Contribution to nanomaterials safety assessment: the need for integrating *in vitro*, *in vivo* and *in silico* strategies

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NANOMATERIALS (NMs) and NANOTECHNOLOGIES (I)

The number of NANOMATERIALS brought to market has exponentially grown in recent years and will continue to grow and evolve to new generation NMs.

NANOTECHNOLOGIES – Key-enabling technologies that use materials and manipulations at the nanoscale and which has a potential influence on almost any technological area.

Fundamental and application-driven research is expected to boost nanosciences and innovation towards development of SAFE-BY-DESIGN NMs and applications

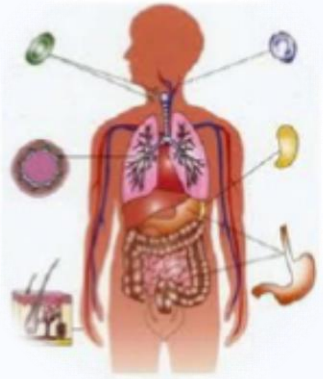
NANOMATERIALS (NMs) and NANOTECHNOLOGIES (II)

Nano-applications in consumer products, medicine and industrial processes are widespread – **SOCIETAL BENEFITS**

WHO, 2012

Human exposure to NMs is growing very fast but…

- Solid information about hazard is limited for the majority of NMs, especially related to chronic exposure to low doses, that is the most likely to occur (e.g., through consumers products)



