NANOGENOTOX

Grant agreement number 2009 21 01

WP 6
In vivo genotoxicity

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WP6 partners

- 9 partners

from 7 countries

French Agency for Food, Environmental and Occupational Health Safety (France)	ANSES	anses
Finnish Institute of Occupational Health (Finland)	FIOH	Finnish Institute of Occupational Health
Roumen Tsanev Institute of Molecular Biology Academy of Sciences (Bulgaria)	IMB-BAS	
Institut national de recherche et de sécurité (France)	INRS	inrs
National Health Institute Doutor Ricardo Jorge (Portugal)	INSA	
Institut Pasteur of Lille (France)	IPL	Institut Pasteur de Lille
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	rivm

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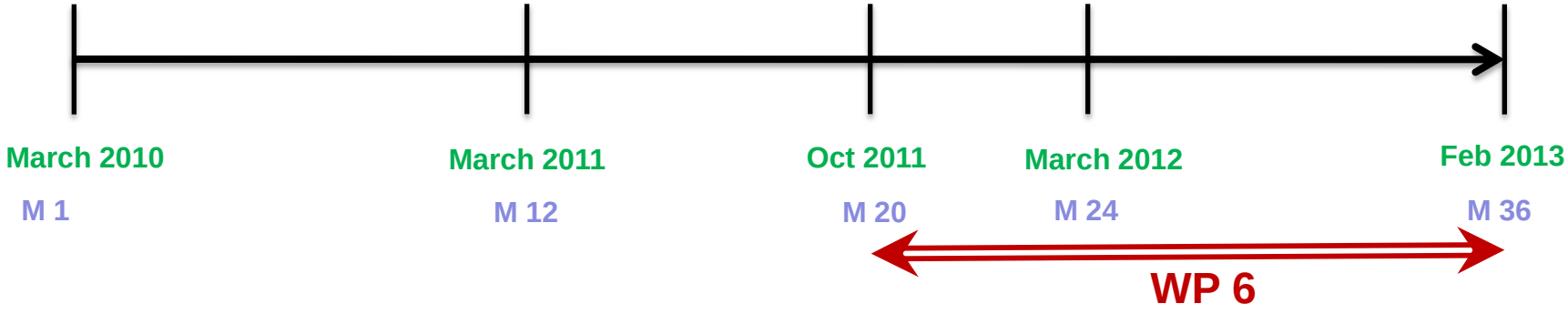
Aims

- Determine the *in vivo* genotoxicity of MNs

(TiO2, SAS and CNT)

- Comparison *in vitro*/*in vivo* /(Physic-chem)



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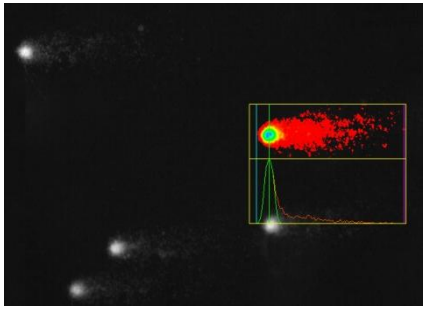
Methodology



- Genotoxicity:
  - 3 complementary tests

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

Comet and micronucleus tests coupled
to reduce the number of animals for ethical point of view

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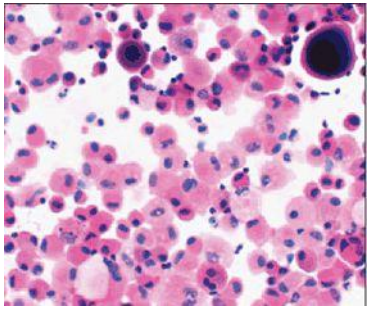
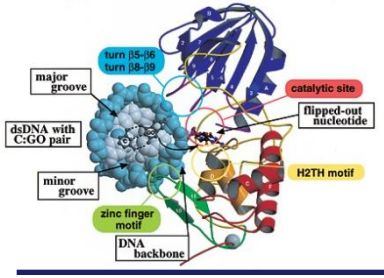
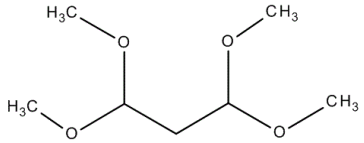
Methodology



- Inflammation and oxidative stress:
  - Broncho alveolar cells count
  - Histology
  - Modified comet assay with FpG enzyme for selective detection of oxidative lesions; some lipid peroxidation measurements in plasma





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Methodology

- **2 routes of exposure: gavage and instillation,**

some iv data also (NM102, 103, 104 and 203)

- **4 MNs per type (4 SAS, 4 TiO_2 and 4 CNT)**

- **Chemical positive control**

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- **Various organs both in site of contact and systemic ones**

Gavage and instillation, 3 doses, 3 administrations each 24h

4 to 5 animals/dose

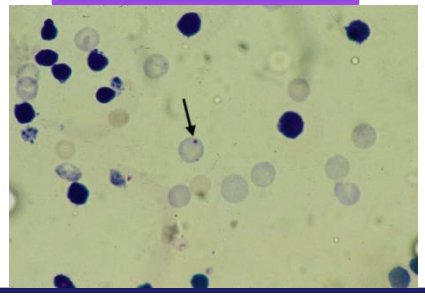
3 to 6 h after the last administration

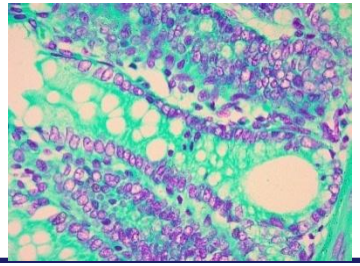
Micronucleus assay

Bone marrow (OECD 474)

Chi-square test

Colon (only for gavage)





Comet assay

lung, BAL, liver, spleen, blood, bone marrow, kidney, intestine, colon

Non parametric Kruskall-Wallis test

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1. Exposure of groups of mice to 0, 10 or 15 mg/kg of NM102, 2x

C57Bl6/J mice harbouring pUR288 plasmid that contains LacZ gene

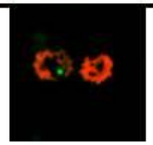
28 days after last i.v. (fixation period)

Removal of Liver and Spleen

3. Comet assay in liver and spleen

42 h after last i.v.

