0,64**	0,02	5	**0,01**
MMS	**25**	**0,00167**	0,00029	3	**0,00017**	**0,64**	0,01	3	**0,01**
ENU	**25**	**0,00200**	0,00050	3	**0,00029**	**0,66**	0,01	3	**0,00**

2.1.2.2 Histopathology findings

Lung tissue from control and all the SAS exposed animals and liver, spleen and kidney from the control and exposed animals to the highest dose of SAS (i.e. 12 mg/kg) were sent for histopathology evaluation. Tissue slide analysis was done following Hematoxylin and eosin staining.

The main findings were the following:

- Lung:

The incidence of minimal (and sometimes slight or moderate) subacute bronchiolitis/peribronchiolitis was slightly higher than controls dosed intratracheally in animals given SAS. The average severity of eosinophilic and/or pyknotic alveolar material was slightly higher than control in some groups given NM-201 or NM-203, although not with a clear dose-realtionship. The possibility cannot be excluded of an augmentation of these changes by these types of particle.
Animals dosed intratracheally with vehicle or particle suspension had minimal or slight alveolar macrophages, granuloma, subacute bronchiolitis/peribronchiolitis or eosinophilic and/or pyknotic alveolar material, which were considered likely to have been due to the intratracheal dosing procedure. Acute perivascular and chronic pleural inflammatory cell infiltrates are seen spontaneously in laboratory rats and are not considered procedure-related.

- L iver, spleen and kidney:

Intratracheal administration of SAS suspensions did not induce any findings in these organs, since the findings observed were similar to those seen in controls and were of the types which are seen spontaneously in laboratory rats.

2.2. Gavage

2.2.1 MATERIAL AND METHODS

2.2.1.1 Animals

Each MN was tested separately. Experiments were conducted in male Sprague-dawley rats 6-8 weeks old (around 200 g). For each MN, the animals were divided into 5 groups with 5 animals per groups. Normal saline was used as a vehicle control whereas methylmethansulfonate (MMS) was used as a positive control.

The animals were treated via oral route (gavage) as three administrations at 0, 24 h and 45 h. In the case of MMS, the last dose was reduced to 80 mg/kg.

Animals were sacrificed 3 h after the last administration.

2.2.1.2 Tissues collect and sample preparation

Animals were anesthetized with intraperitoneal sublethal dose of pentobarbital (60 mg/kg). After collection of blood in heparin tube, the heart was perfused with 60 ml of cold perfusion medium (HBSS medium supplemented with EDTA 1 mM and Hepes 25 mM) at a flow of 10 ml/min. Organs were collected, rinsed with a grinding buffer (perfusion medium supplemented with 10 % DMSO) and kept on ice. For comet assay, liver and kidney were cut in small pieces and cells were mechanically isolated using a medimachine (5 sec in the grinding medium) whereas spleen cells were isolated by flushing small pieces with a pipette. Intestine and colon were rinsed with HBSS medium and cells were collected by scraping with a coverslip. Finally, bone marrow was collected from isolated femur using fetal calf serum. For histology, small pieces of liver, kidney, duodenum, colon and spleen were sampled, fixed in formaldehyde 4 % and embedded in paraffin. Only the sections from rats exposed to NM-203 were then sent for histology analysis.

2.2.1.3 Comet assay

The alkaline version of the Comet assay was performed following the recommendations of Tice *et al.* (Tice et al. 2000). FpG was used to detect oxidative DNA damage.

Briefly, cells were embedded in 0.5 % of low melting point agarose and spread on microscope slides coated with agarose 0.8 % and precoated with agarose 1 %. After lysis for 1 h at 4° (buffer: 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris-HCl, pH10, 10 % DMSO and 1 % Triton X100), cells were washed 2 x 5 min in FpG buffer (40 mM hepes, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH8) . Slides were incubated with either FpG enzyme (3.6 U/slide) or buffer for 30 min at 37 °C. Slides were then immersed in an alkaline solution (NaOH 300 mM, Na_2-EDTA 1 mM) in an electrophoretic tank for 20 min to allow DNA unwinding and electrophoresis was realized at 300 mA and 25 V for 24 min. Slides were neutralized twice during 5 min with neutralization buffer before dehydrating in ethanol 95 °C for 5 min. For scoring, slides were stained with one drop of propidium iodide (PI) at 20 µg/ml. Analyses were performed with a fluorescence microscope using the Comet IV software. 50 nucleoids per slide with 2 slides per condition were scored. The median percentage Tail DNA (% Tail DNA) of 100 nucleoids was chosen to evaluate the quantity of DNA damage and the mean of 5 animals was calculated.

2.2.1.4 Micronucleus assays

- Bone marrow micronucleus assay

Smears were done by spreading a drop of cell suspension on a slide using a coverslip. After fixing with cold methanol, the smears were sent to scoring lab for staining and scoring.

- Colon micronucleus assay

The swiss roll technique was performed. The colon was sampled from the caecum to the rectum, cut longitudinally and washed with HBSS medium. After rolling up from the rectum to the caecum with the mucosa outwards, the tissue was fixed in formaldehyde 4 % and embedded in paraffin. Staining was performed using Schiff's reagent, according to Feulgen method. First, longitudinal sections on slides were deparaffinized and dehydrated with toluene and ethanol. Then slides were stained using Schiff's reagent followed by counterstaining with fast green and dehydration in ethanol (Figure 22).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

For scoring, slides were mounted by adding DPX between slide and coverslip. Intact colon crypts were chosen for scoring at least 1000 cells per rat.

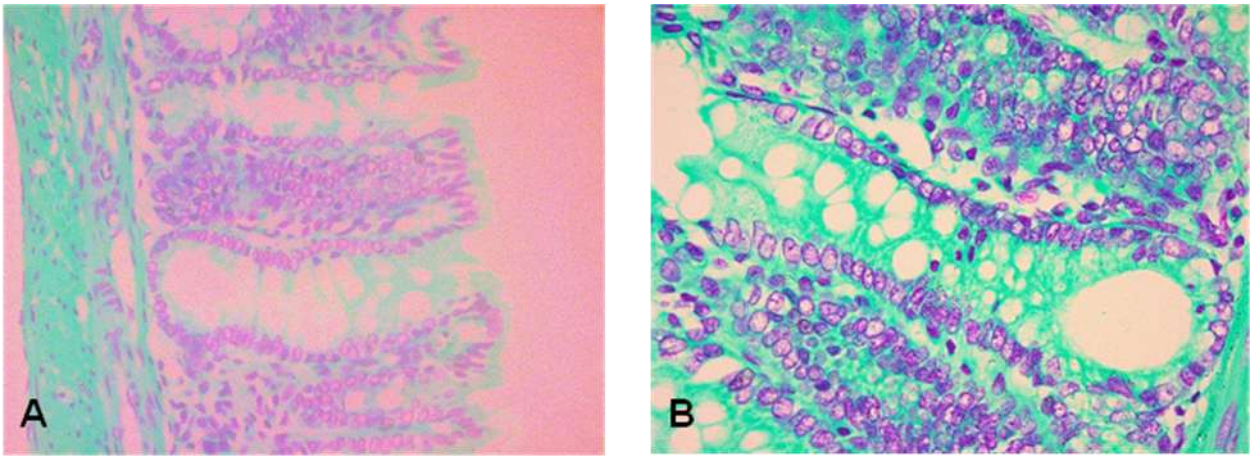


Figure 22: Swiss rolls sections from colon of rats with Feulgen staining and Fast Green counterstain; magnification x400 (A) or x630 (B).

2.2.1.5 LIpid peroxidation

The lipid peroxidation was investigated through the dosage of malondialdehyde (MDA) in the plasma of rats orally exposed to SAS.

- Principle

Oxidative stress may impact all the cellularbiomolecules, especially the lipids. Polyinsaturated fatty acids (PUFA) from the cellular membranes are the main target of hydroxyl radical (•OH). This phenomenon of lipid peroxidation may trigger the formation of secondary metabolites such as malondialdehyde (MDA) which was shown to be cytotoxic and genotoxic. The level of MDA can be measured in biological fluids and was proposed as a biomarker of oxidative stress. In order to add complementary data to the FpG modified comet assay, we measured the levels of plasmatic MDA in the rats orally treated with SAS by gaseous chromatography coupled with mass spectrometry (GC-MS) (Hewlet Packard-Agilent 6890 GC coupled with Agilent 5973 mass spectrometer).

The method consists in derivating aldehydes into pentafluorobenzyloximes (O-PFB oximes) followed by an extraction and sylilation of hydroxyaldehydes to form trimethylsilyl (TMS) esters before GC/MS acquisition. Finally, molecules are identified and quantified using a calibration curve prepared with standard solution of MDA.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

- Method:

MDA was obtained by hydrolysis of TEP (1,1,3,3,-Tetraethoxypropane). TEP was dissolved in 0.1 N hydrochloric acid (HCl) and heated in boiling water for 5 min followed by a rapid cooling. The MDA stock solution was kept in the dark until used. Standard working solutions were prepared by diluting the stock solution in water in order to obtain solutions at 10, 25, 50, 100 and 200 ng/ml.

50 µl of plasma from treated rats were added to 50 µl of water and spiked with 50 µl of tridecanal (internal standard). For the calibration curve, 50 µl of plasma from a rat control were spiked with 50 µl of tridecanal and supplemented with 50 µl of the MDA dilutions (10, 25, 50, 100 and 200 ng/ml)

Following derivatization with O-(2,3,4,5,6-Pentafluorolbenzyl)hydroxylamine hydrochloride (PFBHA.HCl) for 1h, derivative was extracted with methanol followed by hexane. After shaking, the mixture was centrifuged at 3000 g for 5 min at 4°C. The upper layer of hexane was collected and dried. Then, the samples were suspended in 100 µl of hexane and 1 µl of sample was injected for GC/MS analysis using negative-ion chemical ionization.

2.2.1.6 Histological analysis

Samples of liver, kidney, duodenum, colon and spleen were fixed in formaldehyde 4 %. Following dehydration in ethanol then toluene, the samples were embedded in paraffin. Only the sections from rats exposed to NM-203 were then sent for analysis.

2.2 RESULTS

2.2.1 Comet assay

The results are presented in the Figures 23 to 28.
No DNA damage was observed after gavage with SAS irrespective of the MN and the organ investigated.
Using FpG, no oxidative DNA damage was induced in the MN treated animals compared to the control.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-200**

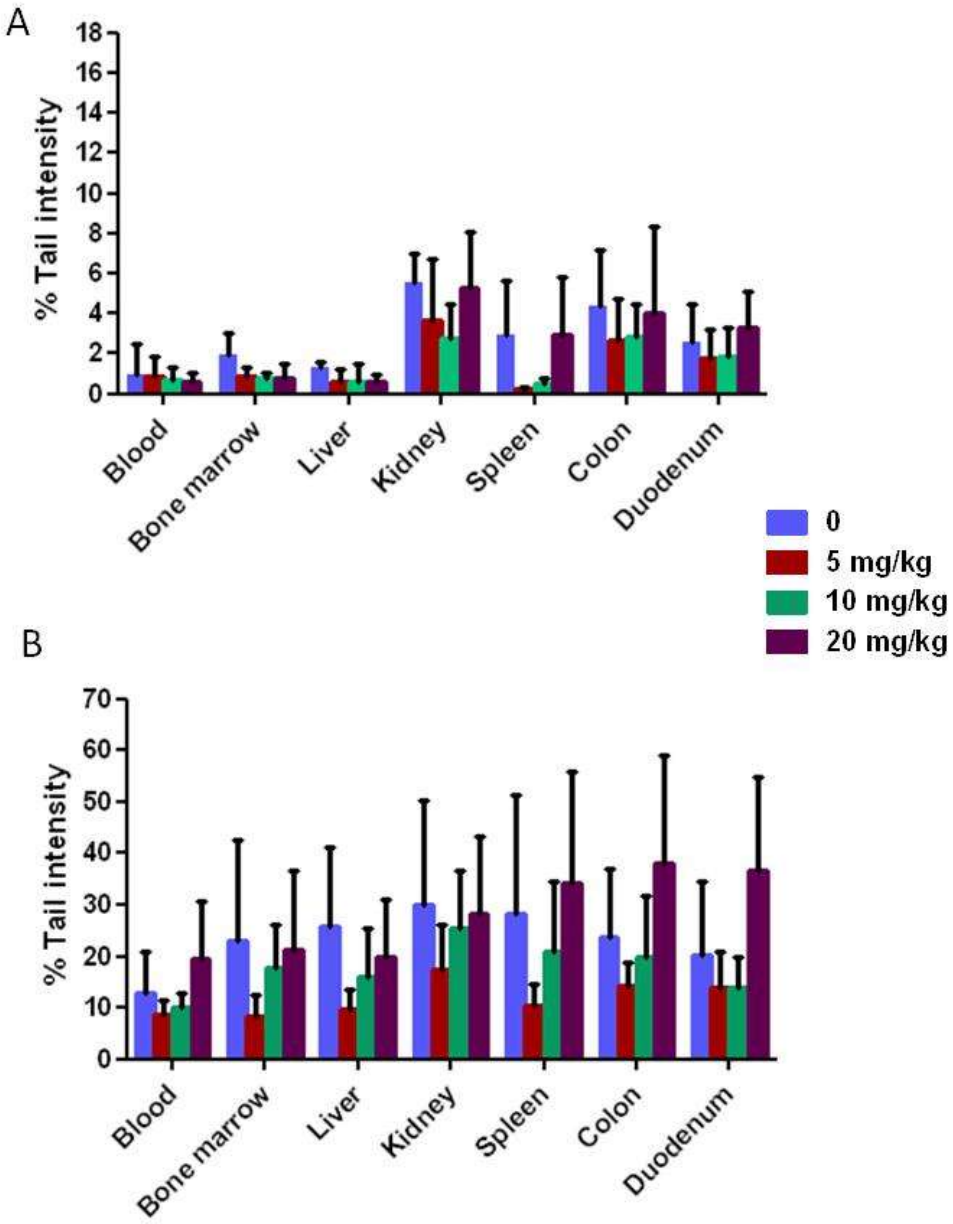


Figure 23: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by gavage to NM-200. Results are expressed as mean ± SD with n=5

❖ **NM-201**

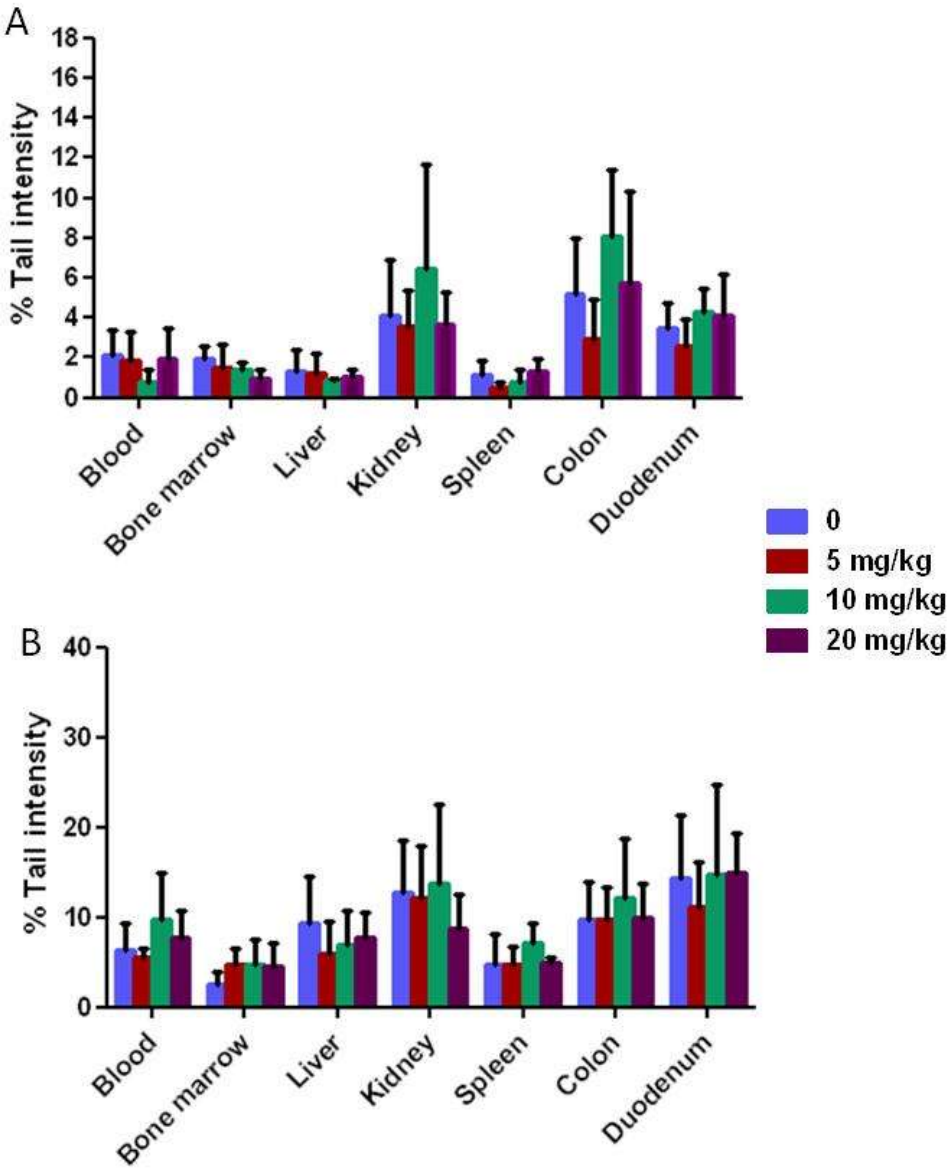


Figure 24: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by gavage to NM-201. Results are expressed as mean ± SD with n=5