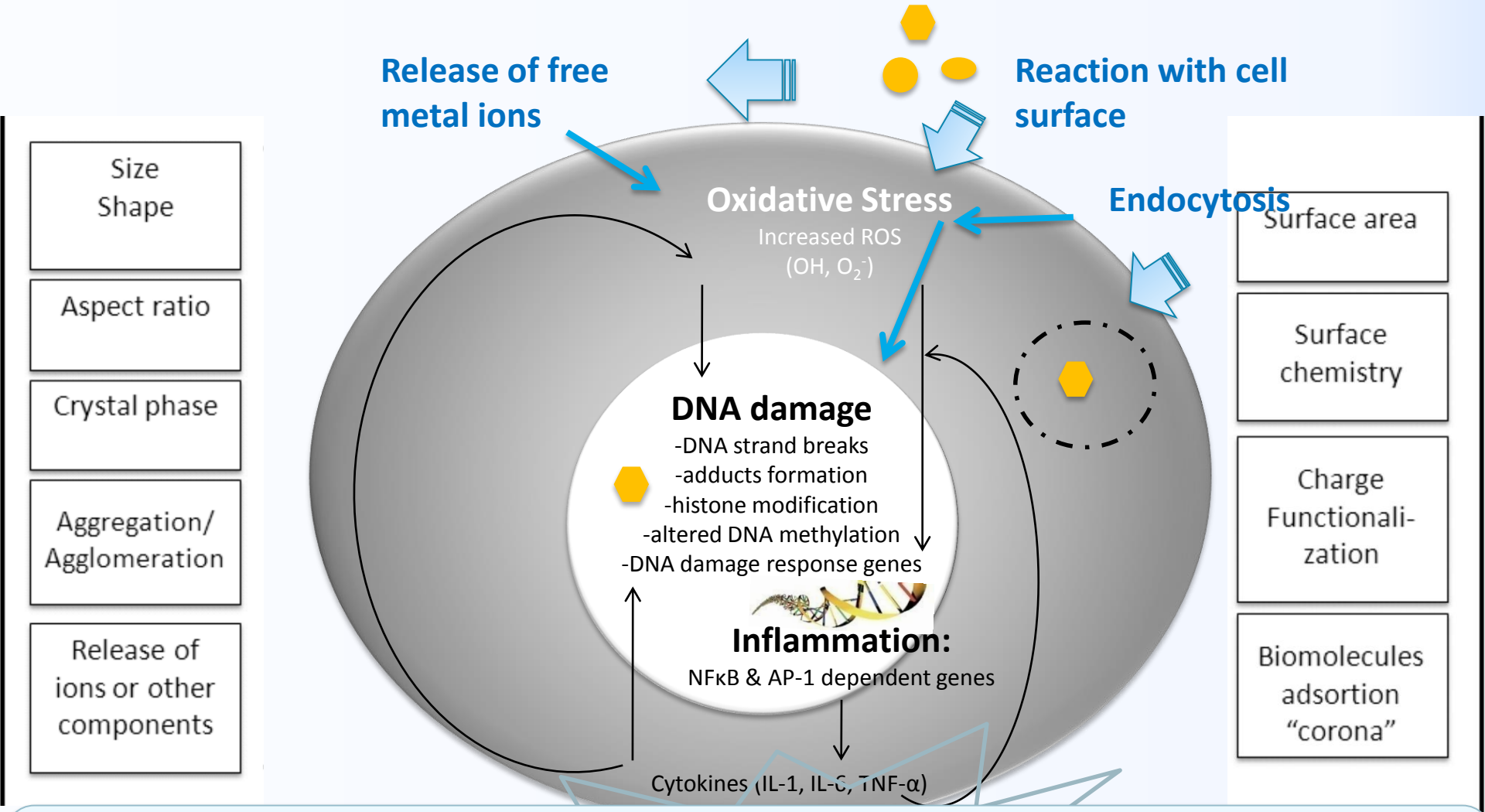
Zhao & Liu, 2012

- Inhalation
- Transdermal
- Oral route
- Intravenous route

Toxicity ?

- The **genotoxic effects of NMs**, which may be linked to carcinogenic effects, are of special concern because cancer has a long latency period and thereby these effects can be less obvious and more difficult to predict than eventual acute effects.

NANO-BIO INTERACTIONS AND POTENTIAL EFFECTS



Assessment of toxicity - use of complementary *in silico*, *in vitro* and *in vivo* assays , taking into account specific physico-chemical properties of NMs

Factors interfering with genotoxicitity tests interpretation and comparability

- **Incomplete description of the NMs physicochemical properties**
- **Coating**
- **Dynamic behavior of NMs** (formation of aggregates and agglomerates, and the kinetics dependent of the medium conditions)
- **Corona formation and composition**
- **Dosing** (difficult to picture a real exposure scenario in *in vitro* or *in vivo* assays - limited human exposure data)
- **Interference with colorimetric assays** (e.g., cytotoxicity assays)
- **Differences in the means of dispersion of insoluble NMs**
- **Different uptake capacity of cell lines**
- **Limited existence/access of SOPs and validated methods**
- **The dose-metrics** (e.g., mass, particle number or surface area)
- **Lack of reliable positive controls at the nanoscale**

IN VITRO AND *IN VIVO* GENOTOXICITY ASSESSMENT OF TiO_2 NMs

- Pigment
  - •Food colorant
  - •Cosmetics
    - •Skin care products
    - •Sunscreen products
- •Photocatalytic properties
  - • Solar panels
  - • paints and construction products





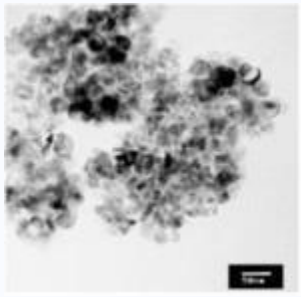

Objectives

To assess genotoxic effects of TiO_2 NMs at cellular, molecular and organism level using a combination of *in vitro* and *in vivo* approaches to allow an integrated understanding of its biological effects

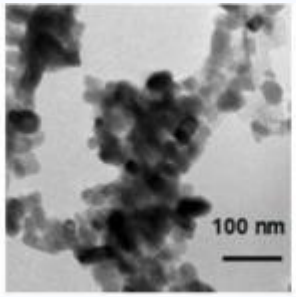
- Minimize variability inherent to NMs and *in vitro* experimental procedures:
  - Benchmark NMs (JRC repository)
  - Characterized physico-chemical properties
  - Standardized method for NMs dispersion and control of particle size distibution
  - MN assay (OECD guideline 487)
  - Comet assay (SOP)
- Use of integrated *in vivo* approach:
  - analysis of several endpoints in the same animals (3Rs)
  - DNA and chromosome damage and somatic gene mutation; inflammation and NPs accumulation in liver (toxicokinetics information)
- Comparison of *in vitro* and *in vivo* data for one TiO2 NM

Experimental strategy

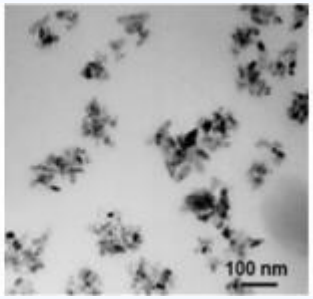
Titanium dioxide nanomaterials (JRC repository)