2. Micronucleus assay in tail blood

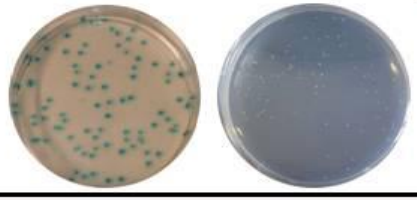


4. LacZ mutation assay

- Genomic DNA extraction

RESCUE PROCEDURE

- Imunomagnetic separation of the transgenic DNA
- Electrotransformation of *E.coli* ΔLacZ
- Determination of the Mutant Frequency in each treatment group



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Methodology

- Training and trials organized before the assays with MNs
  - For the micronucleus assays on bone marrow and colon
  - About positive control: which unique chemical? which dose? What about a nanosized one?
    - MMS and CPA
    - Carbon black

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Gavage

		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)

MMS selected for chemical positive control

Carbon Black not included

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Methodology

Doses selected (from the dispersion protocol and the WP7 results):

- **TiO2:** 4.6, 2.3 and 1.15 mg/kg (x3) instillation
  26, 13.5, 6.5 mg/kg (x3) gavage
  2.3 mg/animal (X5) NM103 and 104 intravenous (WP7)
  10 and 15 mg/kg (x2) NM102 intravenous (LacZ)
- **SAS**: 12, 6 and 3 mg/kg (x3) instillation
  20, 10 and 5 mg/kg (x3) gavage and NM203 intravenous
- **CNT**: 51.2, 25.6 and 12.8 mg/kg (x 3) for gavage except for NM400 (12.8, 6.4, 3.2 mg/kg (x3))
  0.4, 0.2 and 0.1 mg/kg (x3) for instillation except for NM402 (1.6, 0.8 and 0.4 mg/kg (x3))

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Results

TiO2

- **Comet assay:**

Most MNs inducing **no DNA damage** irrespective of the organ except after instillation NM105 in BAL

and after gavage in spleen, intestine (NM103), colon (NM102 and 104) and bone marrow (NM104)

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

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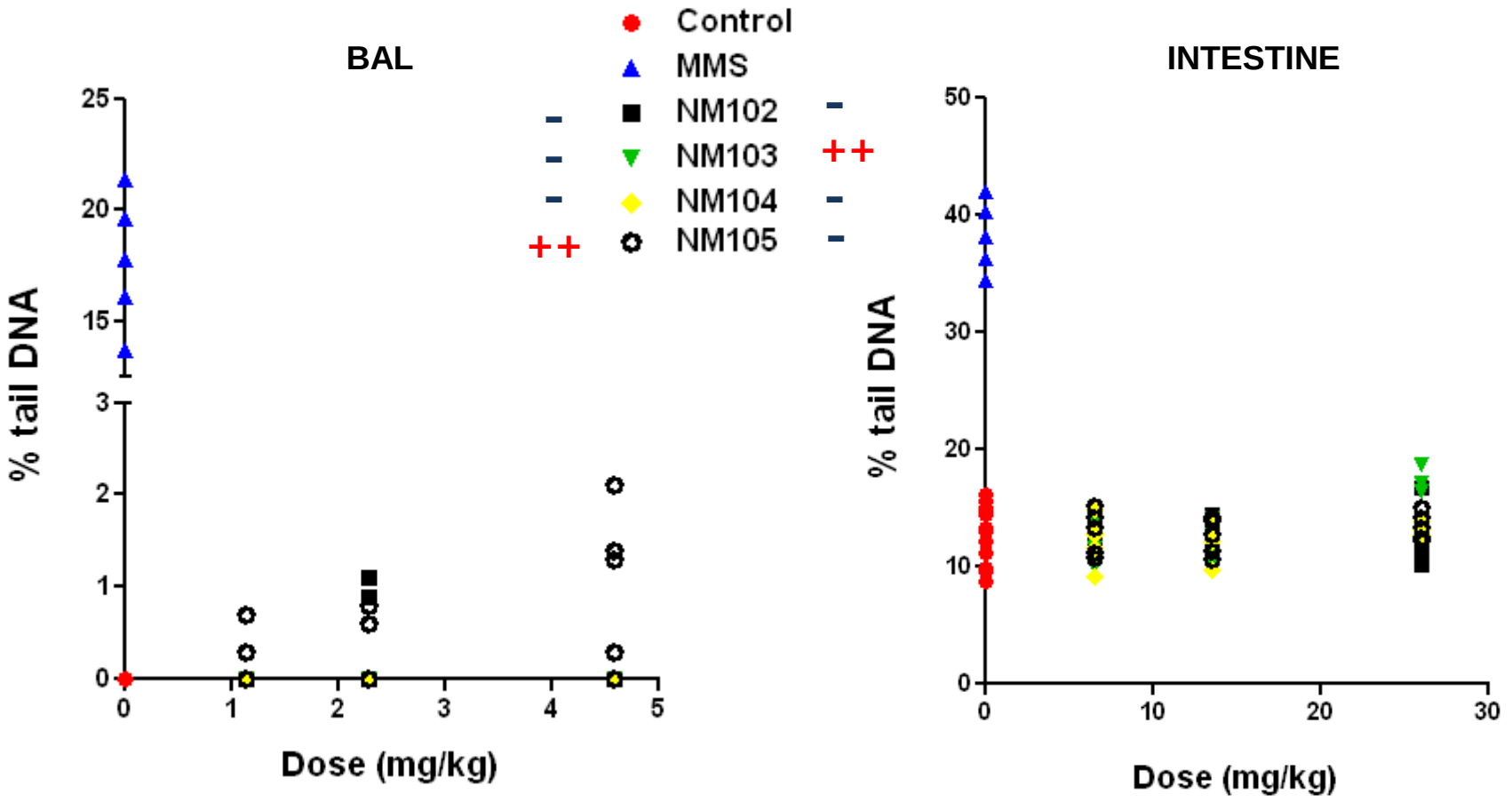
Comet assay: instillation and gavage with TiO2