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-202**

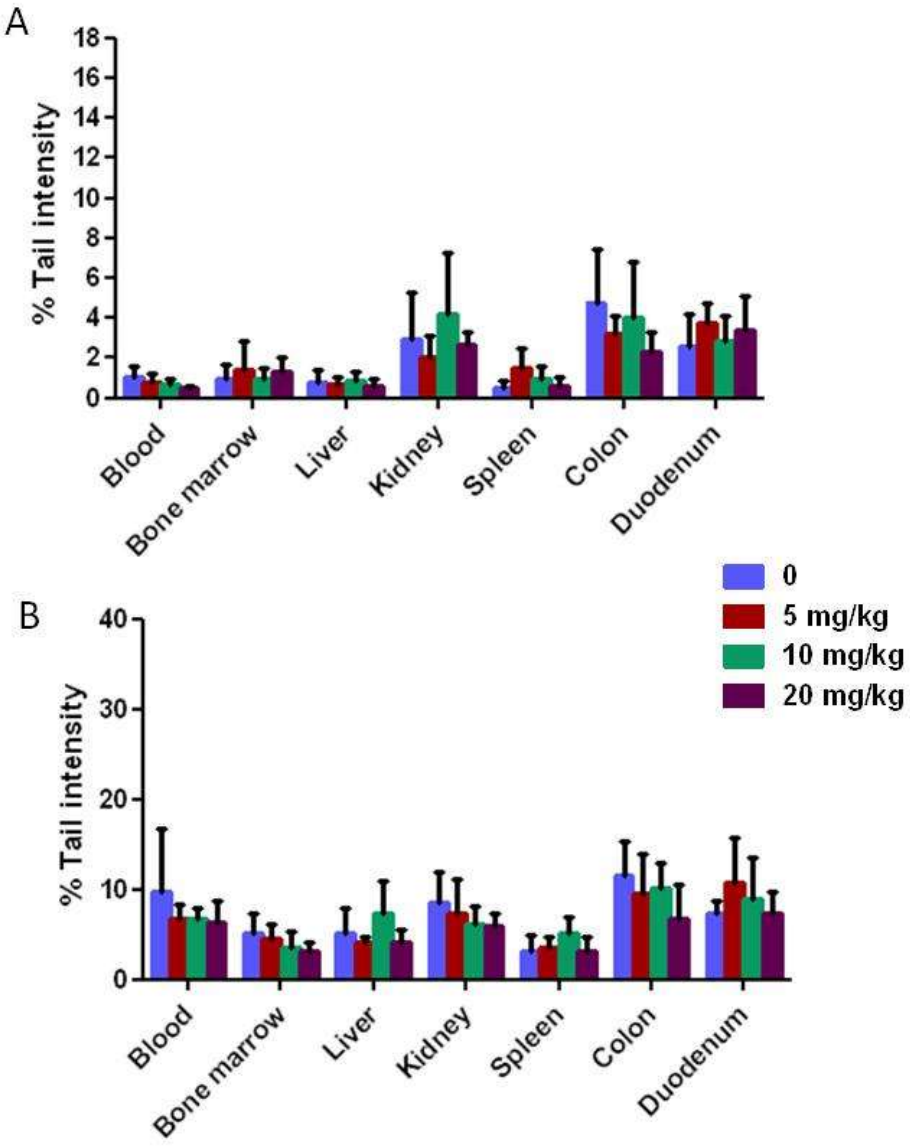


Figure 25: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by gavage to NM-202. Results are expressed as mean ± SD with n=5

❖ **NM-203**

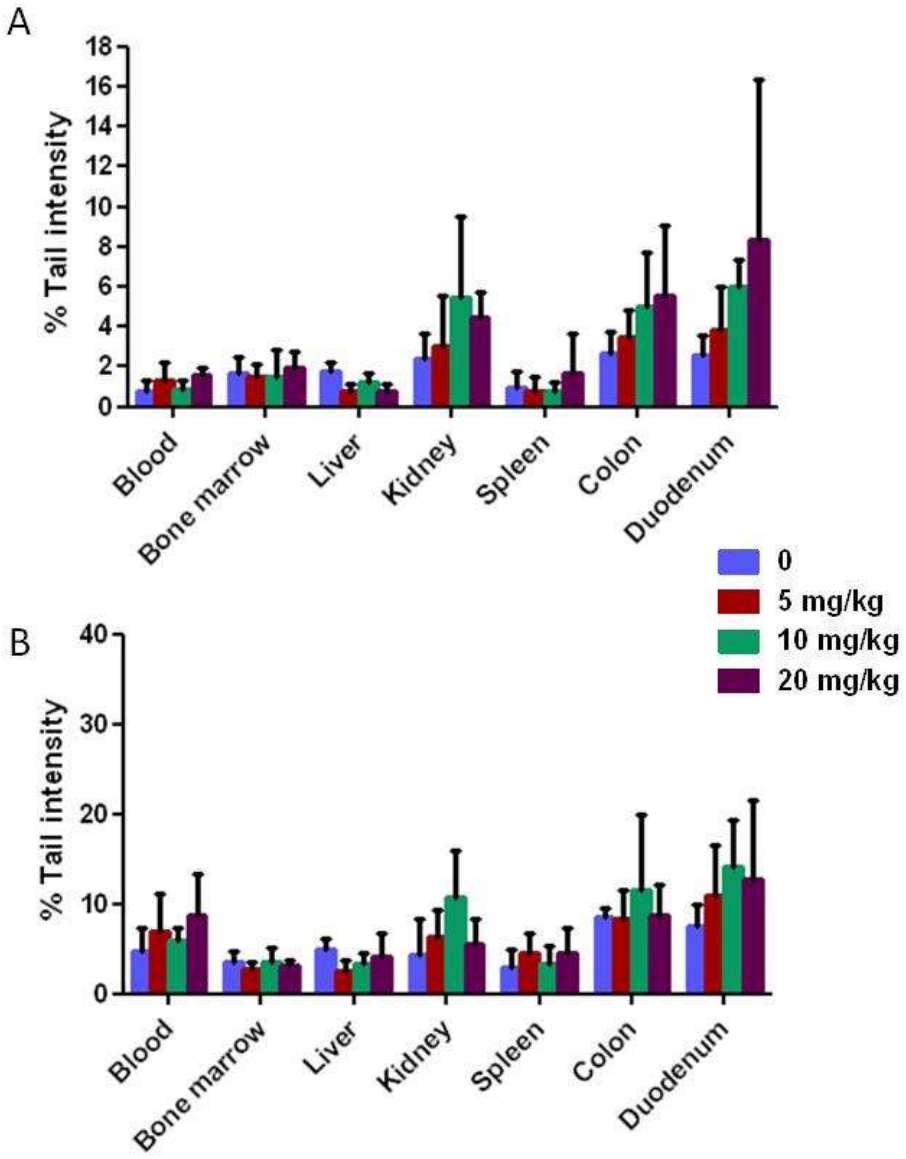


Figure 26: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by gavage to NM-203. Results are expressed as mean ± SD with n=5

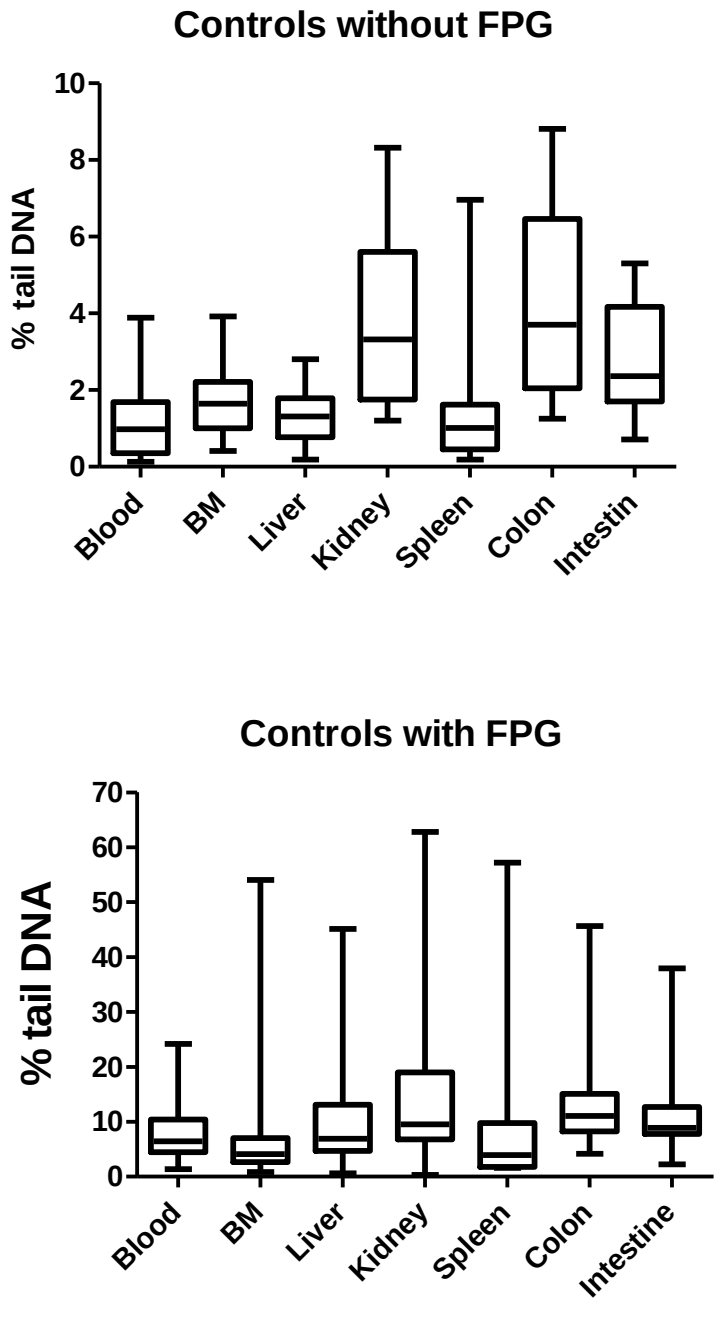


Figure 27: Variability of the comet assay results (%tail DNA) from control rats (n=20) without (A) or with FpG (B) in the organs and tissues investigated after gavage to SAS.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

In the MMS treated animals, the DNA damage was very high, making the slides impossible to score irrespective of the organ.

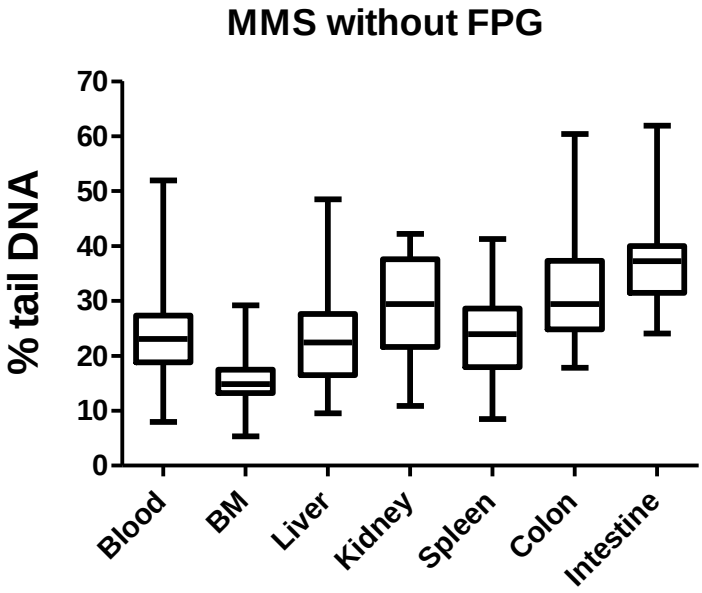


Figure 28: Variability of the comet assay results (%tail DNA) from rats (n=20) treated with the chemical positive control MMS without FpG in the organs and tissues investigated after gavage with SAS.

2.2.2 Micronucleus assay

- Bone marrow

No clastogenicity/aneugenicity was observed in bone marrow with the micronucleus assay irrespective of the SAS MN administered by gavage (Table 10).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Table 10: Genotoxicity (frequency of micronucleated Immature Erythrocytes) and toxicity (PCE/NCE ratio) induced by gavage with NM-200, -201, -202 and -203 in bone marrow. Positive control MMS was administered by gavage as described in the Material & Methods. PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes.

		Genotoxicity				**Toxicity**			
	[mg/kg]	**Micronucleated Cells per 1000 IE Mean**	SD	n	**SE**	**PCE/NCE ratio Mean**	SD	n	**SE**
	0	3,00	1,17	5	0,52	0,59	0,22	5	0,10
	5	1,60	1,47	5	0,66	1,01	1,00	5	0,45
	10	2,00	1,17	5	0,52	0,72	0,48	5	0,21
NM 200	**20**	1,60	1,71	5	0,76	0,79	0,76	5	0,34
MMS	**100**	36,80	17,28	5	7,73	0,30	0,25	5	0,11
	0	2,40	0,82	5	0,37	0,87	0,47	5	0,21
	5	2,40	0,96	5	0,43	1,88	0,87	5	0,39
	10	2,90	2,07	5	0,93	0,76	0,44	5	0,20
NM 201	**20**	2,20	1,48	5	0,66	0,92	0,71	5	0,32
MMS	**100**	24,90	12,11	5	5,41	0,32	0,22	5	0,10
	0	2,20	0,57	5	0,25	3,61	2,14	5	0,96
	5	2,50	0,71	5	0,32	3,20	3,80	5	1,70
	10	3,30	1,64	5	0,73	2,07	2,97	5	1,33
NM 202	**20**	3,80	1,89	5	0,85	3,66	2,82	5	1,26
MMS	**100**	30,30	8,31	5	3,72	0,51	0,19	5	0,08
	0	1,90	1,19	5	0,53	1,82	1,62	5	0,96
	5	2,30	1,64	5	0,73	1,74	1,10	5	1,70
	10	2,40	0,96	5	0,43	1,66	2,06	5	1,33
NM 203	**20**	2,10	1,08	5	0,48	0,84	0,50	5	1,26
MMS	**100**	32,41	12,43	5	5,56	0,29	0,30	5	0,08

- Colon

No induction of micronucleus formation in the colon was observed after gavage with NM-200 and -201up to 20 mg/kg (x3). However, micronculeus formation was induced with NM-202 and -203 but the results were statistically significant only at the lowest dose (5 mg/kg (x3)) (Figure 29).

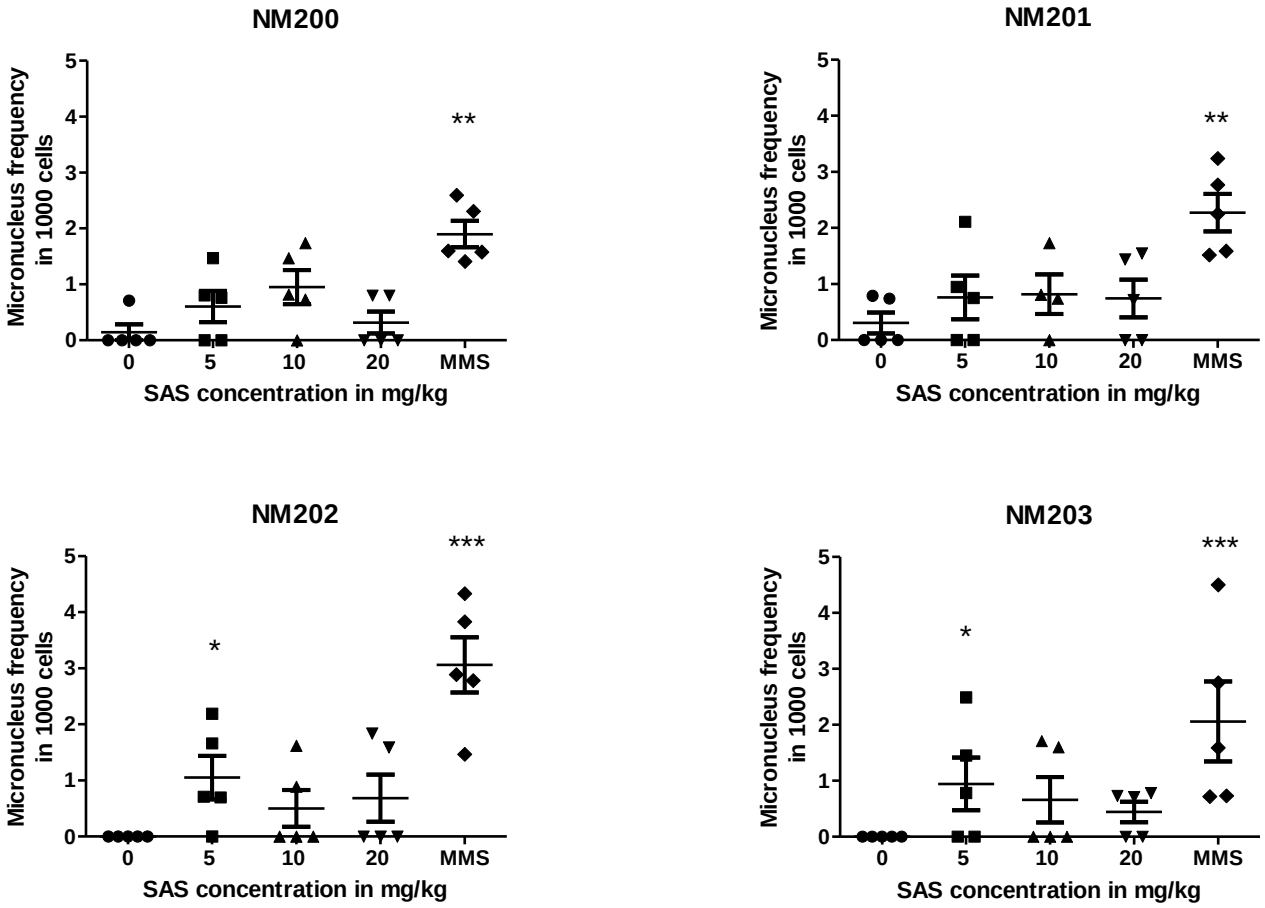


Figure 29: Micronucleus frequency scored from colon swiss rolls of rats treated with SAS by gavage. MMS was used as positive control. * For $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with χ^2 test with Yate's correction

2.2.3 *Lipid peroxidation*

Malondialdehyde (MDA) a product from lipid peroxidation commonly used as a marker of oxidative stress was measured in plasma samples collected from the animals treated by gavage.

No modification of the level of MDA in the plasma of SAS treated animals was observed, irrespective of the MN (Figure 30).

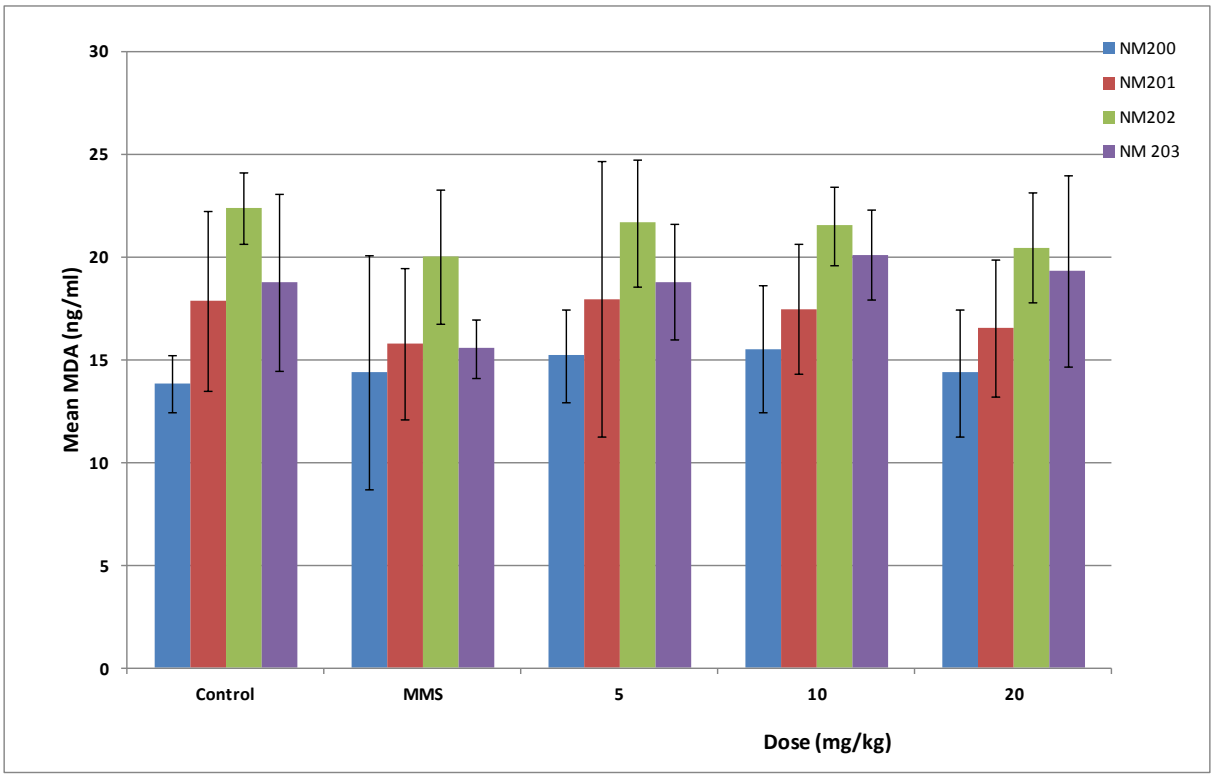


Figure 30: Level of MDA in plasma of rats treated by gavage with NM200, 201, 202 and 203. Mean of 5 animals

2.2.4 *Histopathology*

No histological effects have been observed in liver, kidney, duodenum, colon and spleen from animals treated with NM 203 (5, 10 and 20 mg/kg).
The three other SAS have not been investigated for histology.