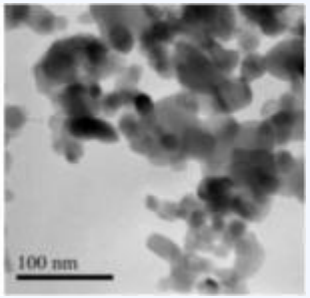
NM-102



NM-103



NM-104



NM-105

Characterization of physico-chemical properties – TEM[1]

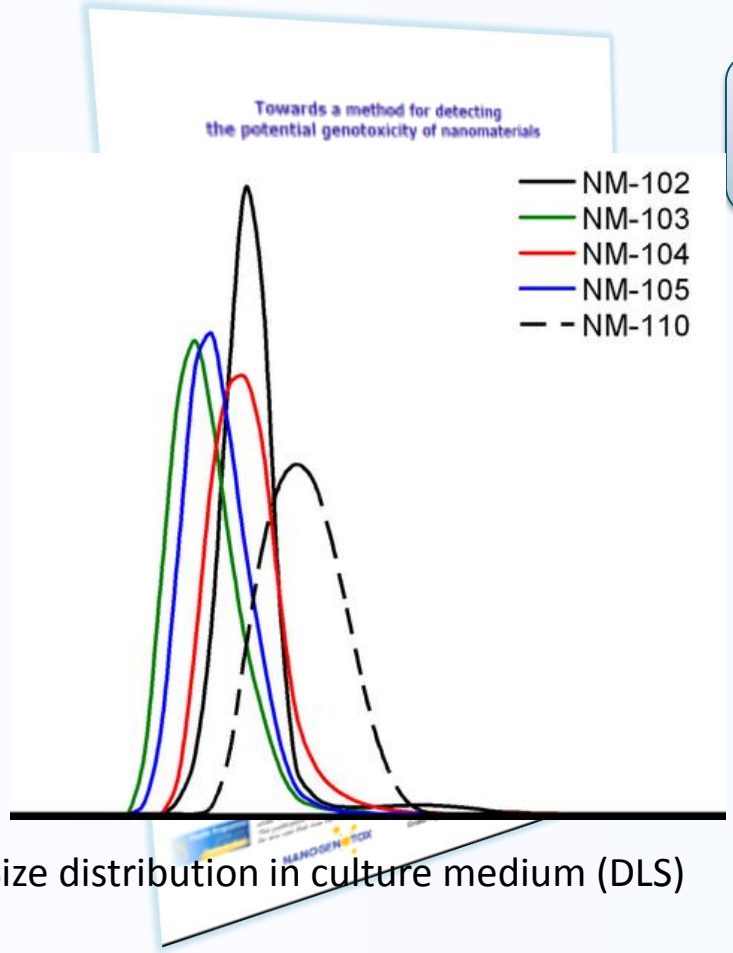
Nanomaterial		Phase (and other information)[a]	Impurities/ coatings (surface modification)	Specific surface area (m^2/g) [a]	Primary particles				Aggregates/agglomerates[d]		
					Feret Min ± SD (nm)[b]	Feret Max ± SD (nm)[b]	Aspect ratio ± SD[b]	N[c]	25% (nm)	Median (nm)	75% (nm)
TiO_2	NM-102	Anatase	–	90	20.8 ± 1.6	33.0 ± 1.5	1.5 ± 1.3	59	43	54	72
	NM-103	Rutile (hydrophobic)	Dimethicone 2%[e]	60	21.9 ± 1.4	37.9 ± 1.6	1.7 ± 1.3	40	33	67	129
	NM-104	Rutile (hydrophilic)	Glycerine[e]	60	19.0 ± 1.5	25.8 ± 1.4	1.4 ± 1.3	47	33	60	112
	NM-105	Rutile-anatase (15–85%)	None[e]	61	20.0 ± 1.3	29.6 ± 1.3	1.4 ± 1.2	42	55	90	144

[1] Jan Mast, Keld A. Jensen *et al., Nanogenotox Deliverable 4.1, 2013; Tavares et al., 2014*

NANOGENOTOX

Experimental strategy

Dispersion of NMs according to a standardized protocol



Size distribution in culture medium (DLS)