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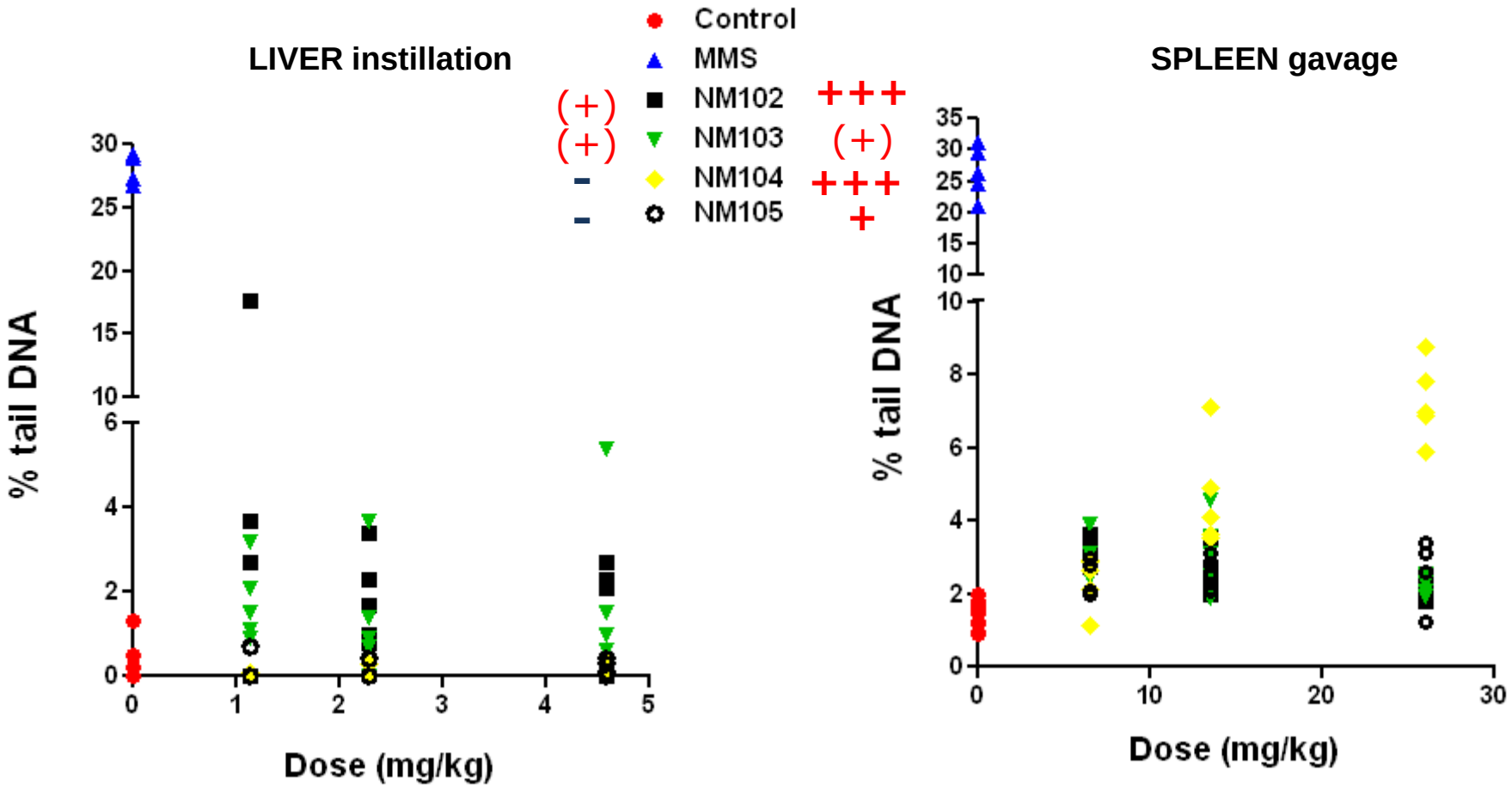
Comet assay: instillation and gavage with TiO2



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Results

TiO2

-**MN assays:**

No mutagenicity in bone marrow
after instillation, gavage or iv

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

no genotoxicity in spleen and liver (comet)
no clastogenicity in blood (micronucleus)
no mutagenicity in liver (lac Z mutation)