2.3 Intravenous administration of NM-203

2.3.1 Materials and Methods

Seven-week old Sprague Dawley rats were purchased from Janvier and were exposed by intravenous injection to NM203 suspensions or vehicle 48, 24 and 3 hours before tissue collection.
Three concentrations were tested 20; 10 and 5 mg/kg b.w.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

For intravenous injection, animals were anesthetized with isoflurane and 330 µL of particle suspensions or vehicles were then administered per 100 g of b.w. through the tail vein using a 24G catheter.

Tissue collection and dissociation were performed the same way as already described for the SAS intratracheal instillation study.
Blood was also collected on in K_2-EDTA- and Li heparin tubes for hematology and biochemistry respectively.
Blood cell count was performed on a Scil Vetabc hematological counter. Leukocyte differential cell count was verified on blood smears stained with Grunwald Giemsa technique.

Paraffin embedded tissue blocks from lung, liver, spleen and kidney from control and all NM203 exposed animals were analyzed for histopathology by a subcontractor (Ricerca Biosciences SAS, France).

2.3.2 RESULTS

2.3.2.1 Toxicity

The highest dose of intravenous NM-203 (20 mg/kg) induced animal death (3 out of 6). NM 203 induced a dose dependent:

- increase of spleen weight (Figure 31)
- increase of liver damage as determined by liver enzymes (glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT)) released into plasma (figure 32).
- thrombocytopenia (Figure 34).

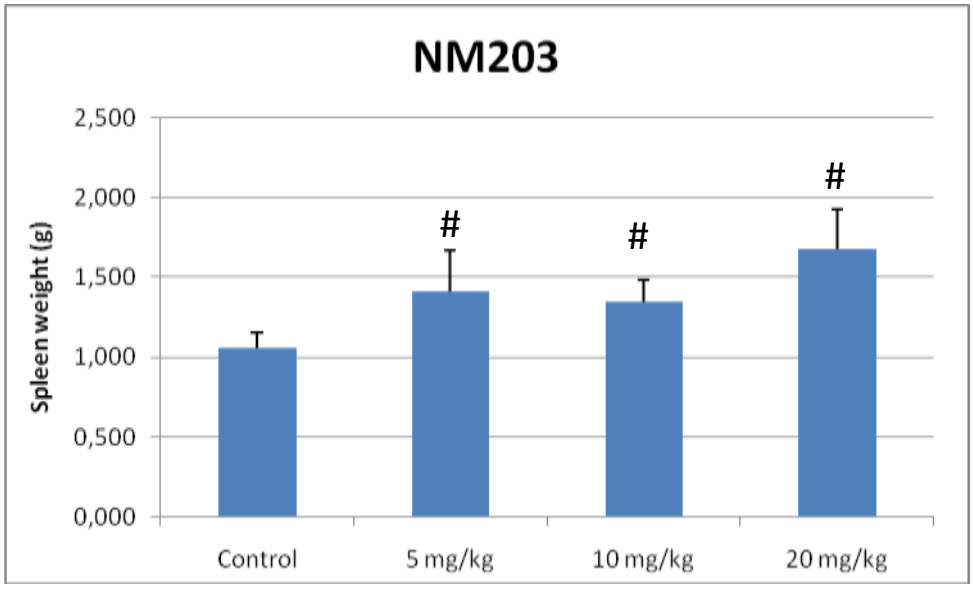


Figure 31: Spleen weight from controls and rats exposed by intravenous injection to NM-203. # Significantly different from the control (Dunnett's test, $p<0.05$).

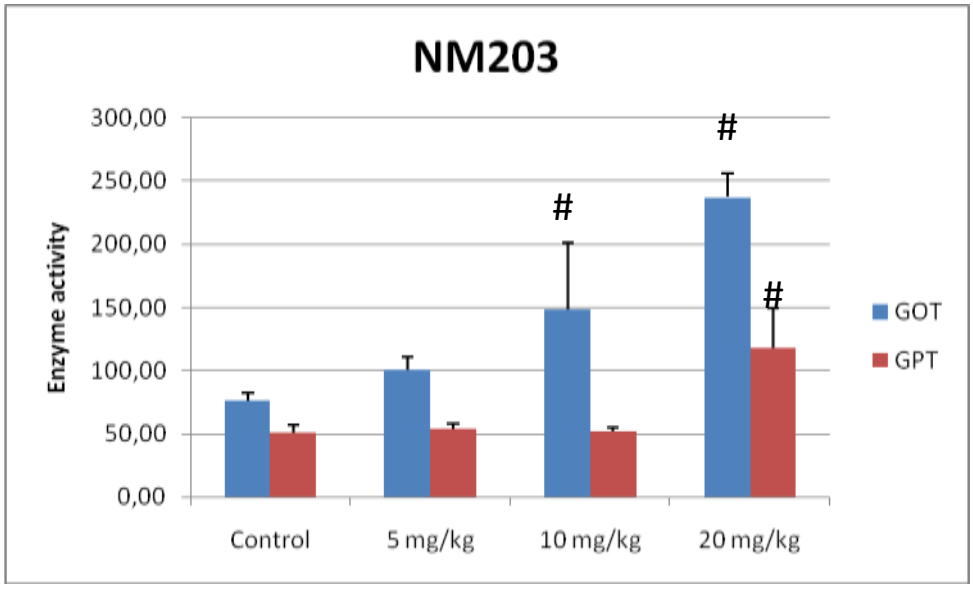


Figure 32: Plasma GOT and GPT activities in controls and rats exposed by intravenous injection to NM-203. # Significantly different from the control (Dunnett's test, $p<0.05$).

2.3.2.1 Blood differential cell count

Intravenous injection of NM-203 induced some modifications of the white blood cell formula and a dose-dependent thrombocytopenia. No change in red blood cell count was however detected (data not shown).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

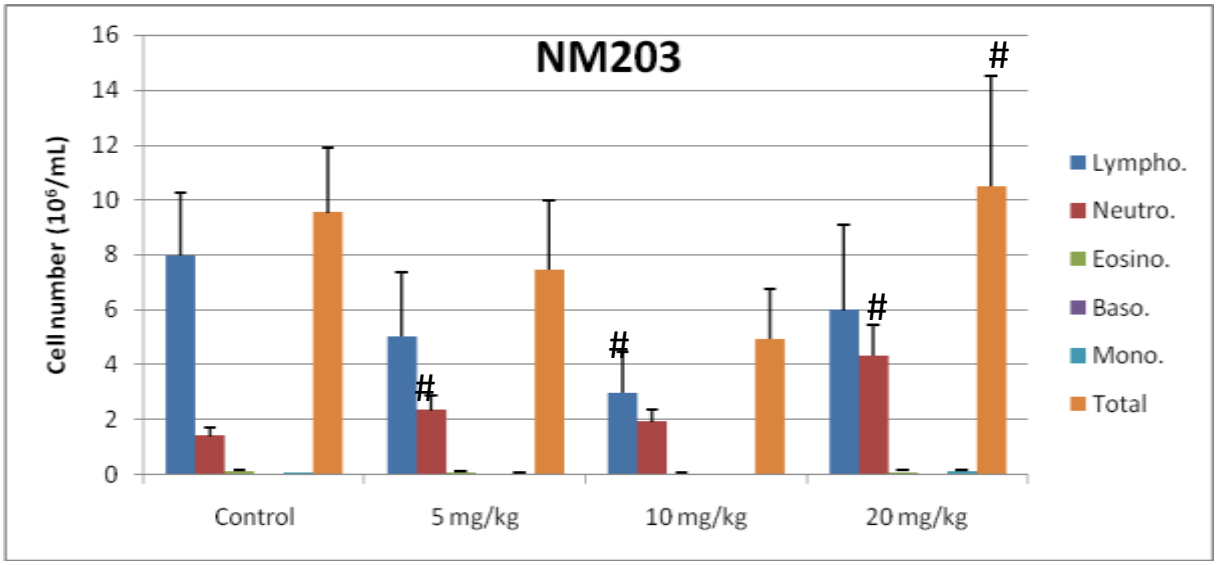


Figure 33: White blood cell differential cell count in controls and rats exposed by intravenous injection to NM-203. # Significantly different from the control (Dunnett's test, $p<0.05$). Lympho: lymphocyte, Neutro: neutrophilic granulocyte, Eosino: eosinophilic granulocyte, Baso: basophilic granulocyte, Mono: monocyte.

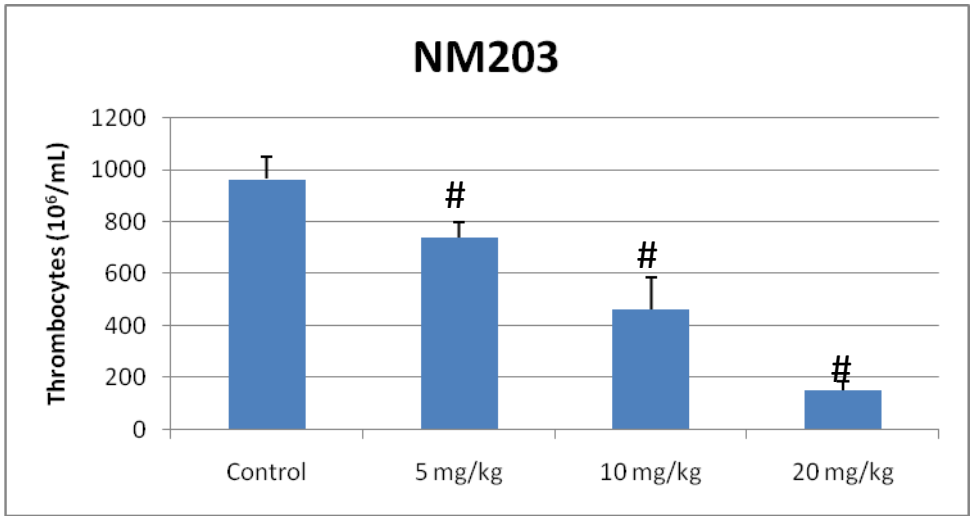
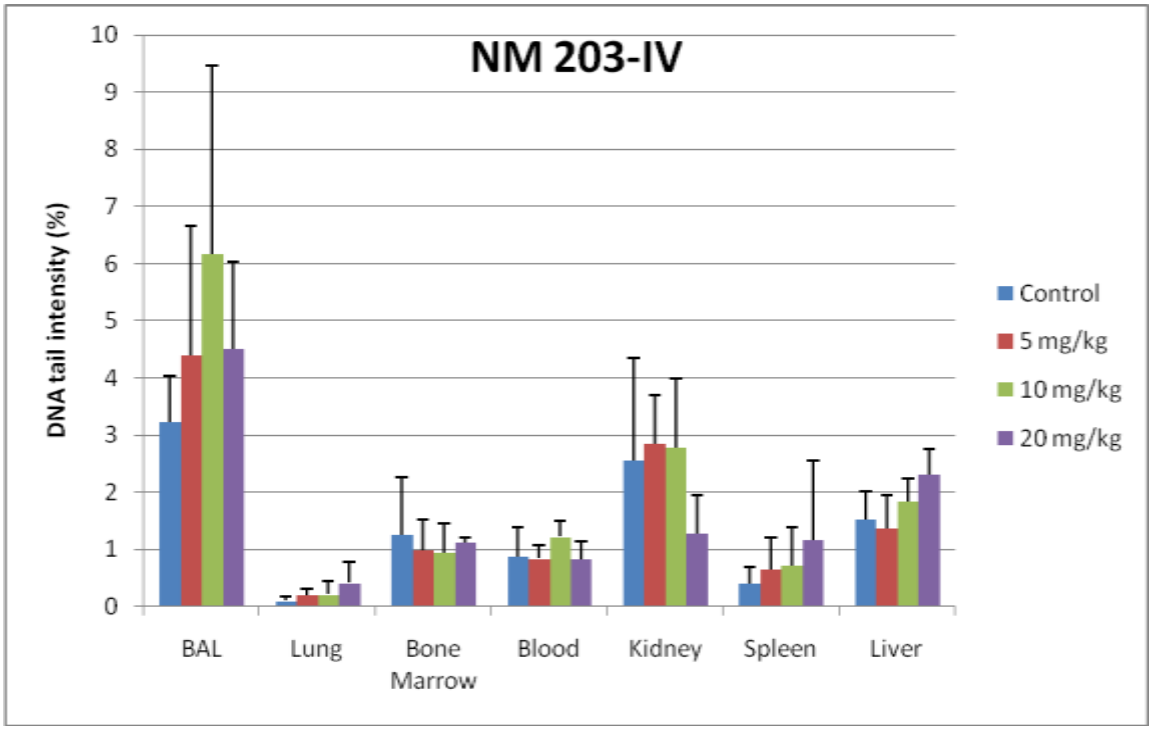


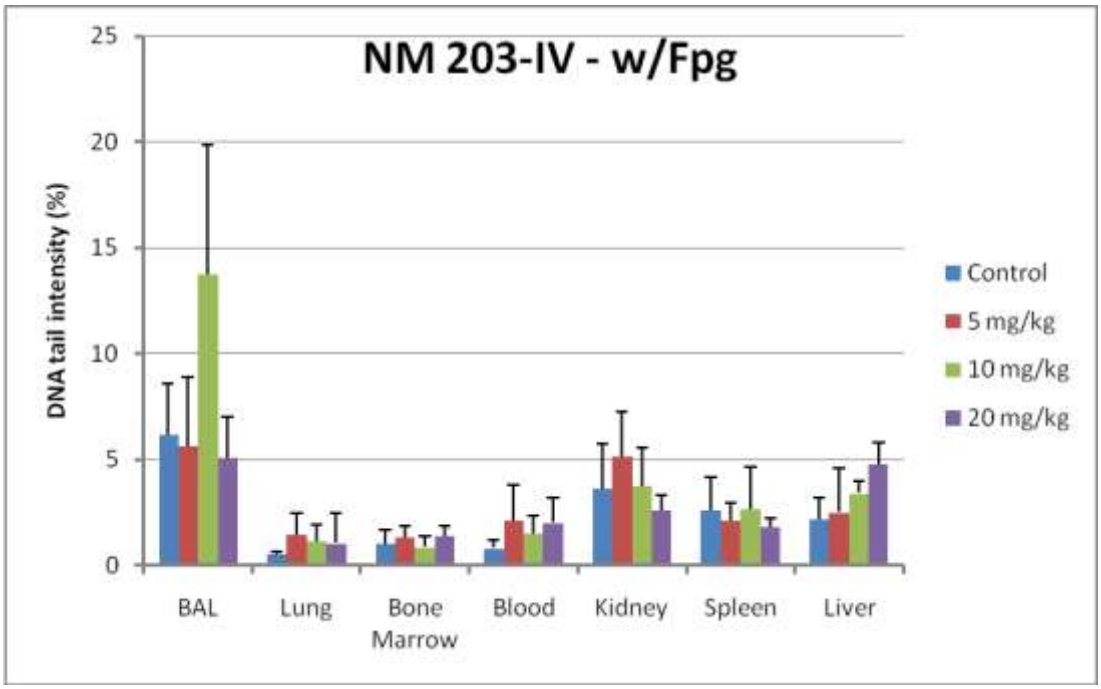
Figure 34: Thrombocytes in blood from controls and rats exposed by intravenous injection to NM-203. # Significantly different from the control (Dunnett's test, $p<0.05$).

2.3.2.2 Comet assay

MMS and/or ENU induced a significant increase of the percentage of tail DNA intensity in all the organs analysed.

In both regular and FpG-modified comet assays, no increase of the percentage of tail DNA intensity was noticed in all the organs tested following intravenous NM-203 treatment (Figure 35).





The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

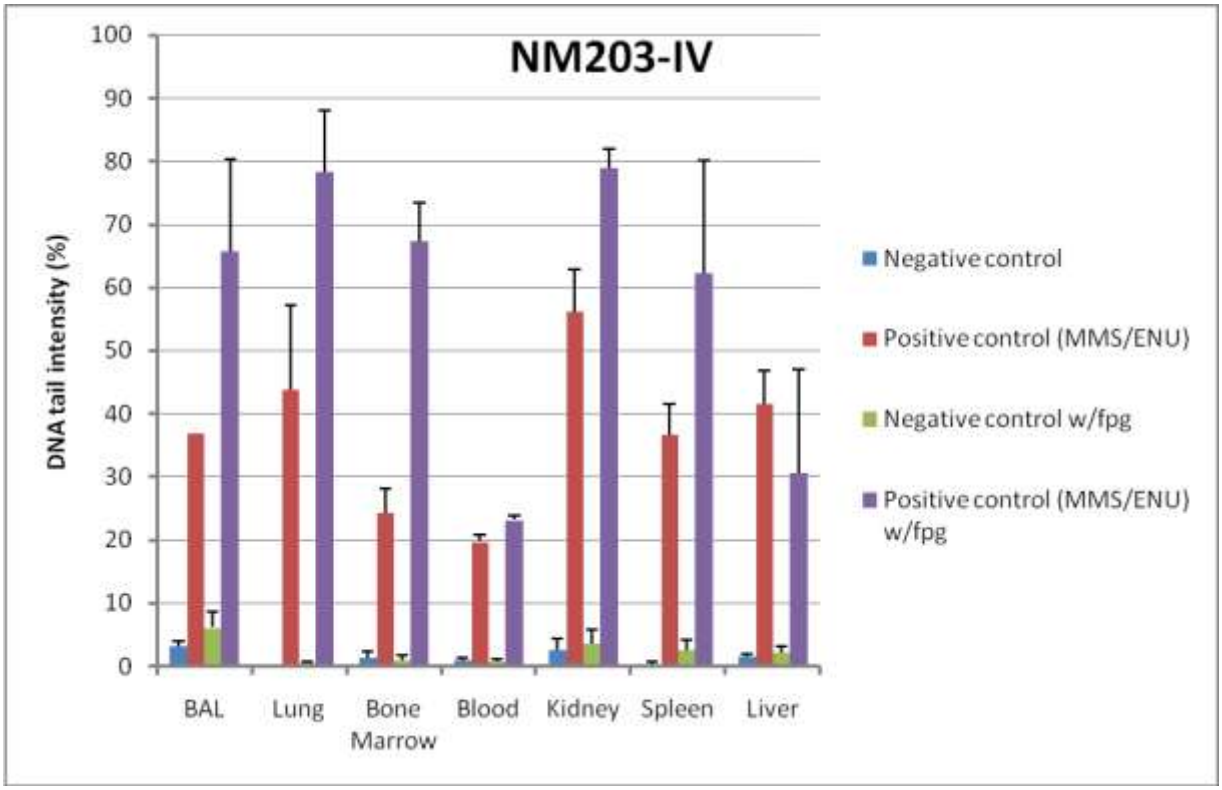


Figure 35: DNA strand breaks assessed by the comet assay without (A) or with FpG (B) in the organs and tissues investigated from rats exposed by intravenous injection to NM-203 or by gavage to MMS or ENU (only for blood) (C). All data from MMS and ENU were significantly different from the control (Mann-Whitney test, $p<0.05$).