Dispersion in BSA/water
Sonication

Keld A. Jensen *et al., Nanogenotox Deliverable 3, 2011; Tavares et al., 2014*

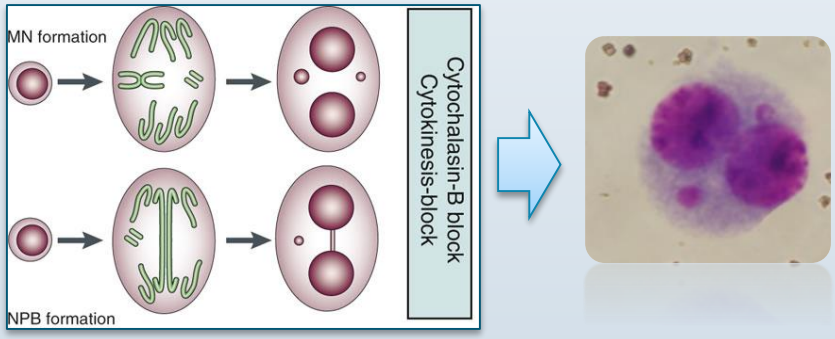
NANOGENOTOX

In vitro testing of TiO_2

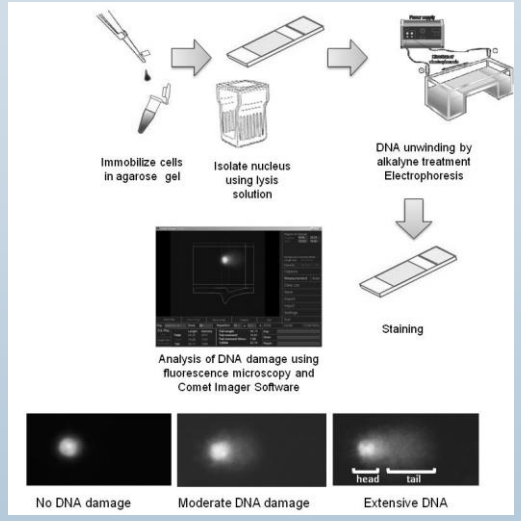
In vivo testing of TiO_2

Micronucleus (MN) assay

48h - exposure to NM (6h before cytB)

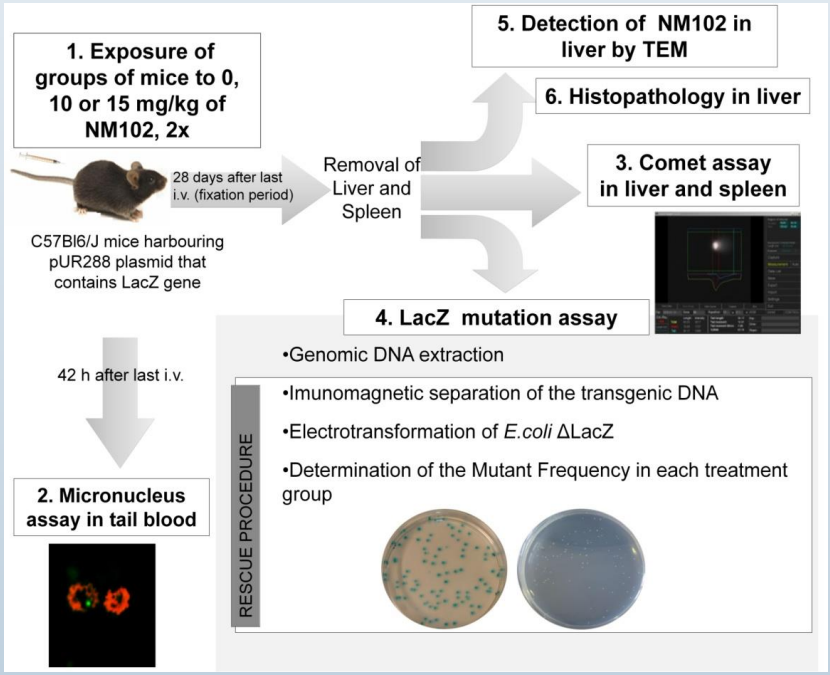


Comet assay



Human lung cell lines (BEAS-2B, A549)
Human lymphocytes

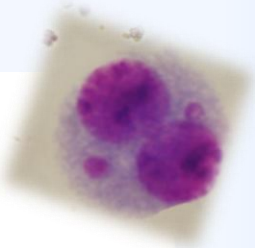
Integrated Approach Using *LacZ* Plasmid-Based Transgenic Mice