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Lac Z assay iv with NM102

Assay	Peripheral Blood	Liver	Spleen
Micronucleus*	NEGATIVE	Not done	Not done
Comet**	Not done	NEGATIVE	NEGATIVE
LacZ mutation***	Not done	NEGATIVE	*Under analysis*
TEM	Not done	*Under analysis*	Not done
Histopathology	Not done	*To be done*	Not done

* Chi-square test; positive control was increased (P<0.0001)
** Kruskall-Wallis test; positive control was increased in liver (P=0.008)
*** Kruskall-Wallis test; positive control was increased in liver (P=0.032)

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Results

SAS

- **Comet assay:**

  No DNA damage

  irrespective of the organ and the route of administration (instillation, gavage and iv for NM203)

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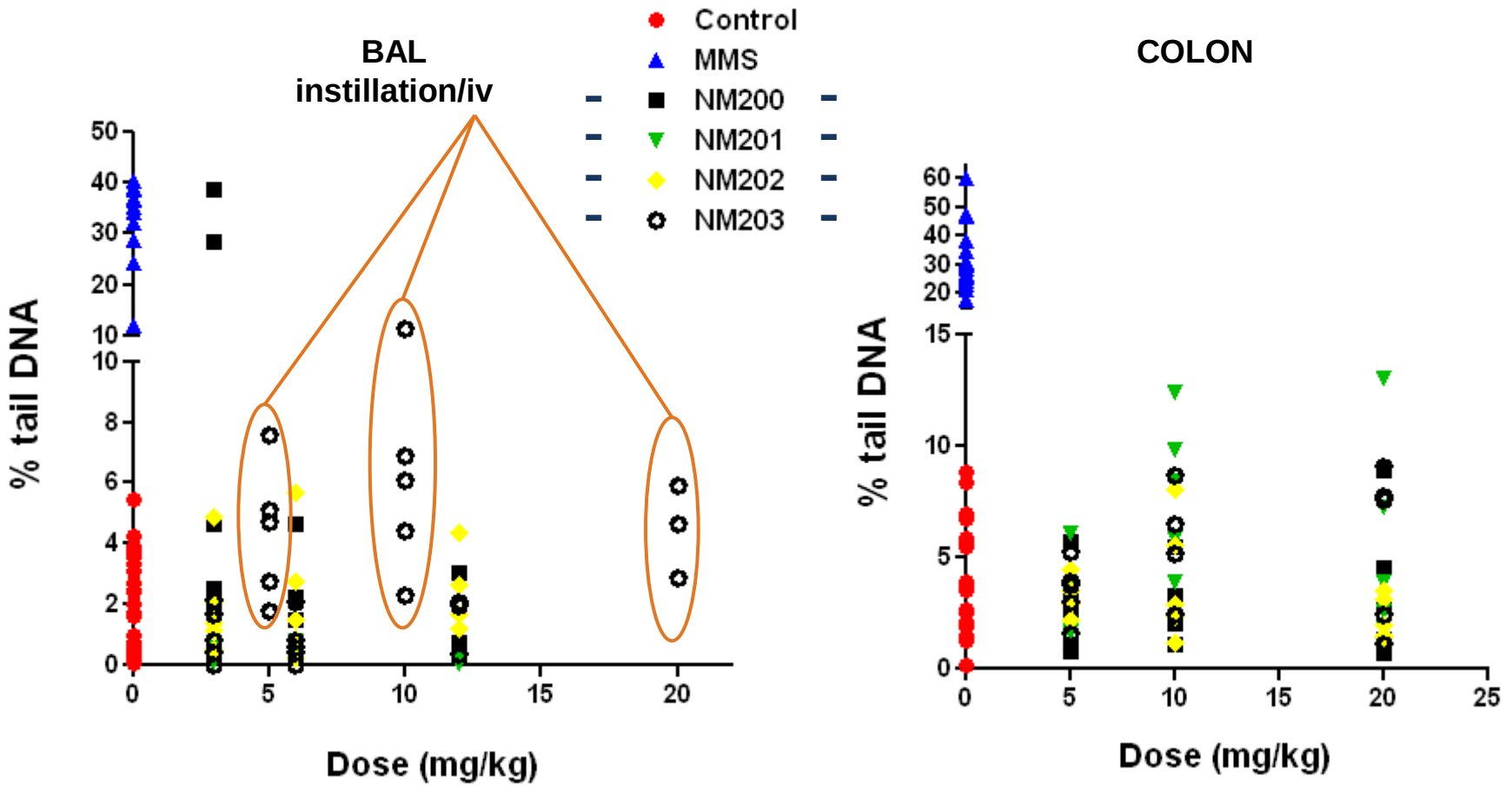
Comet assay: instillation, gavage and IV with SAS