2.3.2.3 Micronucleus assay

Only a significant induction of micronucleus was obtained at the highest dose (20 mg/kg) (Table 11). However, the induction is weak and the results were obtained only from 3 animals due to the lethality induced at this concentration.

Table 11: Genotoxicity (frequency of micronucleated Immature Erythrocytes) and toxicity (PCE/NCE ratio) induced by intravenous NM-203 in bone marrow. Positive controls MMS and ENU were administered by gavage as described in the Material & Methods.

		Genotoxicity				Toxicity			
	[mg/kg]	**micronucleated cells Mean**	SD	n	**SE**	**PCE/NCE ratio Mean**	SD	n	**SE**
NM 203	**0**	**0,00060**	0,00042	5	**0,00019**	**0,66**	0,03	5	**0,01**
	5	**0,00100**	0,00035	5	**0,00016**	**0,64**	0,02	5	**0,01**
	10	**0,00140**	0,00042	5	**0,00019**	**0,64**	0,02	5	**0,01**
	20	**0,00183**	0,00076	3	**0,00044**	**0,64**	0,01	3	**0,00**
MMS	**25**	**0,00250**	0,00050	3	**0,00029**	**0,66**	0,03	3	**0,02**
ENU	**25**	**0,00250**	0,00050	3	**0,00029**	**0,67**	0,04	3	**0,02**

2.3.2.4 Histopathology findings

Lung, liver, spleen and kidney tissues from control and all NM-203 intravenously exposed animals were sent for histopathology evaluation. Tissue slide analysis was done following Hematoxylin and eosin staining.

The main findings were the following:

- Lung

Intravenous administration of NM-203 did not induce any findings in the lungs.

- Liver

All animals dosed intravenously with NM-203 had multiple microgranulomas in the liver, which are considered to be due to NM-203. This is the type of lesion that would be expected from intravenous administration of a particle. This conclusion is reached despite the absence of a dose-response and despite the presence of single microgranulomas in three animals not given particle suspensions intravenously. Minimal microgranuloma formation is seen occasionally in untreated rats. There was a minimal increase in hepatocyte mitotic figures in

some of the animals with these microgranulomas; this is considered to be a secondary response of the hepatocytes to the heightened inflammatory state.

- Spleen

Minimal or slight increased macrophages in the red pulp was considered to have been induced by intravenous administration of all doses of NM-203, since it was present in most treated animals and had a dose-related severity pattern. This is the type of lesion that would be expected from intravenous administration of a particle. There was also an increased incidence of minimal neutrophil infiltrate and of pyknotic material in the splenic red pulp in groups given the medium or high dose of NM-203, which may indicate a minimal injurious effect of the particles in the spleen. Haematopoiesis is often seen in the spleen of rats and was not considered treatment-related.

- Kidney

Intravenous administration of the high dose of NM-203 may have induced minimal tubular dilatation with hyaline casts in the kidneys, although minimal findings such as these can occur in rats at this young age, as part of the development of spontaneous nephropathy.

In brief for SAS

- **Dose ranges:**

Instillation: 12, 6 and 3 mg/kg (x3)

Gavage and intravenous (only NM-203): 20, 10 and 5 mg/kg (x3)

- **Comet assay:**

No genotoxicity irrespective of the organ and the route of administration (instillation, gavage as well as iv administration only for NM-203)

- **Micronucleus assay:**

  - **Bone marrow:**

No mutagenicity irrespective of the route of administration

except after iv with NM-203 at the high dose (but no dose response, small increase as well as animal toxicity)

  - **Colon:**

Mutagenicity for **NM-202 and -203 only at the lowest dose**

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

3) CNT

3.1 MATERIAL AND METHODS

3.1.1 Animals

Young adult male OFA Sprague-Dawley rats (Charles River France origin, Saint-Germain-sur-l'Arbresle; FRANCE) were used for the study. The period of acclimatisation was of at least 5 days. The animals received a clinical examination in order to retain only those which were healthy.

The animals were identified by numbered ear rings.

Body weights in male rats of the main assay were about 200 g. At the start of the main study, the weight range of animals did not exceed ± 20 % of the mean weight when compared to the vehicle group. Indeed, the weight homogeneity of the animals used in this test after random-distribution was checked, by comparing the mean weight in each treatment group with that in the control group using Student's t test.

The bedding consisted of dust-free, irradiated softwood pellets.

The animals were dispatched in polypropylene cages by random-distribution.

The cages were placed in a ventilated system in the animal room, which was ventilated 20 times per hour. A timer provides lighting 12 hours a day (8 a.m. - 8 p.m.) in all the animal room. The temperature in the ventilated animal cupboard was 22 ± 3 °C, and humidity was 55 ± 15 %.

The animals were not fasted at the treatment time. Drinking water, softened by reverse osmosis and filtered on 0.20 µm membrane, was provided *ad libitum*. The feedstuff used was A04C irradiated from Safe.

3.1.2 Nanoparticle preparation

- *Oral route*:

NM-400: dispersions at 0.64 mg/mL were prepared, *i.e.* function of the maximum of solubility of the nanomaterial

NM-401 – NM-402 – NRCWE-006: dispersions at 2.56 mg/mL were prepared in accordance with the recommendations from WP4.

- *Endotracheal route*:

The highest concentration leading to a satisfactory dispersion and compatible with the route of exposure

NM-400: max initial concentration: 0.64 mg/mL

NM-401: max initial concentration: 0.32 mg/mL

NM-402: max initial concentration: 1.28 mg/mL

NRCWE-006: max initial concentration: 0.32 mg/mL

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

MNs were dispersed in sterile water (Fresenius) containing 0,05% of RSA and 0,5% of Ethanol according to the WP4 dispersion protocol.

3.1.3 Treatments

Rats were treated 3 times with the NMs by either oral or endotracheal route at different dose levels. Specifically, the animals were treated for 3 days at 24-hour interval. Three to six hours after the third treatment, the rats were deeply euthanized and cells from the selected target organs were isolated for both the micronucleus test and the comet assay.

3.1.4 Tissue collection

For tissue collection, animals were anesthetised with pentobarbital (60 mg/kg)

- *Colon*:

For NM-400, colon was opened and the internal side was scratched with a scrapper. Little pieces were prepared and put in a 50 µm-medicon with 1mL PBS for 10 seconds. Liquid was collected and medicon was rinsed with 1 mL of PBS. Liquid was added to 3 mL of PBS in a tube and then centrifugated at 1000 rpm for 5 minutes. Supernatant was removed and 3 mL of PBS were added. Cells were suspended and filtered through a 60 µm-filter. Finally, cells were enumerated in presence of Trypan blue.

For the other MNs, colon was opened and the internal side was scratched with a scrapper. 8 mL of a mix collagenase/dispase were added and incubated at 37°C during 20 minutes. 0.8mL Fetal Bovine Serum was added and cells were suspended. Centrifugation was performed during 1000 rpm for 5 minutes. Supernatant was pumped out and 4 mL of HBSS were added. Cells were enumerated in presence of Trypan blue.

- *Liver*:

For NM-400 (oral and endotracheal route), liver was cut in little pieces and then put in a 50 µm-medicon and crushed for 5 seconds with 1 mL of PBS. Liquid was collected and medicon was washed with 1 mL of PBS. Liquid was added to 3 mL of PBS in a tube and then centrifugated at 400 rpm for 3 minutes. Supernatant was removed and 3 mL of PBS were added. Cells were suspended and filtered through a 60 µm-filter. Finally, cells are enumerated in presence of Trypan blue.

For the others MNs, liver was opened and gently cut with a scalpel in 5 mL of PBS. All the liquid was collected in a tube and cells were directly enumerated in presence of Trypan blue.

- *Spleen*:

A gentle incision was done with a scalpel and then scratched carefully in 5 mL of PBS. 500 µL of the liquid were collected and added to 10 mL of PBS. Counting was done in presence of Trypan blue.

- *Kidney*:

The fibrous layer was taken off and kidney was cut in little pieces and then put in a 50 µm-medicon and crushed for 5 seconds with 1 mL of PBS. Liquid was collected and medicon was washed with 1 mL of PBS. Liquid was added to 3 mL of PBS in a tube and then centrifugated at 400 rpm for 3 minutes. Supernatant was removed and 3 mL of PBS were added. Cells were suspended and filtered through a 60 µm-filter. Finally, cells were enumerated in presence of Trypan blue.

- *Lung*:

This organ was cut in little pieces and then put in a 50 µm-medicon and crushed for 5 secondes with 1 mL of PBS. Liquid was collected and medicon was washed with 1 mL of PBS. Liquid was added to 3 mL of PBS in a tube and then centrifugated at 400 rpm for 3 minutes. Supernatant was removed and 3 mL of PBS were added. Cells were suspended and filtered through a 60 µm-filter. Finally, cells were enumerated in presence of Trypan blue. For the additional experiment, PBS was replaced by PBS + 2% Fetal Calf Serum and previous to enumeration cells were filtered through a 150 µm-filter.

3.1.5 Comet assay

Before use, a volume of 85 µL of 0.8% of Normal Agarose (NA) was added on the microscope slide pre-layered with 1.5% of NA and then covered with a glass coverslip. Slides were placed at room temperature until the agarose layer hardened (3 to 5 minutes). Around 3 x 10^4 cells of the different doses tested were mixed with 75 µL of 0.5% of Low Melting Point Agarose (LMPA) kept at 37 °C and added on the microscope slide after gentlly sliding off the coverslip. The slides were then covered with a new glass coverslip, and were placed once again at room temperature for 3 to 5 minutes.

Six slides were prepared for the comet assay: 2 for the regular single gel electrophoresis assay and 2 for the formamidopyrimidine DNA glycosylase (FpG)-modified comet assay. Two other slides were prepared for the non-denaturating fast halo assay

After the top layer of agarose has solidified, the glass coverslips were removed and the slides were immersed for at least 1 hour at + 4 °C in the dark in a lysing solution consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, to which 1% Triton X-100 and 10% DMSO were freshly added (pH adjusted to 10 with NaOH).

For the FpG-modified comet assay, following lysis, slides were washed two times 5 min with the FpG incubation buffer (Hepes 40 mM, KCl 0.1 M, EDTA 0.5 mM, bovine serum albumin 0.2 mg/mL; pH 8) at room temperature and then incubated for 35 min at 37°C with **0.6 U/mL** of FpG (Sigma Aldrich ref F3174) in the FpG incubation buffer. For the standard comet assay, slides were not treated with FpG.

After this incubation period, the slides were then removed and placed on a horizontal gel electrophoresis unit and the unit filled with freshly prepared alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13) to around 0.25 cm above the slides. In order to avoid excessive variation across the groups during each electrophoretic run, only one of the replicate slides was processed in each run for each animal (DNA – unwinding and electrophoresis). The cells were exposed to the alkali for 20 minutes to allow the DNA unwinding, and expression of single-strand breaks and alkali-labile sites. Next, electrophoresis was conducted for 20 minutes at 0-4°C by applying an electric current of 0.7 V / cm (25 V / 300 mA). All these steps were conducted protected from daylight to prevent the occurrence of additional DNA damage. After electrophoresis at pH >13, the slides were neutralized

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

twice for 5 minutes with 0.4 M Tris (pH 7.5) and the DNA was exposed for 5 minutes to absolute ethanol in order to preserve all the comet assay slides. Subsequently, the slides were airdried and then stored at room temperature until they were scored for DNA migration.

Just prior to scoring, the DNA was stained using propidium iodide (final concentration of 20 µg/mL in distilled water; 25 µL/slide).

Slides were examined with a 200 x magnification, using a fluorescent microscope (Leica Microsystems SAS - DM 2000, Heerbrugg, Switzerland), equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm, connected through a gated monochrome CCD IEEE1394 FireWire video camera (Allied Vision Technologies), to the Comet Assay IV Image Analysis System, version 4.11 with Windows XP Pro Software (Perceptive Instruments Ltd, Suffolk, UK).

6 slides were prepared for each animal and each organ, with 4 animals per group. 100 cells per slide were randomly scored, *i.e.* 200 cells per animal (800 analysed cells per group).

For the Fast Halo Assay, the slides were immersed in the lysis solution at pH 10.1, for 10 minutes at + 4 °C in the dark. The slides were then rinsed in PBS for less than 30 seconds and neutralized for 15 minutes in PBS (pH 7.4) containing 0.1 mg/mL RNase. The DNA was then rinsed in distilled water and exposed for 5 minutes to absolute ethanol in order to preserve all the halo assay slides. Subsequently, the slides were airdried and then stored at room temperature until they were scored for the presence of halos.

Just prior to scoring, the DNA was stained using propidium iodide (final concentration of 20 µg/mL in distilled water; 25 µL/slide).

In order to quantify the test item effects on DNA, the following statistical analysis strategy was applied, using the statistical software Stat view®, version 5.

As the median of percentage of DNA in tail and other tail parameters do not follow a Gaussian distribution (E. Bauer *et al.*, 1998), the non-parametric, one-way Kruskall-Wallis test was performed. This method is based on the analysis of variance by ranks for testing equality of population medians among groups, in order to display a possible dose-response relationship.

The non-parametric Mann-Whitney U-test was applied to compare each of the doses tested with the vehicle control in order to determine statistical significance of differences in group median values between each group versus the vehicle control. This test was also used to compare vehicle control and positive control to determine acceptable criteria of a valid test.

3.1.6 Micronucleus test on bone marrow

At the sampling time, the femurs were removed and the bone marrows were extracted with fetal calf serum (1 mL per animal). The cell suspensions were centrifuged for 5 minutes at 1000 rpm. The supernatants were removed. The centrifugate was spread on slides. The smears were stained using a technique, derived from the May Grunwald Giemsa technique which makes it possible to distinguish between polychromatic (PCE) and normochromatic erythrocytes (NCE): PCE are purple whereas NCE are red.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

After blind coding the slides by a person not involved in the study, two slides per animal were read by two independent operators; for each animal, the number of polychromatic erythrocytes having one or more Howell-Jolly bodies (micronuclei) was determined from the microscopic examination of 2000 polychromatic erythrocytes. As divergence was noted, 2000 new polychromatic erythrocytes were examined. The retained value was the mean of all readings.

The polychromatic/normochromatic erythrocyte ratio was determined from the microscopic examination of 1000 erythrocytes per animal.

The statistical comparison for the polychromatic/normochromatic erythrocyte ratio was performed using Student's t test.

Statistical analysis was performed for micronucleated PCE number using the Chi^2 test.

3.1.7 Micronucleus test on colon

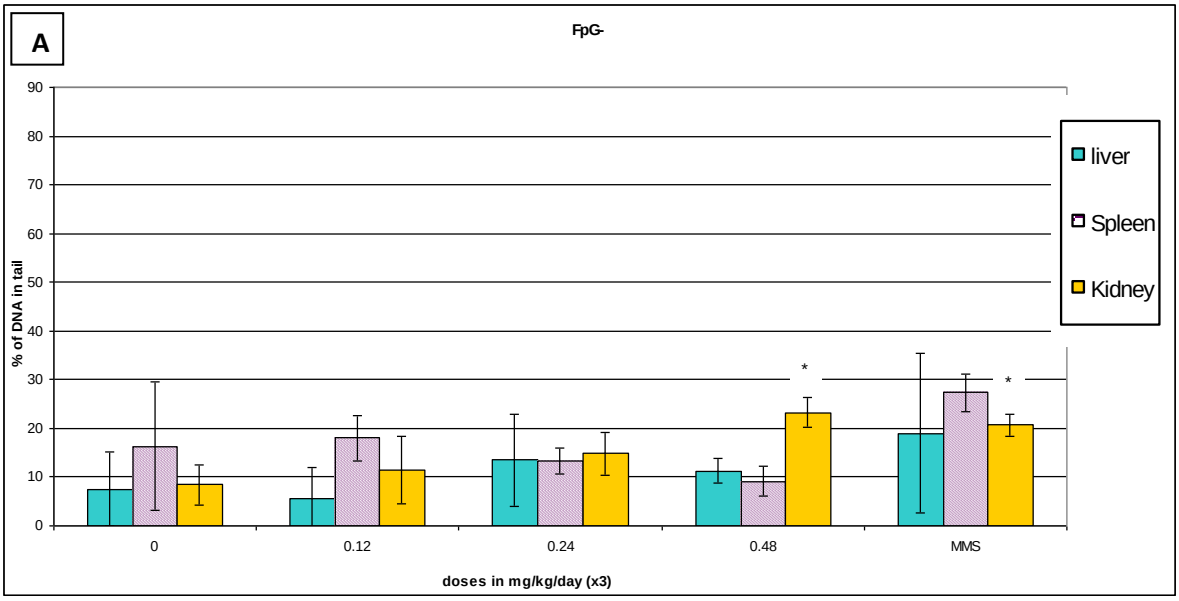
Micronucleus test on colon was performed according to the protocol already described for TiO_2 and SAS.