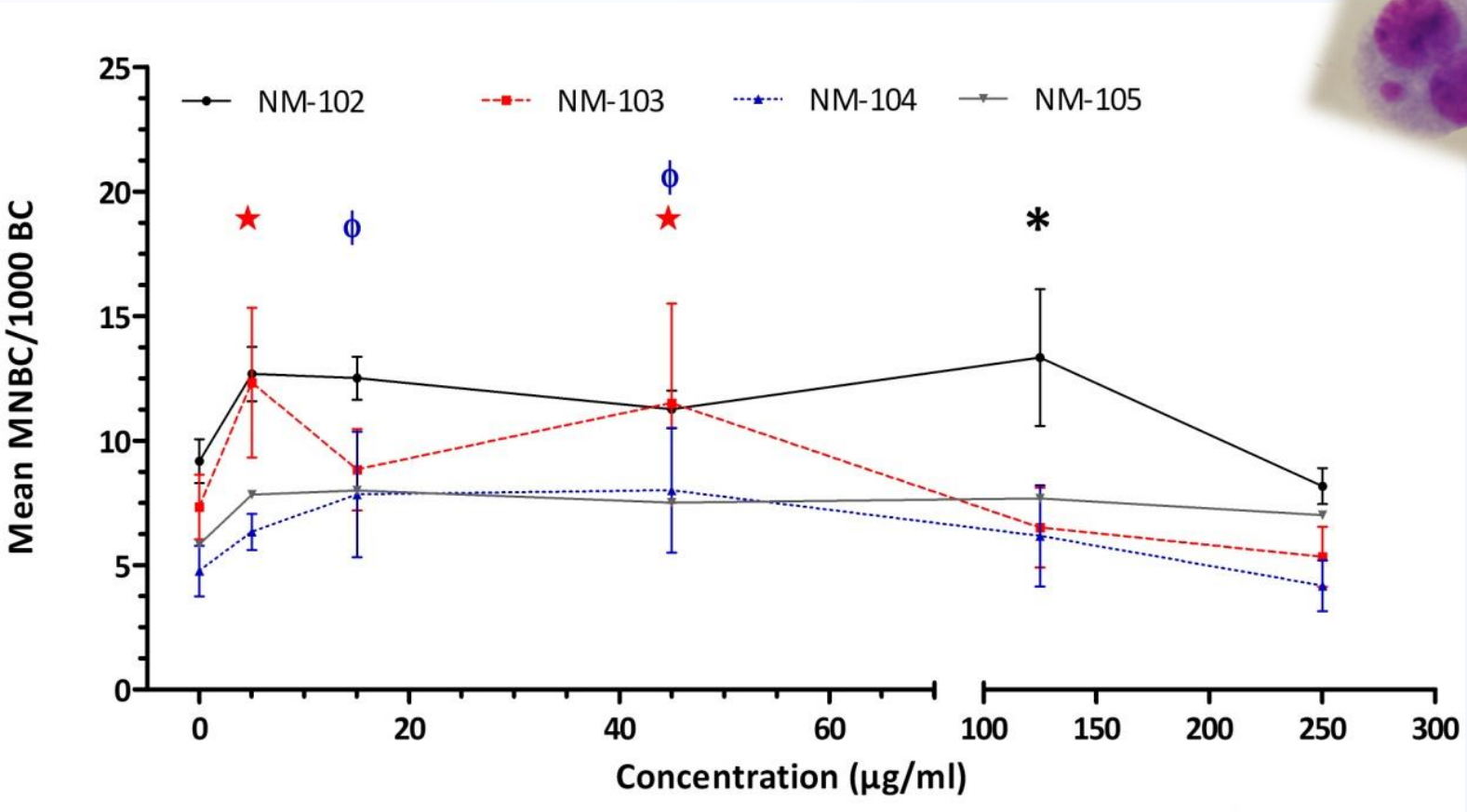


Results

In vitro testing of TiO_2

1. MN assay in human *lymphocytes*





No monotonic dose-response relationship; Significant increase in the micronucleus frequency :