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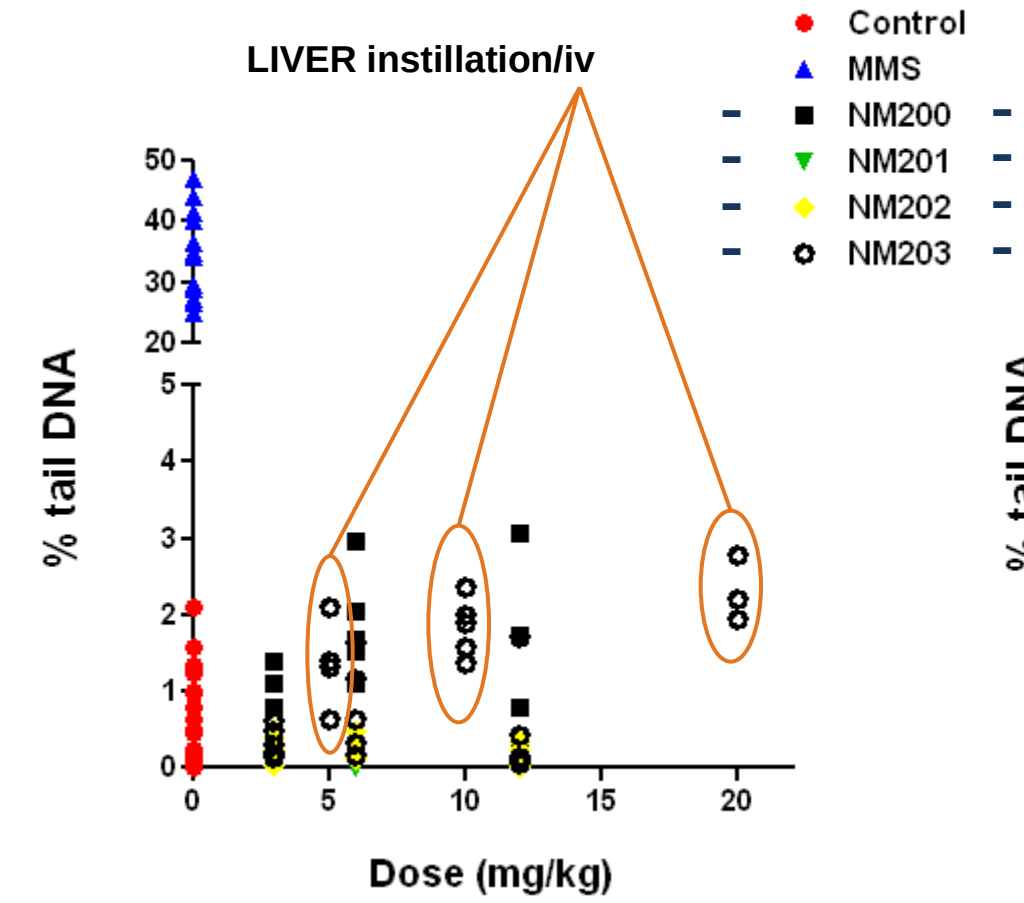
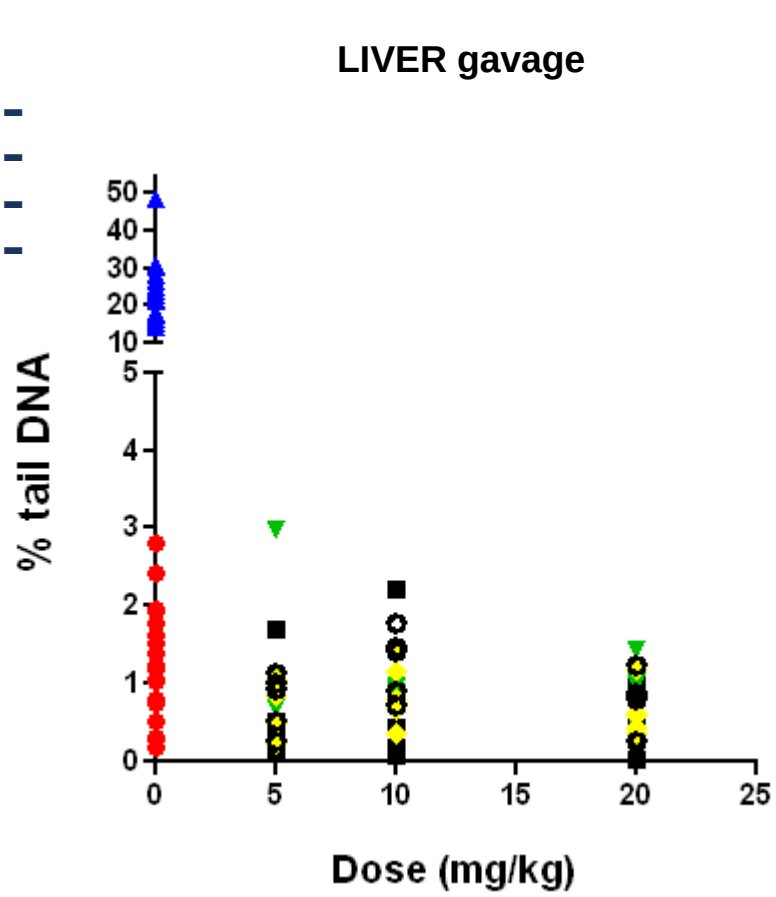
Comet assay: instillation, gavage and IV with SAS





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Results

SAS

- **Micronucleus assay:**

  - **Bone marrow:**

    no induction of micronuclei

    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:**

    increase of micronuclei formation

    for **NM202 and 203 only at the lowest dose**

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Colon micronucleus assay with SAS