3.2 RESULTS

3.2.1 Intratracheal instillation

3.2.1.1 Comet assay

❖ **NM-400**



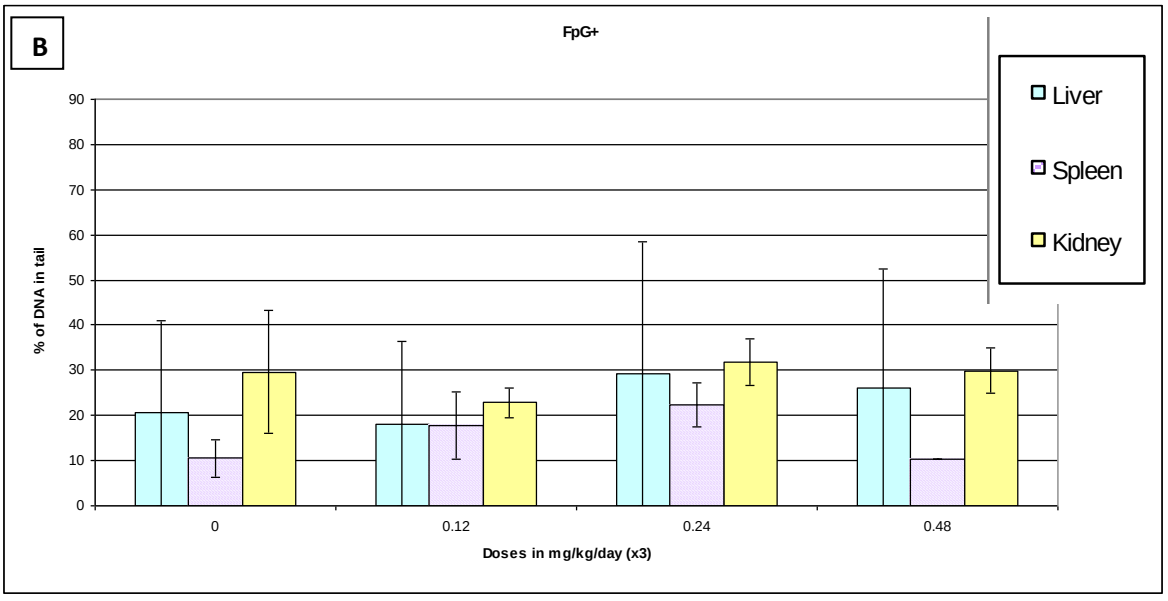


Figure 36: Comet assay with NM-400 without (A) or with FpG (B)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Comments without FpG

As no satisfactory DNA migration was noted in the spleen, the results were invalidated.

A statistically significant increase in the percentage of DNA in tail was noted in kidney at the highest dose of 0.48 mg/kg/day (x3).

Regarding the results noted for the positive control, **MMS**, in the **liver**, the results were considered as biologically significant (high inter-animals heterogeneity).

With FPG

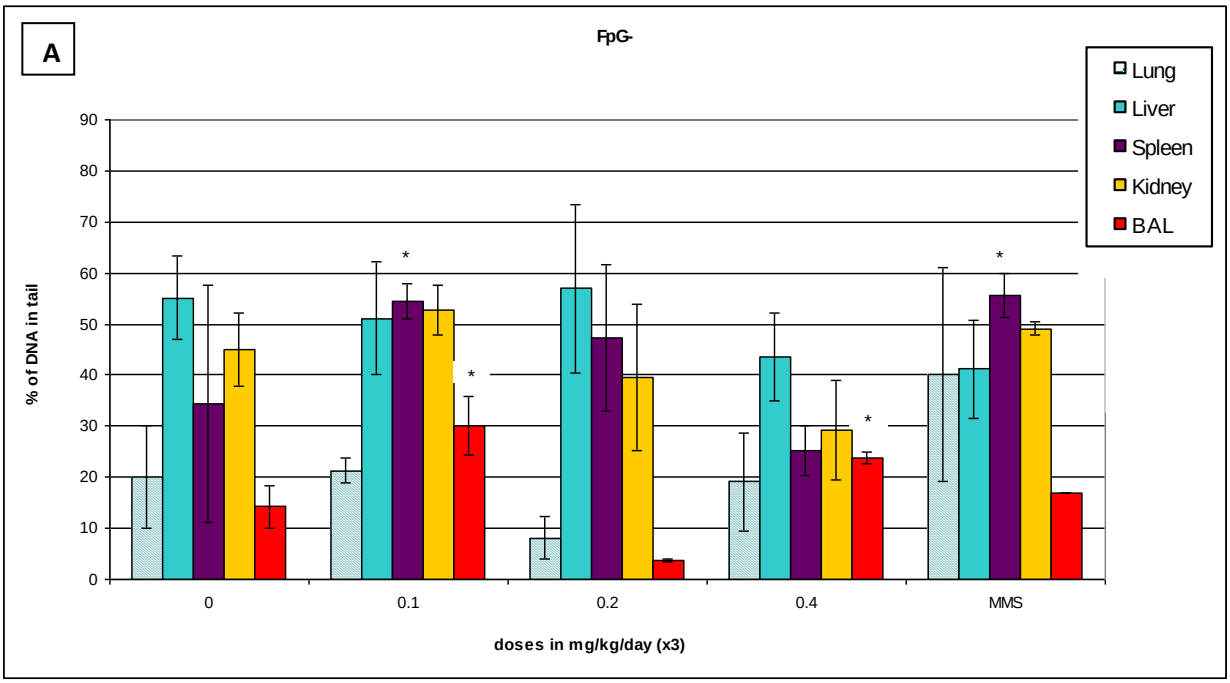
As no satisfactory DNA migration was noted in the **spleen**, the results were invalidated.

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analyzed and the dose tested.

Conclusion NM-400 - Instillation

No statistically significant increase in the percentage of tail DNA with or without FpG, except in the kidney at the highest dose of 0.48 mg/kg/day (x3), in the presence of FpG.

❖ **NM-401**



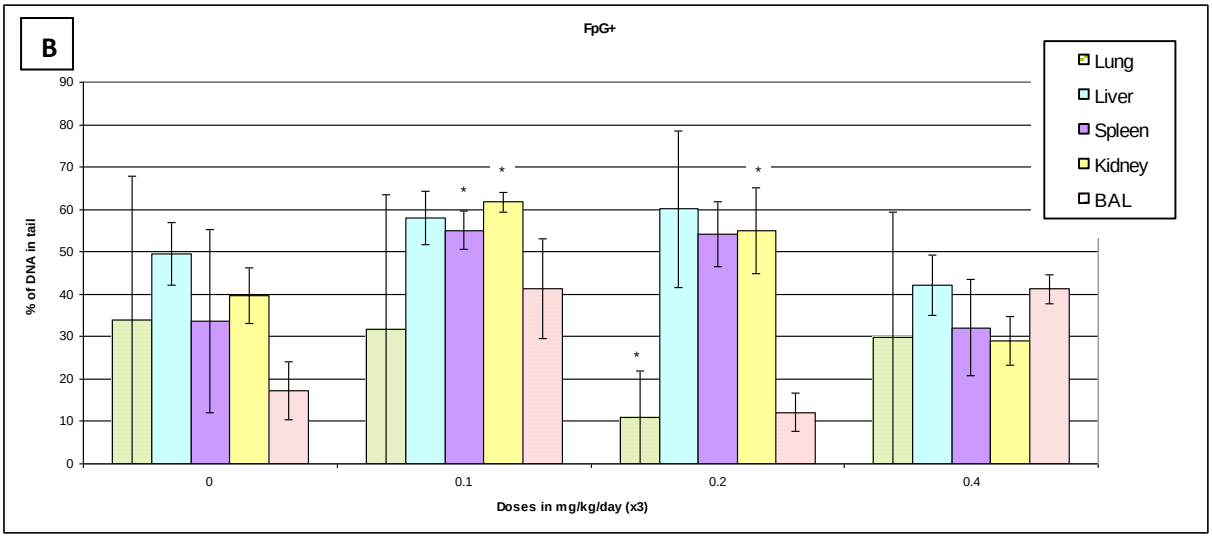


Figure 37: Comet assay with NM-401 without (A) or with FpG (B)

Comments without FpG

The quality of the slides prepared for the **lung** was unsatisfactory and too few cells were analysable in all groups. The results were thus invalidated.

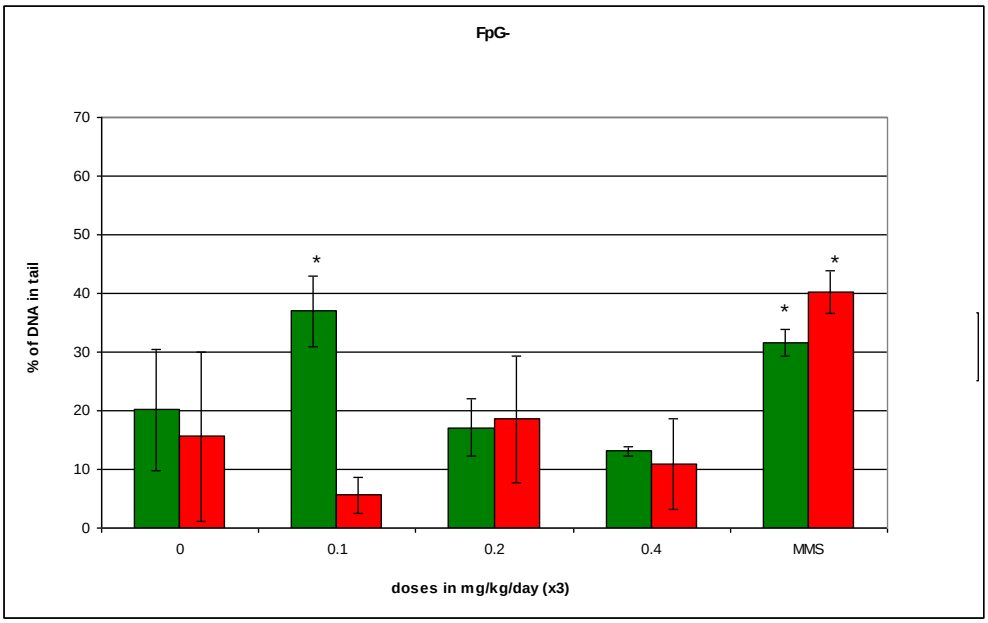
Statistically significant increases in the percentage of DNA in tail were noted in the **spleen** at the lowest dose of 0.1 mg/kg/day (x3) and in the **BAL** at 0.1 and 0.4 mg/kg/day *(x3)*.

Comments with FpG

Statistically significant increases in the percentage of DNA in tail were noted in the **spleen** at the lowest dose of 0.1 mg/kg/day (x3), and in the **kidney** at 0.1 and 0.2 mg/kg/day (x3).

Regarding the results for the **lung** and the **BAL**, they had to be invalidated.

Therefore, an additional experiment was carried out to investigate the NM 401 effect only on those organs with some modification for lung cell collection (see Material & Methods section).



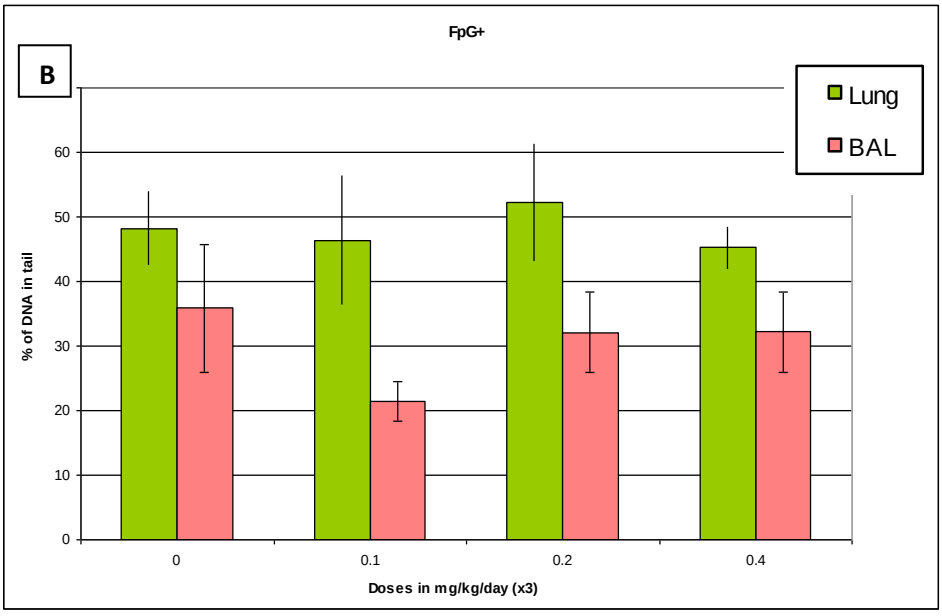


Figure 38: Comet assay with NM-401, additional experiment without (A) or with FpG (B)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Statistically significant increases in the percentage of DNA in tail were noticed in lung at the lowest dose of 0.1 mg/kg/day (x3), in the absence of FpG only.

In order to verify if any effect could be observed with lower doses in lung, a third assay was performed only on lung with a dose range between 0.05 and 0.2 mg/kg/day.

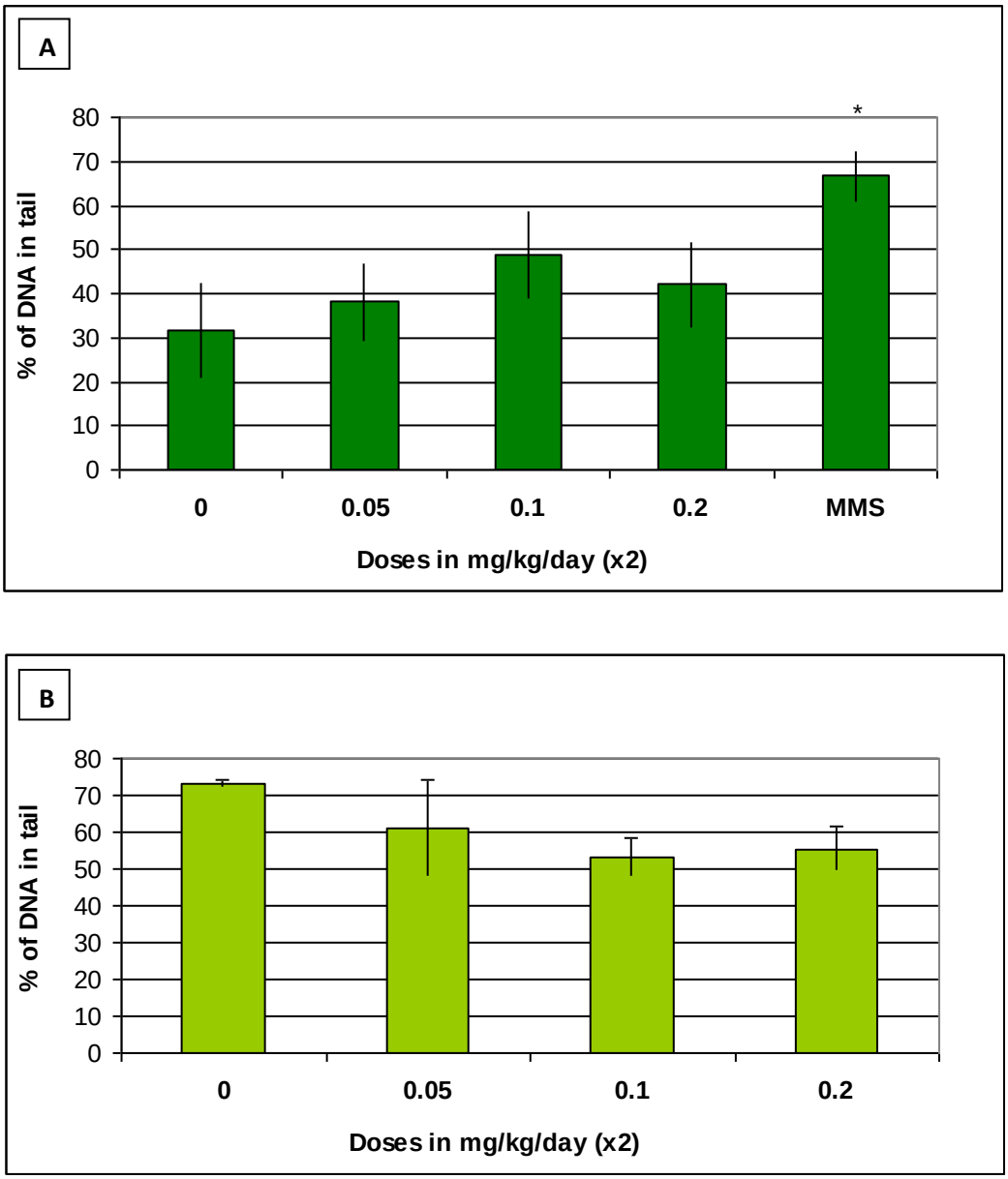


Figure 39: Comet assay with NM-401, additional experiment on lung with lower doses exposure without (A) or with FpG (B)

No statistically significant increase in the percentage of DNA in tail was noted at the doses tested from 0.05 to 0.2 mg/kg/day (x2), either in the absence or in the presence of FpG.

Conclusion NM-401 – Instillation

The manufactured nanomaterial NM-401 induced statistically significant increase in the percentage of tail DNA in the spleen both with or without FpG at the lowest dose of 0.1 mg/kg/day (x3) and in the kidney at the 2 lowest doses of 0.1 and 0.2 mg/kg/day (x3), in the presence of FpG but also in the lung at the lowest dose only without FpG as shown in the 2nd assay described above.

❖ **NM-402**

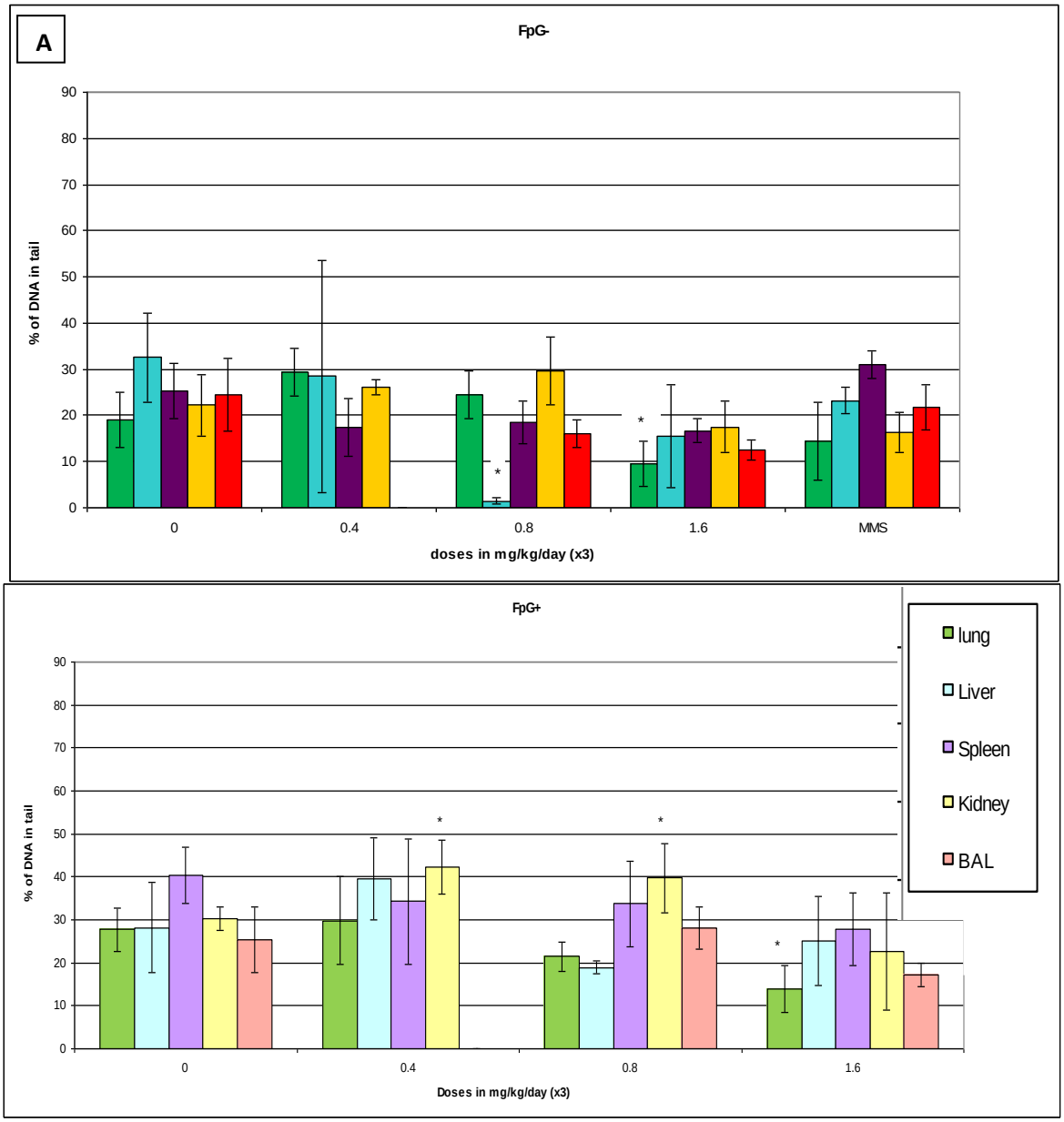


Figure 40: Comet assay with NM-402 – endotracheal route – without (A) or with FpG (B)

Comments without FpG

No statistically significant increase in the percentage of tail DNA was noticed irrespective of the organ and the dose tested.