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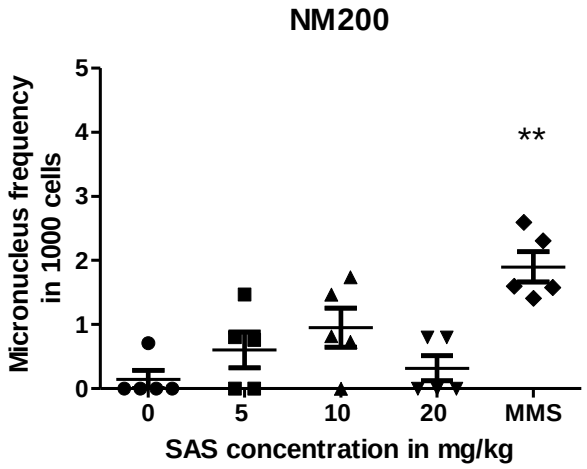

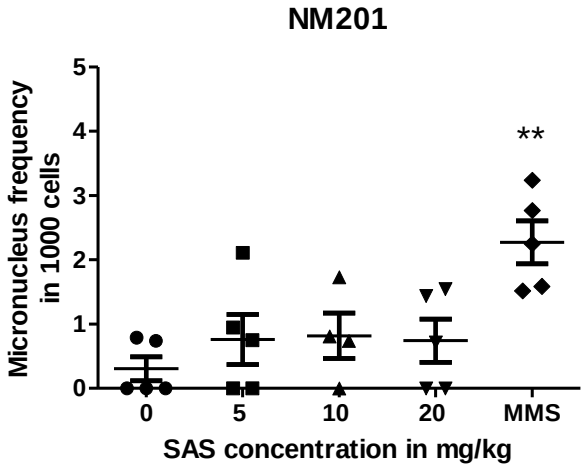

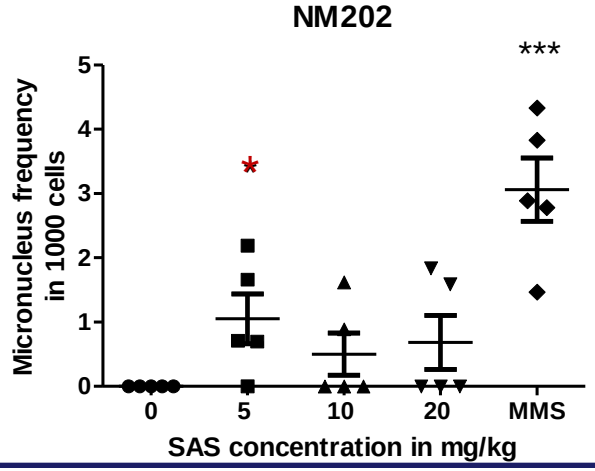

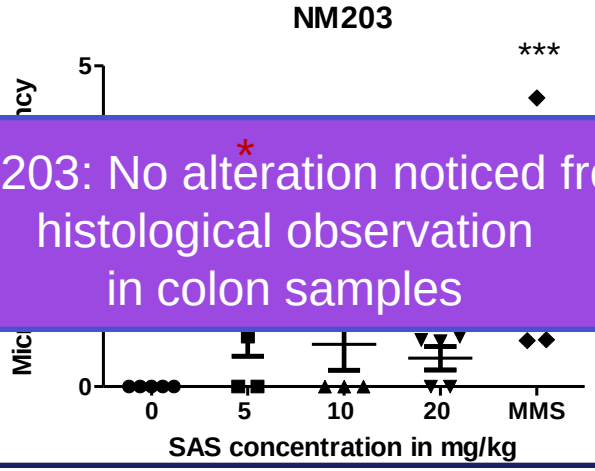

NM 203: No alteration noticed from histological observation in colon samples

MMS: 2 first doses 100 mg/kg, the 3rd 80 mg/kg

* For $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$ with χ^2 test with Yate's correction

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Results

CNT

- **Comet assay:**

Some genotoxicity induced in various organs

After gavage NM401 in liver and kidney

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

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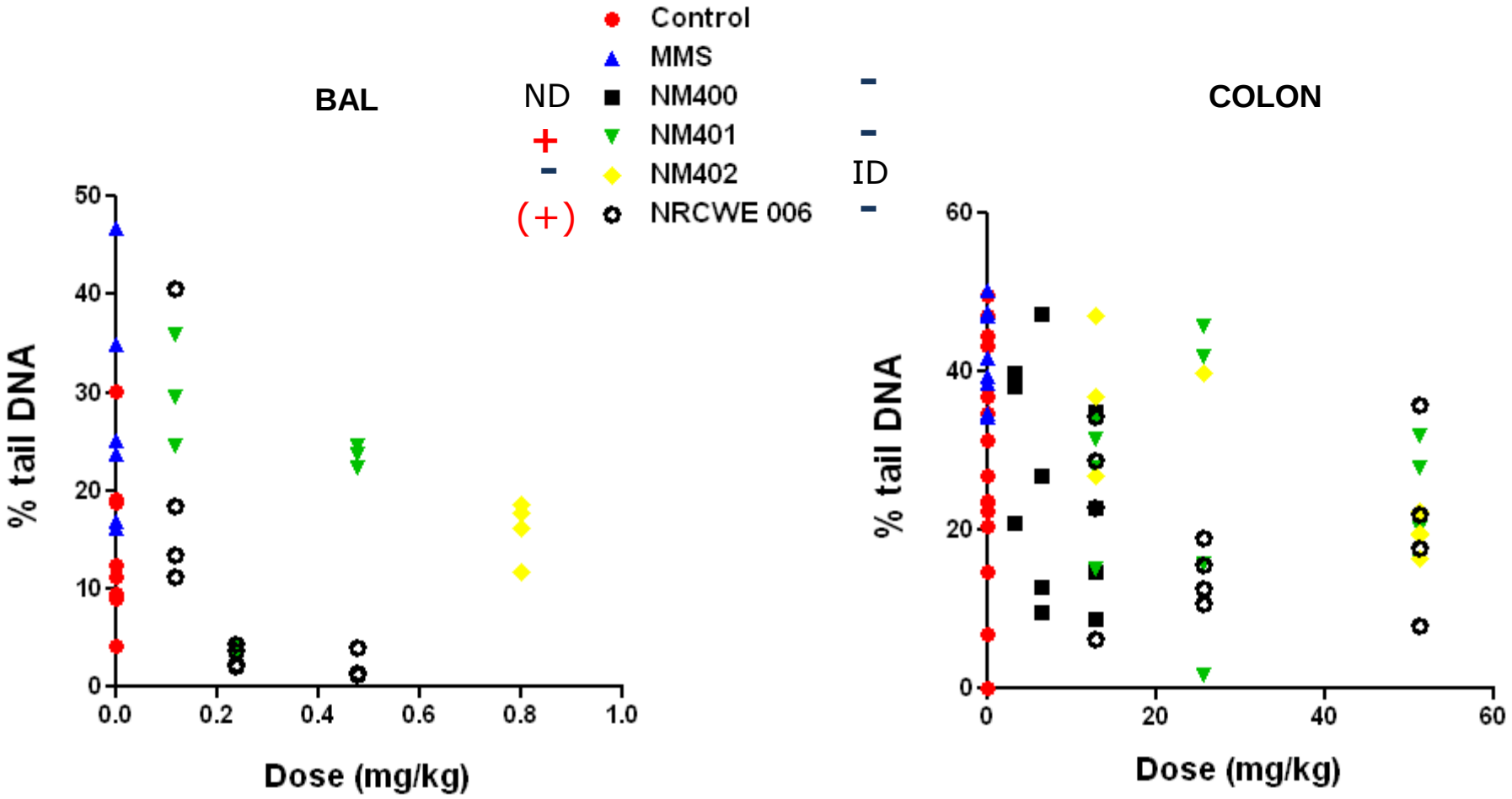
Comet assay: instillation and gavage with CNT



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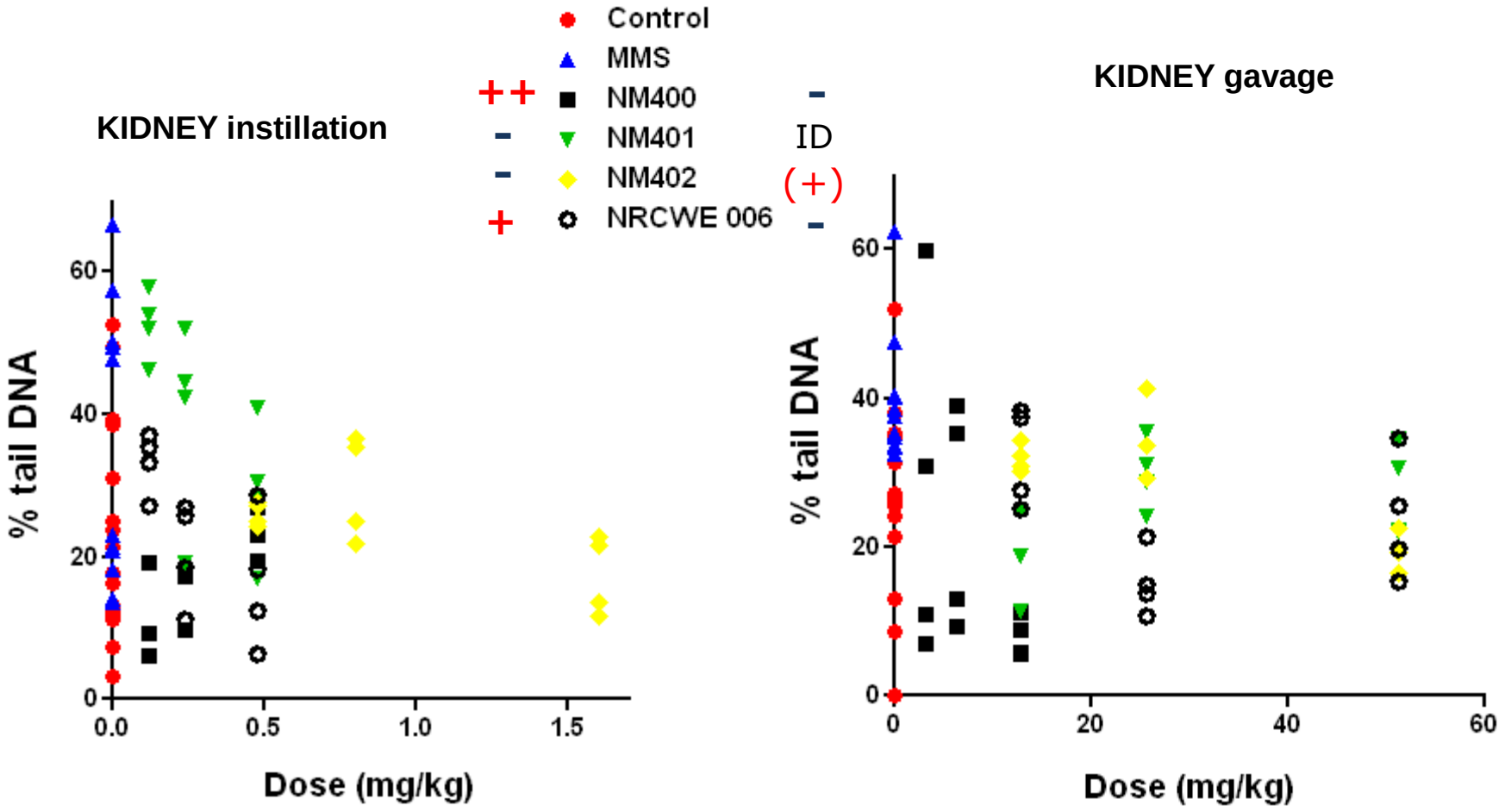
Comet assay: instillation and gavage with CNT