A decrease in the percentage of DNA in tail was noticed, in the liver at 0.8 mg/kg/day (x3) and in the lung at 1.6 mg/kg/day (x3). They are devoid of genotoxic hazard

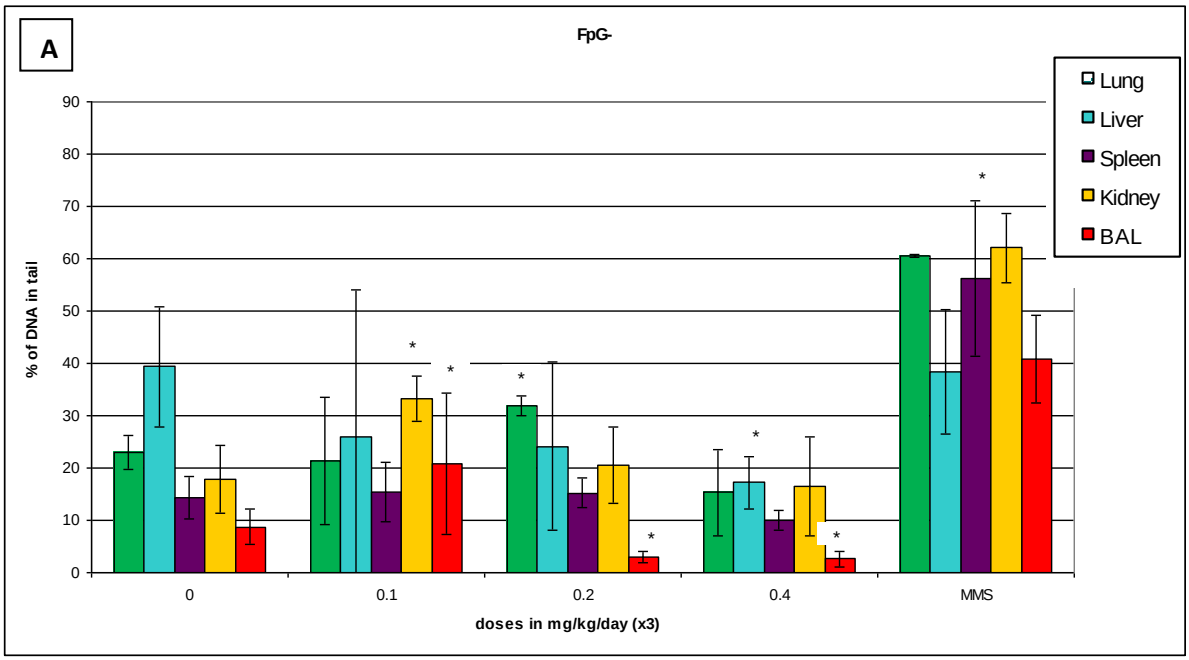
Numerous ghost cells in **all organs** were observed from the analysis of the slides of the positive control, MMS.

Comments with FpG

Statistically significant increases in the percentage of DNA in tail were noted in the **kidney** at 0.4 and 0.8 mg/kg/day (x3).

NB: A decrease in the percentage of DNA in tail was noted, in the lung at 1.6 mg/kg/day (x3). It is devoid of genotoxic hazard.

❖ **NRCWE-006**



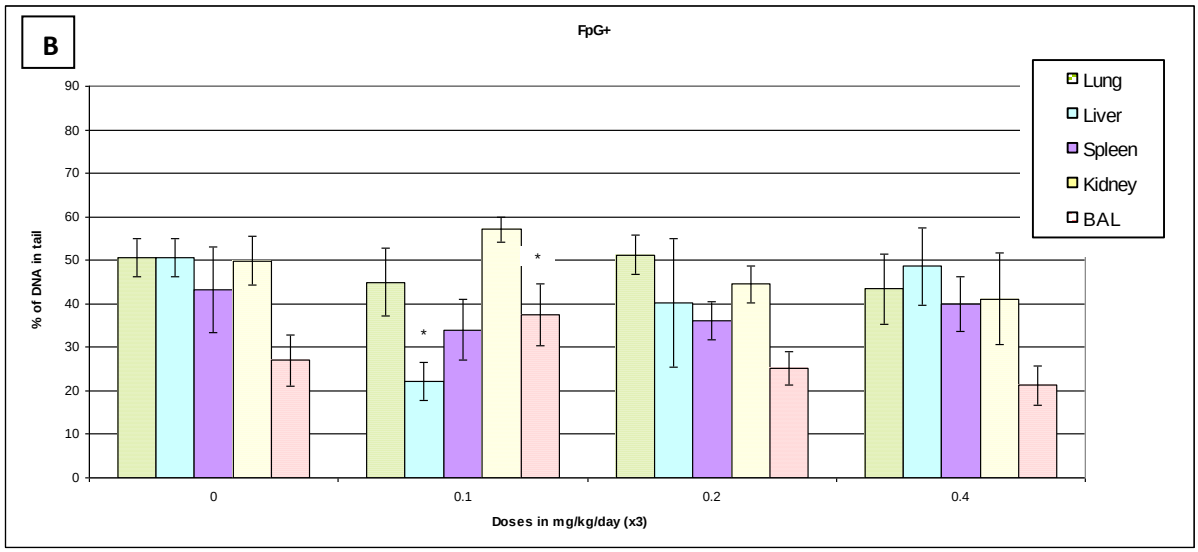


Figure 41: Comet assay with NRCWE-006 without (A) or with FpG (B)

Comments without FpG

The results for the **kidney** of rats treated with **0.1 mg/kg/day (x3)** demonstrated a statistically significant increase in DNA migration. However, slides from 2 animals out of 4 were actually analysed. Results must be taken with caution and considered as **equivocal**

An increase in the percentage of DNA in tail was noted in the **kidney** and in the **BAL** at 0.1 mg/kg/day (x3), and in the lung at 0.2 mg/kg/day (x3).

NB: Decreases in the percentage of DNA in tail were noted, in the BAL at 0.2 and 0.4 mg/kg/day (x3)*. They are devoid of genotoxic hazard.*

Regarding the results obtained with the positive control, MMS, in the **kidney**, the **lung** and the **BAL**, they were considered as biologically significant.
Otherwise, the slides of the **liver** showed numerous ghost cells.

Comments with FpG

As the quality of the slides was unsatisfactory in the **kidney**, the **lung** and the **BAL**, the results were invalidated.

No statistically significant increase in the percentage of tail DNA was noticed irrespective of the organ and the dose tested,
A decrease in the percentage of DNA in tail was noticed, in liver at 0.1 mg/kg/day (x3)*. It is devoid of genotoxic hazard.*

3.2.1.2 Micronucleus assay

❖ **NM-400**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM400 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 12).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM400 at any dose when compared with the control group (Table 12).

Therefore, NM400 was considered as **not genotoxic** under these experimental conditions.

Table 12: Micronucleus test on bone marrow with NM-400 – endotracheal route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test	
					χ^2	p
0.05% RSA	**0**	0.95 +/- *0.34*		**0.80** +/- *0.57*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.72 N.S *0.19*	**N.S.**	**6.00** +/- *1.17*	39.9	**<0.001**
NM 400	**0.48**	1.02 +/- *0.15*	**N.S.**	**1.10** +/- *0.74*	0.474	**N.S.**
	0.24	0.98 +/- *0.21*	**N.S.**	**0.50** +/- *0.35*	0.693	**N.S.**
	0.12	0.99 +/- *0.17*	**N.S.**	**0.60** +/- *0.42*	0.286	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NM-401**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM401 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 13).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM401 at any dose when compared with the control group (Table 13).

Therefore, NM401 was considered as **not genotoxic** under these experimental conditions.

Table 13: Micronucleus test on bone marrow with NM-401 – endotracheal route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test	
					χ^2	p
0.05% RSA	**0**	1.05 +/- *0.51*		**0.90** +/- *0.74*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.43 N.S *0.08*	**<0.05**	**7.60** +/- *4.41*	53.037	**<0.001**
NM 401	**0.4**	1.08 +/- *0.16*	**N.S.**	**0.80** +/- *0.67*	0.059	**N.S.**
	0.2	0.85 +/- *0.16*	**N.S.**	**0.50** +/- *0.50*	1.144	**N.S.**
	0.1	0.82 +/- *0.17*	**N.S.**	**0.60** +/- *0.55*	0.6	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05

PCE: Polychromatic erythocytes

NCE: Normochromatic erythocytes

SD: Standard Deviation

❖ **NM-402**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM-402 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 14).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM402 at any dose when compared with the control group (Table 14).

Therefore, NM402 was considered as **not genotoxic** under these experimental conditions.

Table 14: Micronucleus test on bone marrow with NM-402 – endotracheal route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test	
					χ^2	p
0.05% RSA	**0**	0.92 +/- *0.29*		**0.50** +/- *0.35*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.63 +/- *0.11*	**N.S.**	**19.40** +/- *5.89*	181.31	**<0.001**
NM 402	**1.6**	0.88 +/- *0.14*	**N.S.**	**0.30** +/- *0.45*	0.13	**N.S.**
	0.8	1.02 +/- *0.28*	**N.S.**	**0.60** +/- *0.42*	0.09	**N.S.**
	0.4	1.00 +/- *0.19*	**N.S.**	**0.30** +/- *0.45*	0.13	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

❖ **NRCWE-006**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NRCWE-006 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 15).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NRCWE-006 at any dose when compared with the control group (Table 15).

Therefore, NRCWE-006 was considered as **not genotoxic** under these experimental conditions.

Table 15: Micronucleus test on bone marrow with NRCWE-006 – endotracheal route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test χ^2	p
0.05% RSA	**0**	1.11 +/- *0.31*		**0.80** +/- *0.84*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.75 N.S *0.39*	**N.S.**	**14.70** +/- *8.42*	125.625	**<0.001**
NRCWE 006	**0.4**	1.03 +/- *0.44*	**N.S.**	**0.50** +/- *0.50*	0.693	**N.S.**
	0.2	0.83 +/- *0.34*	**N.S.**	**0.50** +/- *0.35*	0.693	**N.S.**
	0.1	0.90 +/- *0.24*	**N.S.**	**0.70** +/- *0.76*	0.067	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

3.2.2 Gavage

3.2.2.1 Comet assay

❖ **NM-400**

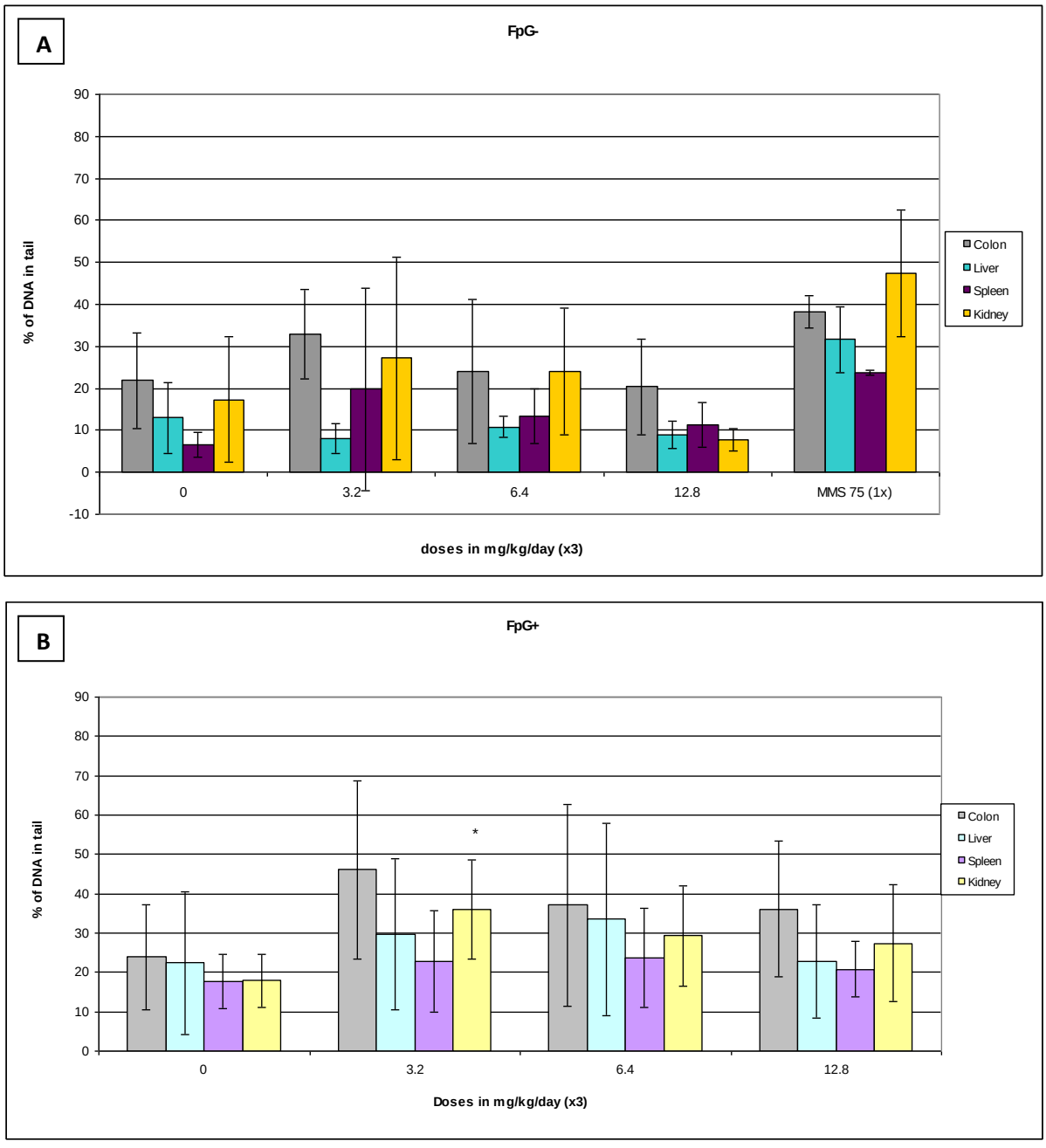


Figure 42: Comet assay with NM-400 without (A) or with FpG (B)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Comments without FpG

The slides for **spleen** from only 2 animals in negative control were analysed (Failure in DNA migration). Moreover, great inter-animals heterogeneity was noted. Results must be taken with caution and considered as **equivocal**.

Otherwise, no statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested**.**

The results obtained with MMS demonstrated biological significance for all organs (high inter-animals heterogeneity).

Comments with FpG

A statistically significant increase in the percentage of DNA in tail was noted in kidney at the lowest dose of 3.2 mg/kg/day (x3).

Conclusion NM-400 - Oral route

No statistically significant increase in the percentage of tail DNA with or without FpG, except in the kidney at the lowest dose of 3.2 mg/kg/day (x3), in the presence of FpG.

❖ **NM-401**

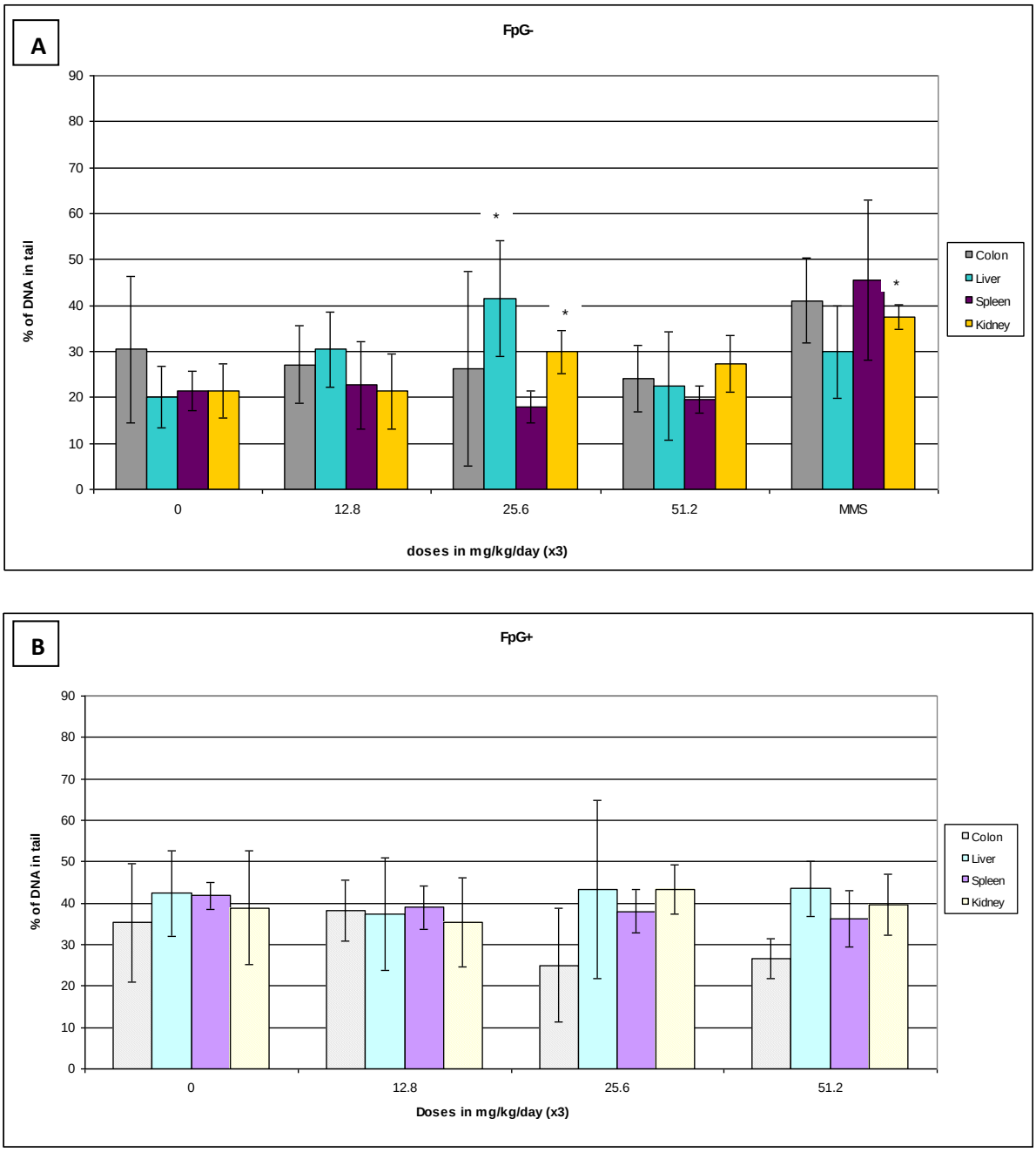


Figure 43: Comet assay with NM-401 without (A) or with FpG (B)

Comments without FpG

A statistically significant response observed at 25.6 mg/kg/day (x3) in the **kidney**. However, it is noteworthy that for one series of slides, unsatisfactory DNA migration was noted (no data for this series). Results must be taken with caution, and considered as **equivocal**

Otherwise, a statistically significant increase in the percentage of DNA in tail was noted in the liver at the intermediary dose of 25.6 µg/kg/day (x3).

The results obtained with the positive control (MMS) in the **spleen** demonstrated biological significance (high inter-animals heterogeneity).

Comments with FpG

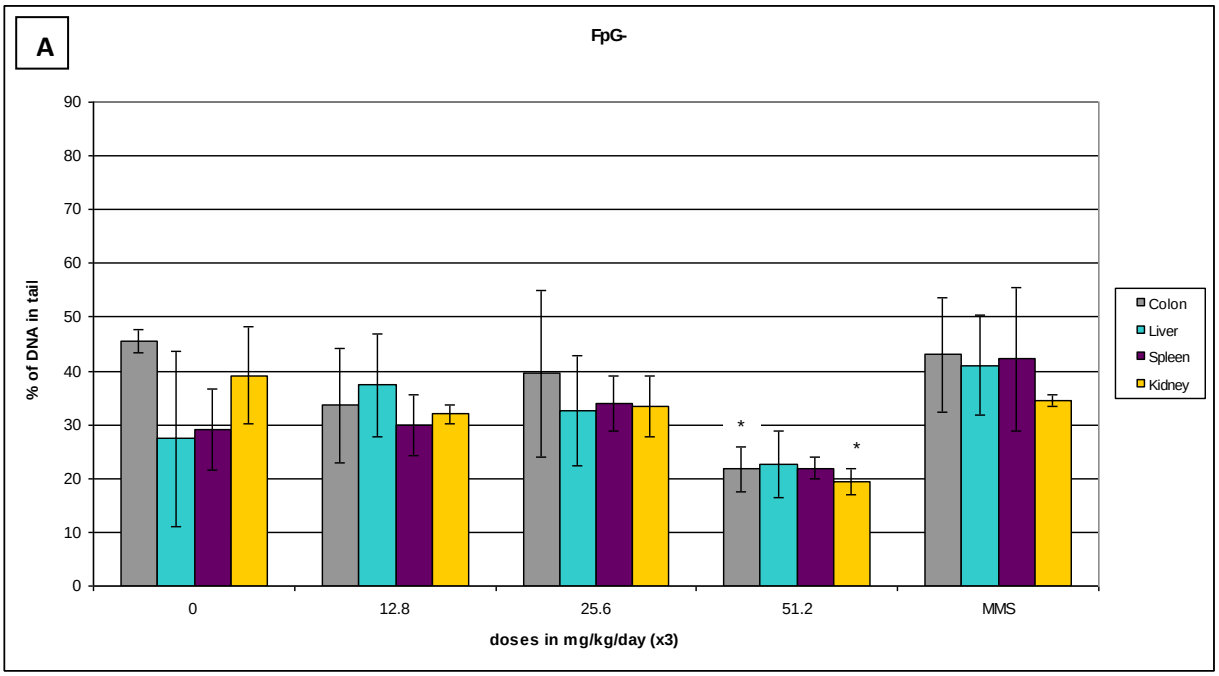
The quality of slides prepared for the **kidney** and the **colon** was unsatisfactory. The results were thus invalidated.

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested**.**

Conclusion NM-401 - Oral route

Statistically significant increases in the percentage of tail DNA were noted in liver and kidney at the intermediary dose of 25.6 µg/kg/day (x3) only in absence of FpG. This effect was considered as equivocal in the kidney

❖ **NM-402**



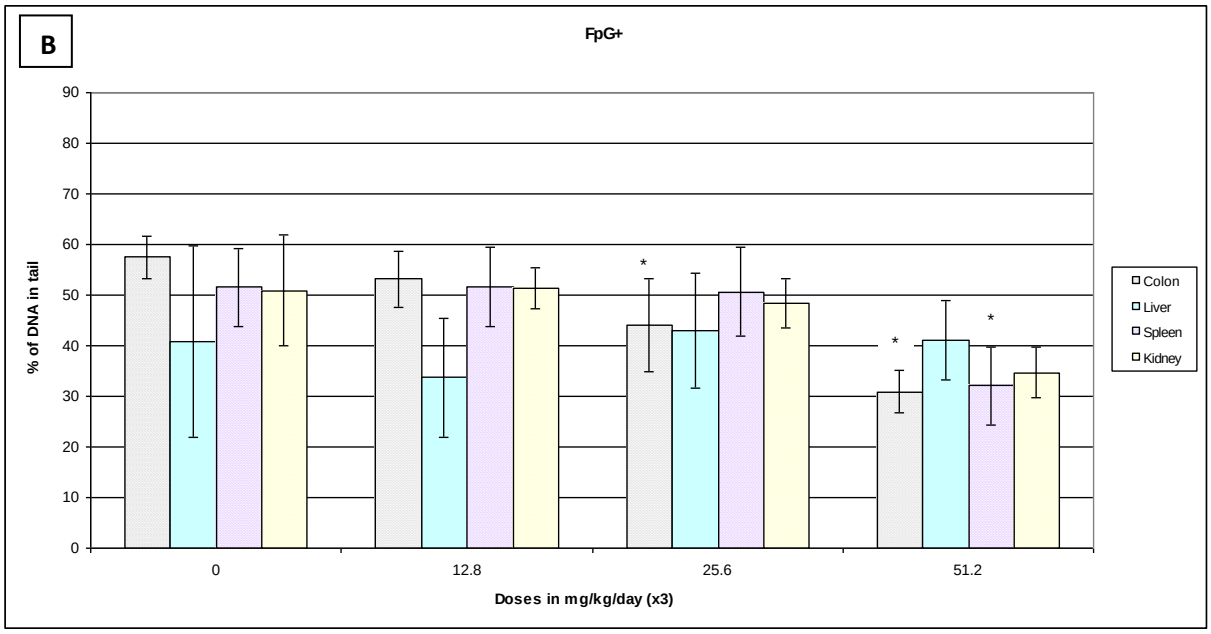


Figure 44: Comet assay with NM-402 without (A) or with FpG (B)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Comments without FpG

The quality of slides prepared for the **colon** was unsatisfactory and too few cells were analysable in all groups. The results were thus invalidated.

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested.

Decreases in the percentage of DNA in tail were noted. They are devoid of genotoxic hazard.

Regarding the results obtained with the positive control, **MMS**, in the **liver**, they were considered as biologically significant.

Otherwise, at the analysis of the slides of the **kidney** and the **spleen**, it was noted too numerous ghost cells, and the results were invalidated.

Comments with FpG

The quality of slides prepared for the **kidney**, the **spleen** and the **colon** was unsatisfactory. The results were thus invalidated.

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested.

Conclusion NM-402 - Oral route

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested, either in the absence or in the presence of FpG.

❖ **NRCWE-006**

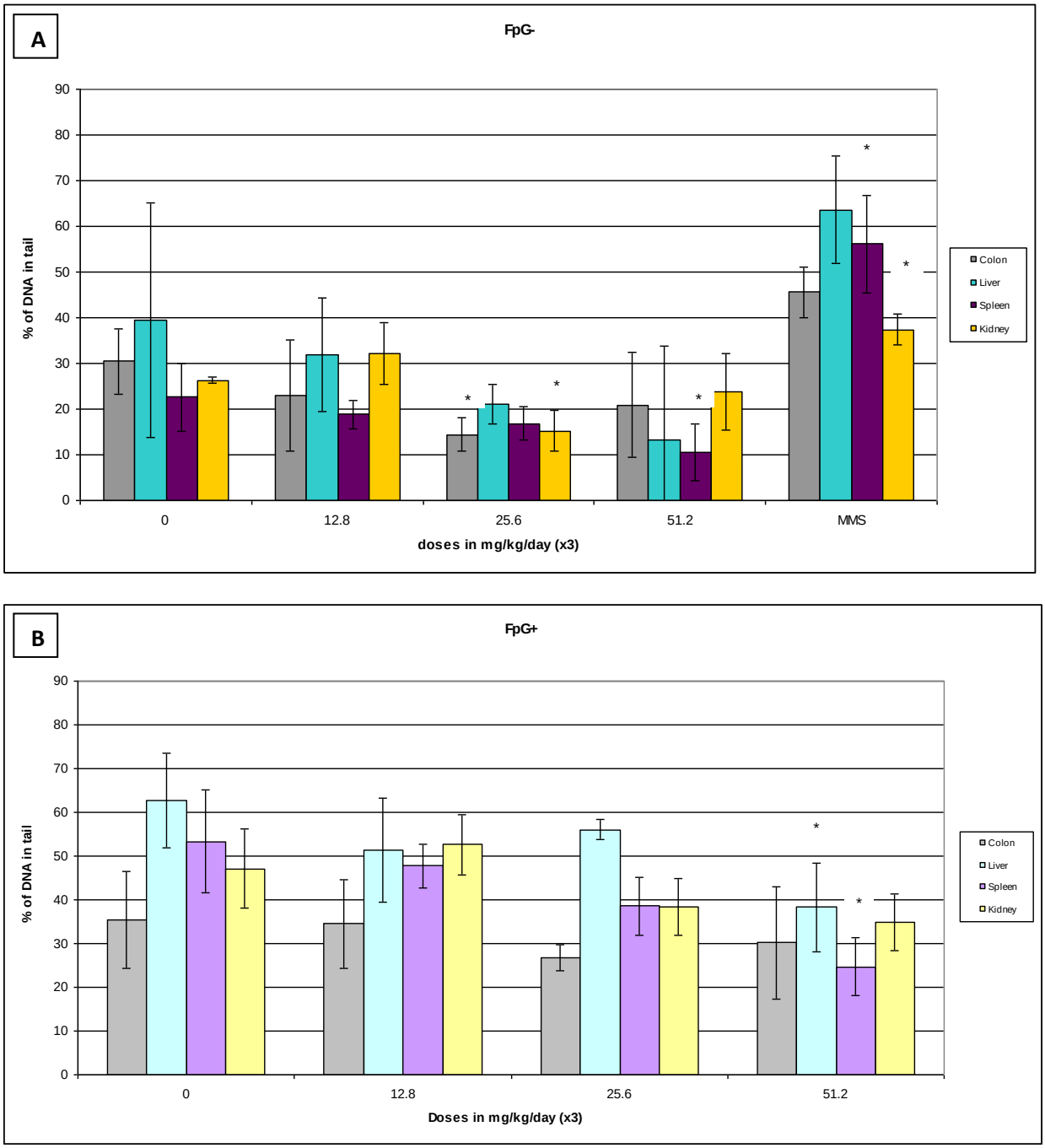


Figure 45: Comet assay with NRCWE-006 without (A) or with FpG (B)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Comments without FpG

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested.

Decreases in the percentage of DNA in tail were noted. They are devoid of genotoxic hazard.

Regarding the results obtained with the positive control, **MMS**, in the **liver**, they were considered as biologically significant.
Otherwise, at the analysis of the slides of the **colon**, it was noted too numerous ghost cells.

Comments with FpG

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested.

Decreases in the percentage of DNA in tail were noted. They are devoid of genotoxic hazard.

Conclusion NRCWE-006 - Oral route

No statistically significant increase in the percentage of DNA in tail was noted whatever the organ analysed and the dose tested, either in the absence or in the presence of FpG.

3.2.1.2 Bone marrow micronucleus assay

- **NM-400**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM400 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 16).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM-400 at any dose when compared with the control group (Table 16).

Therefore, NM-400 was considered as **not genotoxic** under these experimental conditions.

Table 16: Micronucleus test on bone marrow with NM-400

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test	
					$\chi 2$	p
0.05% RSA	**0**	0.57 +/- *0.24*		**2.40** +/- *0.82*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.37 +/- *0.20*	**N.S.**	**46.70** +/- *11.51*	409.752	**<0.001**
NM 400	**12.8**	0.43 +/- *0.13*	**N.S.**	**3.60** +/- *2.25*	2.407	**N.S.**
	6.4	0.74 +/- *0.39*	**N.S.**	**2.60** +/- *0.65*	0.08	**N.S.**
	3.2	0.64 +/- *0.43*	**N.S.**	**2.00** +/- *0.79*	0.364	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

- **NM-401**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM-401 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 17).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM-401 at any dose when compared with the control group (Table 17).

Therefore, NM-401 was considered as **not genotoxic** under these experimental conditions.

Table 17: Micronucleus test on bone marrow with NM-401 – Oral route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test χ^2	Chi² test p
0.05% RSA	**0**	0.96 +/- *0.11*		**2.60** +/- *0.89*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.34 +/- *0.07*	**<0.001**	**35.20** +/- *8.79*	286.57	**<0.001**
NM 401	**51.2**	1.03 +/- *0.20*	**N.S.**	**3.50** +/- *1.12*	1.332	**N.S.**
	25.6	1.90 +/- *2.14*	**N.S.**	**1.80** +/- *0.91*	1.458	**N.S.**
	12.8	1.05 +/- *0.17*	**N.S.**	**2.20** +/- *0.76*	0.334	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05

PCE: Polychromatic erythocytes

NCE: Normochromatic erythocytes

SD: Standard Deviation

- **NM-402**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NM-402 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 18).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, a statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NM-402 at the highest dose (51.2 mg/kg) when compared with the control group (Table 18).

However, the number of micronucleated cells was within the interval of historical data for negative control obtained in our lab (*i.e.* 0-3). Therefore, NM-402 was considered as **not genotoxic** under these experimental conditions.

Table 18: Micronucleus test on bone marrow with NM-402 – Oral route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test χ2	Chi² test p
0.05% RSA	**0**	0.82 +/- *0.16*		**1.00** +/- *0.94*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.51 +/- *0.19*	**<0.05**	**22.20** +/- *6.96*	195.998	**<0.001**
NM 402	**51.2**	1.74 +/- *0.53*	**<0.01**	**2.10** +/- *0.65*	3.909	**<0.05**
	25.6	1.32 +/- *0.34*	**<0.05**	**2.00** +/- *0.79*	3.338	**N.S.**
	12.8	1.06 +/- *0.49*	**N.S.**	**1.90** +/- *1.24*	2.797	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

- **NRCWE-006**

PCE / NCE ratio

The ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) was established at each dose level. No statistically significant decrease in the ratio PCE to NCE was noted in the 3 NRCWE-006 treatment groups when compared to the negative control group. In consequence, no proof of systemic exposure was evidenced (Table 19).

Frequency of micronucleated PCE

Regarding the frequency of micronucleated polychromatic erythrocytes, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was found in the animals treated with NRCWE-006 at any dose when compared with the control group (Table 19).

Therefore, NRCWE-006 was considered as **not genotoxic** under these experimental conditions.

Table 19: Micronucleus test on bone marrow with NRCWE 006 – Oral route

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	PCE / NCE RATIO		MICRONUCLEI FOR 1000 PCE		
		Mean +/- SD	Student's t Test (p)	*Mean +/- SD*	Chi² test	
					χ^2	p
0.05% RSA	**0**	0.99 +/- *0.22*		**1.40** +/- *0.74*		
POSITIVE	Cyclophosphamide 10 mL/kg 25 mg/kg/day (x1)	0.42 +/- *0.12*	**<0.001**	**4.70** +/- *2.71*	17.907	**<0.001**
NRCWE 006	**51.2**	1.52 +/- *0.61*	**N.S.**	**0.60** +/- *0.65*	3.203	**N.S.**
	25.6	1.30 +/- *0.30*	**N.S.**	**0.70**+/- *0.67*	1.638	**N.S.**
	12.8	0.85 +/- *0.48*	**N.S.**	**1.40** +/- *0.55*	0	**N.S.**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05

PCE: Polychromatic erythocytes

NCE: Normochromatic erythocytes

SD: Standard Deviation

.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

3.2.1.3 Colon micronucleus assay

No mutagenic effect was detected with NM-400, -402 and NRCWE-006 in the colon (Tables 20 to 23).