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Results

CNT

-**Micronucleus assay:**

- **Bone marrow**:

  no mutagenicity
  irrespective of the route of administration

- **Colon:**

  no mutagenicity with NRCWE 006

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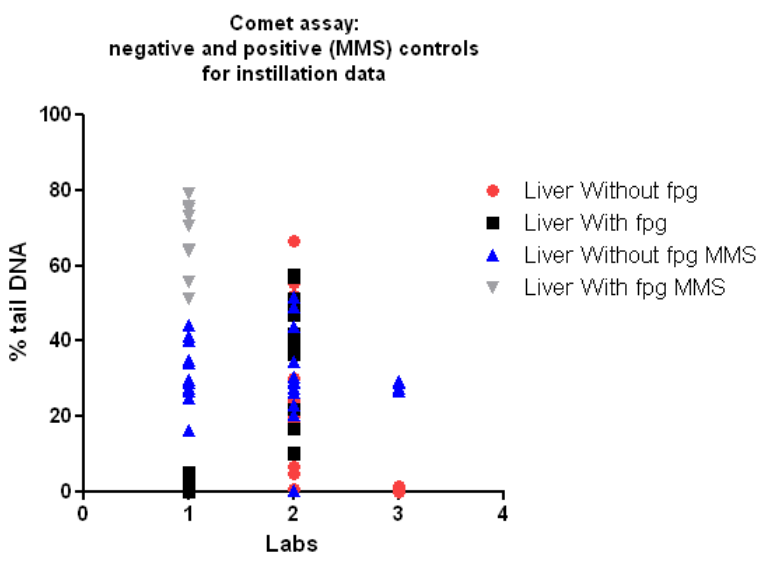
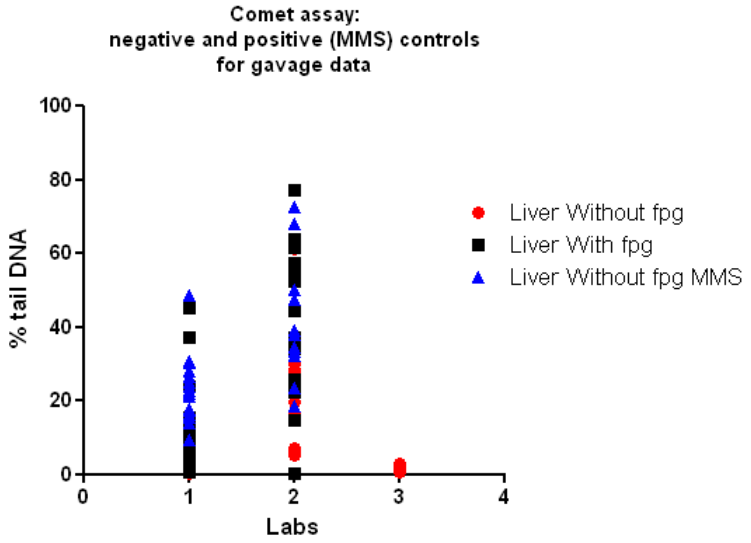
Intra and inter laboratory variabilities

Results





Variabilities due to the assay leading in few cases to invalidated data and inconclusive results

Provide some criteria of acceptability for the non-OECD tests (EFSA 2012)

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Results

- Not much effect from oxidative damage due to MN exposure when it had been measured by the modified Fpg comet assay

- Some toxicity observed:
  - death after iv exposure with NM203 (2/5; 20 mg/kg)
  - diarrhea after gavage with NM105 (3/5; 26 mg/kg)

- Some data still expected (especially micronucleus on colon)

- Comparison with *in vitro* and phys-chem to be performed

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Conclusions

- Most data indicating no genotoxicity
- However some genotoxic effect observed in few organs that need to be confirmed, few dose response
- Apparently, within the same family, the toxic effect varies according to the MN (genotoxicity but also toxic injuries)
- Negative results with the OECD guideline 474 on bone marrow
  (except after iv with NM 203 at the high dose)
- Use of non-OECD tests which would require to set up some criteria of acceptability because some variability from lab to lab highlighted
- Comparison with the other WP results

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WP6 comments of external experts

Laetitia Gonzalez, Micheline Kirsch-Volders

Vrije Universiteit Brussel

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Strengths

- Comparison of 3 exposure routes (it instillation, iv injection, gavage)
- Use of two complementary assays
  - Comet assay
  - MN assay
- Collaborative experiments with clear protocols
- Training
- Critical assessment of the results

Weaknesses

- Tissue type choice after specific route of exposure (e.g. bone marrow after gavage or it instillation)
- Acceptability criteria and historical controls

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Recommendations for future research

- Focus on relevant organs depending on route of exposure
  - Colon after gavage
  - Bone marrow after iv injection (OECD validated)
  - Epithelial lung cells after it instillation
- Validation of in vivo genotoxicity assays in colon and lung cells

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