Table 20: Micronucleus test on colon with NM-400. NS = non-significant at the threshold of p=0.05; SD = standard deviation

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	MICRONUCLEI FOR 1000 cells			APOPTOSIS FOR 1000 cells			MITOSIS FOR 1000 cells		
		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test	
			χ^2	p		χ^2	p		χ^2	p
0.05% RSA	**0**	**3.67** +/- *2.36*			**1.17** +/- *0.58*			**15.17** +/- *4.31*		
POSITIVE	DMH 10 mL/kg 30 mg/kg/day (x1)	**12.00** +/- *4.50*	44.91	**<0.001**	**11.50** +/- *2.18*	85.20	**<0.001**	**8.33** +/- *2.25*	20.27	**<0.001**
NM 400	**12.8**	**4.50** +/- *1.80*	0.86	**N.S.**	**1.67** +/- *0.58*	0.88	**N.S.**	**7.83** +/- *5.86*	23.84	**<0.001**
	6.4	**4.33** +/- *1.04*	0.56	**N.S.**	**2.00** +/- *0.50*	2.20	**N.S.**	**6.50** +/- *1.50*	35.30	**<0.001**
	3.2	**3.83** +/- *1.26*	0.04	**N.S.**	**2.33** +/- *0.58*	3.90	**<0.05**	**7.50** +/- *3.12*	26.43	**<0.001**

** except for positive control: 24h after unique treatment*

Table 21: Micronucleus test on colon with NM-401. NS = non-significant at the threshold of p=0.05; SD = standard deviation

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	MICRONUCLEI FOR 1000 cells			APOPTOSIS FOR 1000 cells			mitosis FOR 1000 cells		
		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test	
			χ^2	p		χ^2	p		χ^2	p
0.05% RSA	**0**	**2.30** +/- *1.48*			**1.00** +/- *0.61*			**9.80** +/- *5.51*		
POSITIVE	DMH 10 mL/kg 30 mg/kg/day (x1)	**7.50** +/- *4.87*	35.35	**<0.001**	**8.90** +/- *5.24*	74.84	**<0.001**	**8.40** +/- *5.16*	0.02	**N.S.**
NM 401	**51.2**	**1.70** +/- *0.57*	2.77	**N.S.**	**0.60** +/- *0.65*	2.11	**N.S.**	**5.20** +/- *1.82*	26.73	**<0.001**
	25.6	**1.80** +/- *0.57*	2.26	**N.S.**	**0.60** +/- *0.42*	2.11	**N.S.**	**9.60** +/- *3.40*	2.93	**N.S.**
	12.8	**3.30** +/- *1.10*	0.26	**N.S.**	**1.20** +/- *0.76*	0.01	**N.S.**	**6.40** +/- *2.46*	17.05	**<0.001**

** except for positive control: 24h after unique treatment*

N.S.: Non-significant at the threshold of p=0.05
PCE: Polychromatic erythocytes
NCE: Normochromatic erythocytes
SD: Standard Deviation

Table 22: Micronucleus test on colon with NM-402. NS = non-significant at the threshold of p=0.05; SD = standard deviation

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	MICRONUCLEI FOR 1000 cells			APOPTOSIS FOR 1000 cells			MITOSIS FOR 1000 cells		
		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test	
			χ2	p		χ2	p		χ2	p
0.05% RSA	**0**	**10.75** +/- *1.32*			**4.00** +/- *1.58*			**9.75** +/- *1.04*		
POSITIVE	DMH 10 mL/kg 30 mg/kg/day (x1)	**33.00** +/- *7.74*	71.72	**<0.001**	**25.90** +/- *8.14*	118.85	**<0.001**	**14.80** +/- *3.55*	2.26	**N.S.**
NM 402	**51.2**	**10.70** +/- *3.51*	2.83	**N.S.**	**6.10** +/- *3.05*	9.92	**<0.01**	**9.00** +/- *4.24*	4.38	**<0.05**
	25.8	**8.80** +/- *2.20*	8.90	**<0.01**	**6.40** +/- *2.46*	6.04	**<0.05**	**8.00** +/- *2.69*	7.98	**<0.01**
	12.6	**10.60** +/- *2.22*	3.05	**N.S.**	**5.30** +/- *0.97*	29.82	**<0.001**	**12.90** +/- *4.55*	0.18	**N.S.**

** except for positive control: 24h after unique treatment*

Table 23: Micronucleus test on colon with NRCWE-006. NS = non-significant at the threshold of p=0.05;
SD = standard deviation

SAMPLING TIME (3-6 H after last treatment*)	TEST ITEM DOSES in mg/kg/day (x3)	MICRONUCLEI FOR 1000 cells			APOPTOSIS FOR 1000 cells			mitosis FOR 1000 cells		
		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test		Mean +/- SD	Chi² test	
			χ2	p		χ2	p		χ2	p
0.05% RSA	**0**	**2.00** +/- *2.06*			**2.80** +/- *1.15*			**15.10** +/- *7.72*		
POSITIVE	DMH 10 mL/kg 30 mg/kg/day (x1)	**13.00** +/- *0.79*	81.28	**<0.001**	**22.20** +/- *5.30*	152.45	**<0.001**	**6.20** +/- *1.15*	37.59	**<0.001**
NRCWE 006	**51.2**	**1.60** +/- *1.02*	0.45	**N.S.**	**1.30** +/- *1.20*	5.50	**<0.05**	**8.60** +/- *3.66*	18.04	**<0.001**
	25.8	**2.30** +/- *0.91*	0.21	**N.S.**	**1.40** +/- *1.08*	4.68	**<0.05**	**8.60** +/- *4.38*	18.04	**<0.001**
	12.6	**2.40** +/- *1.02*	0.36	**N.S.**	**3.00** +/- *0.35*	0.07	**N.S.**	**16.60** +/- *6.17*	0.72	**N.S.**

** except for positive control: 24h after unique treatment*

General conclusions

In the assays performed by **instillation,** statistically significant effects were noted with:

- **NM-400** in the **Kidney** in the **absence of FpG**,
- **NM-401** in the **Spleen** both in the **absence and the presence of FpG**. Moreover, it induced a statistically significant effect in the **Kidney** in **the presence of FpG** and in the

BAL in the **absence of FpG.** It also induced a statistically significant increase in the percentage of DNA in tail in the **lung** at low dose in **absence of FpG** that was not confirmed in a further experiment with decreased doses.

- **NM-402** in the **Kidney** in **the presence of FpG.**
- **NRCWE-006** in the **Kidney,** the **Lung** and in the **BAL** in the **absence of FpG.**

In the assays performed by the **oral** route, statistically significant increases in the percentage of DNA in tail were observed with:

- **NM-400** in the **Kidney** in the **presence of FpG**,
- **NM-401** in the **Liver** and the **Kidney** in the **absence of FpG**.

In brief for CNT

- **Dose ranges:**

Instillation: 0.4, 0.2 and 0.1 mg/kg (x3) except for NM-402 (1.6, 0.8 and 0.4 mg/kg (x3))

An assay was also done only with NM401 at lower doses: 0.05, 0.1 and 0.2 mg/kg (x3)

Gavage: 51.2, 25.6 and 12.8 mg/kg (x 3) except for NM-400 (12.8, 6.4, 3.2 mg/kg (x3))

- **Comet assay:**

Some genotoxicity induced in various organs

After gavage NM-401 in liver and kidney

After instillation, depending on the MN, in kidney, spleen, lung and BAL

- **Micronucleus assay:**

  - **Bone marrow:**

No mutagenicity irrespective of the route of administration

  - **Colon:**

No mutagenicity with NM-400, -401, -402 and NRCWE-006.

Outcome tables

The outcome tables present an overview of the statistic analysis of all the results provided within the WP. The statistics were performed as described in the Material & Methods with the chi-square test for the micronucleus assays (both colon and bone marrow) and the non-parametric Kruskall-Wallis test for the comet assay.

The following

+++, POSITIVE - Significant dose-dependent increase, ≥2 significant doses.
++, POSITIVE - Significant dose-dependent increase, high dose significant.
+, POSITIVE - No significant dose-dependent increase, ≥2 significant doses.
(+), EQUIVOCAL - No significant dose-dependent increase, 1 significant dose.
-, NEGATIVE;

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Table 24. Outcome of the ***in vivo*** **micronucleus assay** after gavage or instillation with Nanogenotox MNs.

Route	Gavage		Instillation	Intravenous	
Organ of origin	Bone marrow	Colon	Bone marrow	Bone marrow	Bone marrow (LacZ mice)
TiO_2					
NM-101	ND	ND		ND	
NM-102	-	*	-	ND	-
NM-103	-	*	-	-	ND
NM-104	-	*	-	-	ND
NM-105	-	*	-	ND	ND
SAS					
NM-200	-	-	-	ND	ND
NM-201	-	-	-	ND	ND
NM-202	-	(+)	-	ND	ND
NM-203	-	(+)	-	(+)	ND
MWCNT					
NM-400	-	-	-	ND	ND
NM-401	-	-	-	ND	ND
NM-402	-	-	-	ND	ND
NRCWE-006	-	-	-	ND	ND

ND, NO DATA (not performed); * = technical problem

Table 25. Outcome of the ***in vivo*** **comet assay after instillation** with Nanogenotox NMs.

Tissue	BAL fluid	Lung	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-101	++	-	ND	ND	-	-	-
NM-102	-	-	ND	ND	-	(+)	-
NM-103	-	-	ND	ND	-	(+)	-
NM-104	-	-	ND	ND	-	-	-
NM-105	++	-	ND	ND	-	-	-
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	ND	**	ND	ND	ID	-	++
NM-401	-	(+)	ND	ND	(+)	-	-
NM-402	-	-	ND	ND	-	-	-
NRCWE-006	(+)	(+)	ND	ND	-	-	(+)

ND, NO DATA (not performed).
ID: Invalidated data
** Technical problem: no cells were analysable

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Table 26. Outcome of the ***in vivo*** **comet assay after gavage** with Nanogenotox MNs.

Tissue	Intestine	Colon	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	-	++	-	-	+++	-	-
NM-103	++	-	-	-	(+)	-	-
NM-104	-	-	-	++	+++	-	-
NM-105	-	++	-	-	+	-	-
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	ND	-	ND	ND	-	-	-
NM-401	ND	-	ND	ND	-	(+)	(+)
NM-402	ND	ID	ND	ND	-	-	-
NRCWE-006	ND	-	ND	ND	-	-	-

ND, NO DATA (not performed).

ID: Invalidated data

Table 27. Outcome of the ***in vivo*** **comet assay for oxidative DNA damage (FpG) after instillation** with Nanogenotox MNs.

Tissue	BAL fluid	Lung	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	ND	ND	ND	ND	ND	ND	ND
NM-103	ND	ND	ND	ND	ND	ND	ND
NM-104	ND	ND	ND	ND	ND	ND	ND
NM-105	ND	ND	ND	ND	ND	ND	ND
SAS							
NM-200	-	-	-	-	(+)*	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	ND	**	ND	ND	ID	-	-
NM-401	-	-	ND	ND	(+)	-	+
NM-402	-	-	ND	ND	-	-	+
NRCWE-006	ID	ID	ND	ND	-	-	ID

*intermediate dose significantly different from control

ND, NO DATA (not performed).

ID: Invalidated data

** Technical problem: no cells were analysable

Table 28. Outcome of the ***in vivo*** **comet assay for oxidative DNA damage (FpG) after gavage** with Nanogenotox MNs.

Tissue	Intestine	Colon	Blood (not compulsory)	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	-	-	ND	ND	ND	ND	ND
NM-103	-	-	ND	ND	ND	ND	ND
NM-104	-	-	ND	ND	ND	ND	ND
NM-105	-	-	ND	ND	ND	ND	ND
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	ND	-	ND	ND	-	-	(+)
NM-401	ND	ID	ND	ND	-	-	ID
NM-402	ND	ID	ND	ND	ID	-	ID
NRCWE-006	ND	-	ND	ND	-	-	-

ND, NO DATA (not performed)

ID: Invalidated data

Data correlations

The correlations between *in vivo* and *in vitro* responses as well as between *in vivo* and physic-chem properties could not be done during the framework as some results from the *in vivo* workpackage are still expected and numerous data were only reported by January and February 2013. Some investigations on those correlations are planned in the upcoming months.

Conclusions

With the comet assay, the responses were largely negative in the tested organs for most of the investigated MNs. When positive results were obtained, in most cases, no dose response relationship could be established which makes it difficult to conclude on the *in vivo* genotoxicity of the MNs tested.

The WP6 results herein presented show that no chromosome damage was observed in bone marrow after gavage, intratracheal and intravenous administrations, irrespective of the MN. As generally a low bioavailability for internal organs after gavage was observed in the toxicokinetics workpackage (WP7), the MNs are likely not to have reached the bone marrow. Therefore, the bone marrow micronucleus test may not be appropriate for MN *in vivo* genotoxicity detection, unless some uptake in this tissue could be demonstrated. Some mutagenicity was observed in colon with the micronucleus assay for two SAS MNs but only at the lowest dose without a dose-response. However, no micronucleus induction was reported with three CNT. The results from TiO_2 and one CNT are still expected.

Apparently, within the same family, the toxic effect may vary according to the MN (genotoxicity but also toxic effects). The genotoxic effects observed in few organs during this WP need however to be confirmed. Moreover, some low dose effects were reported and additional investigations would be required to explain these results.

A critical analysis of the *in vivo* genotoxicity of MNs must be carried out as soon as the last results will be provided. Correlations with *in vitro* and physic-chemical properties are also planned.

Recommendations

We highlighted during this workpackage some intra and inter laboratory variabilities as presented in Figure 46 for the comet assay on liver. However, it must also be outlined that most of the labs involved in the comet assay showed a weak variability of the results obtained with controls (negative and positive) considering that neither the isolation nor the protocol of migration were harmonised before running the comet assay.

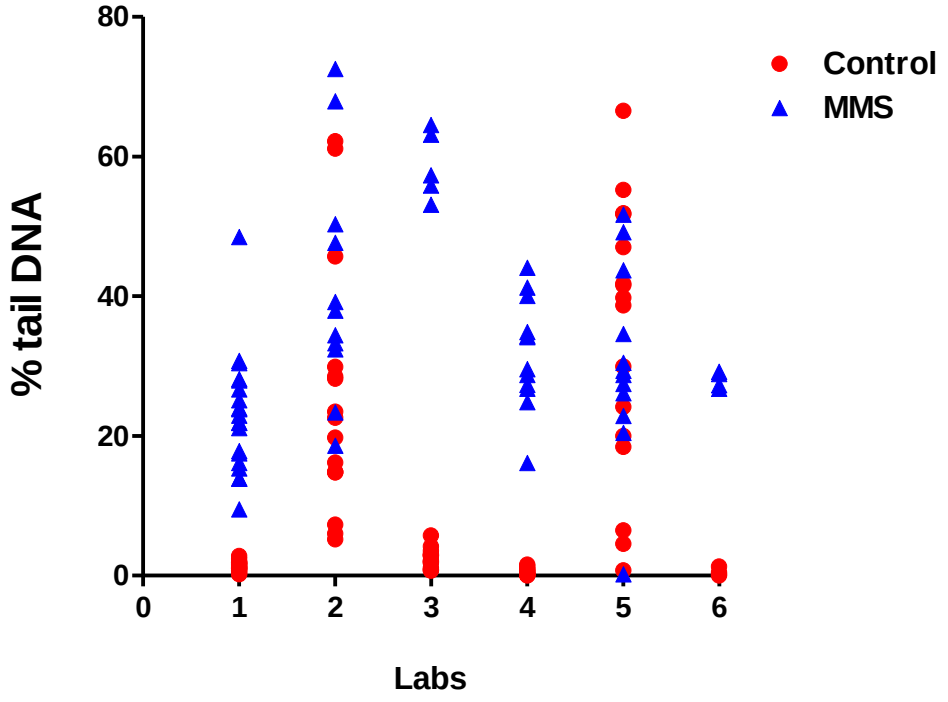


Figure 46: Variability of comet assay responses in liver for negative (vehicle) and positive (MMS) controls among the different laboratories involved in the WP. Each point is the median of one animal.

Unfortunately, as this project was innovative in the protocol used (3 administrations) and in the number of organs to be investigated (at least 5 per animal), the laboratories did not have historical data available or only on few specific organs (mostly liver). Moreover, the comet assay and the colon micronucleus assay do not have OECD guidelines. Although some recommendation papers exist for the *in vivo* comet assay (Tice et al 2000, Hartmann et al 2003, EFSA 2012), the criteria of acceptability are not well established yet.

The bone marrow micronucleus test may not be appropriate for MN *in vivo* genotoxicity detection since the uptake in this tissue has not been confirmed. Investigating sites of contact (lung and gut) for genotoxic effect of MNs should be also recommended. Complementary tests to the OECD 474 guideline including comet assay, colon or lung micronucleus assay and lacZ mutation assay could be promising tools that need to be pushed through the validation process.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

References

Burlinson et al. Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup. Mutation Research 627 (2007) 31–35

Collins et al. The comet assay: topical issues. Mutagenesis 23 (2008) 143–151

EFSA Journal Minimum Criteria for the acceptance of in vivo alkaline Comet Assay Reports 2012;10(11):2977 [12 pp.]. (http://www.efsa.europa.eu/fr/efsajournal/pub/2977.htm)

Guidance on a Strategy for Testing of Chemicals for Mutagenicity (2000), Crown Copyright, (Website: http://www.doh.gov.uk/com.htm)

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR; Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop.Mutagenesis. 2003 Jan;18(1):45-51.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-21.

Acknowledgements

Many persons were involved in this WP and we would like to thanks them all for their help in carrying out

Supervisor	Participants
Laurent Gaté	Cristina Langlais, Sylvie Sébillaud, Caroline Fontana, Yves Guichard, Christian Darne, Jean Claude Micillino, Stéphane Binet, Marie-Aline Maire
Margarita Apostolova	Irina Karadjova, Rahila Kozarova, Iva Ugrinova, Julian Kirilov, Iliyan Kolev, Nadya Ivanova, Margarita Dubrovina, Nina Kyneva, Leni Markova, Maria Mitreva
Sophie SIMAR	Alizée Brient, Céline Defrain, Alexandre Delobel, Gwendoline Dewaele, Gonzague Dourdin, Séverine Heumel, Doris Lagache, Lauren Nakab, Fabrice Nesslany, Stéphanie Quarré, Aurélie Stachowiak, Smaïl Talahari, Eric Vercauteren
Maria João Silva	Ana Tavares, Henriqueta Louro, Nádia Vital
Nicklas Raun Jacobsen	Håkan Wallin, Petra Jackson, Zdenka Kyjowska, Lisbeth Meyer Petersen, Lourdes Pedersen
Wim de Jong	

Maciej Stepnik	Marek Nocuń, Barbara Pawlak, Joanna Roszak, Konrad Rydzyński, Anna Smok-Pieniążek, Mariola Włodarek
Hannu Norppa Finnish Institute of Occupational Health	
Adeline Tarantini anses	Sylvie Huet, Kevin Hogeveen, Gérard Jarry, Rachelle Lanceleur, Ludovic Le Hégarat, Martine Poul, Jean-Guy Rolland

NANOGENOTOX

Grant Agreement

• Contact •

Website: www.nanogenotox.eu

E-mail: nanogenotox@anses.fr

Coordinator: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

27-31, avenue du Général Leclerc
94701 Maisons-Alfort Cedex France

anses
French agency for food, environmental and occupational health & safety

Partners

French Agency for Food, Environmental and Occupational Health Safety (France)	ANSES
Federal Institute of Risk Assessment (Germany)	BfR
French Atomic Energy Commission (France)	CEA
Institute of Mineralogy and Crystallography (Bulgaria)	IMC-BAS
Veterinary and Agrochemical Research Centre (Belgium)	CODA-CERVA
Finnish Institute of Occupational Health (Finland)	FIOH
Roumen Tsanev Institute of Molecular Biology Academy of Sciences (Bulgaria)	IMB-BAS
Institut national de recherche et de sécurité (France)	INRS
National Health Institute Doutor Ricardo Jorge (Portugal)	INSA
Scientific Institute of Public Health (Belgium)	IPH
Institut Pasteur of Lille (France)	IPL
Istituto superiore di sanità (Italy)	ISS
The Nofer institute of Occupational Medicine (Poland)	NIOM
National Research Centre for the Working Environment (Denmark)	NRCWE
National Institute for Public Health and the Environment (The Netherlands)	RIVM
Universitat Autònoma de Barcelona (Spain)	UAB

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Co-funded by the Health Programme of the European Union