***NM-102: 125 µg/ml** (*p*=0.038);

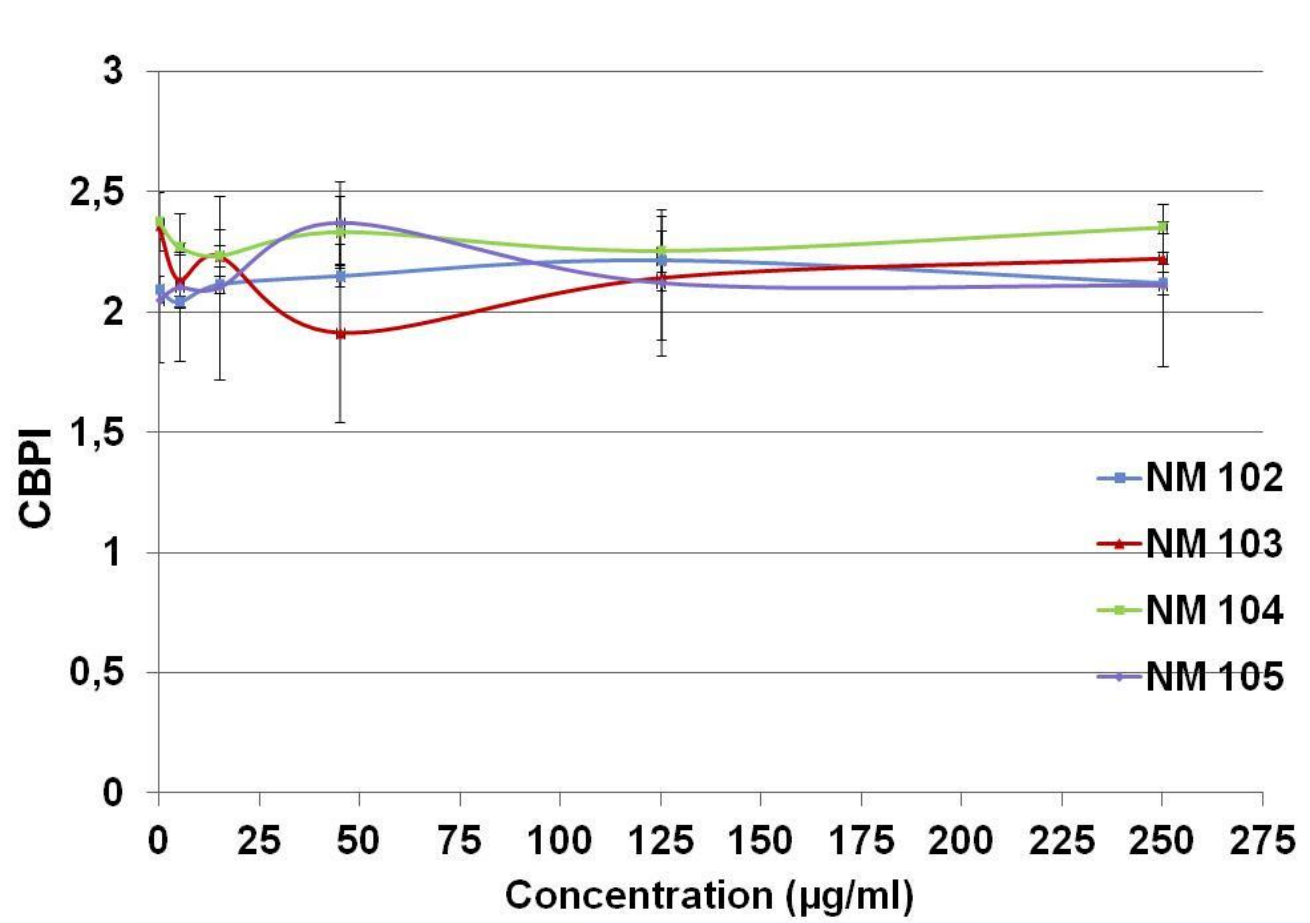
★**NM-103: 5 e 45 µg/ml** (*p*=0.007 and 0.039)

ϕ**NM-104: 15 e 45 µg/ml** (*p*= 0.037 and 0.048)

Results

In vitro testing of TiO_2

1.1. Cytokinesis-block proliferation index in human *lymphocytes*

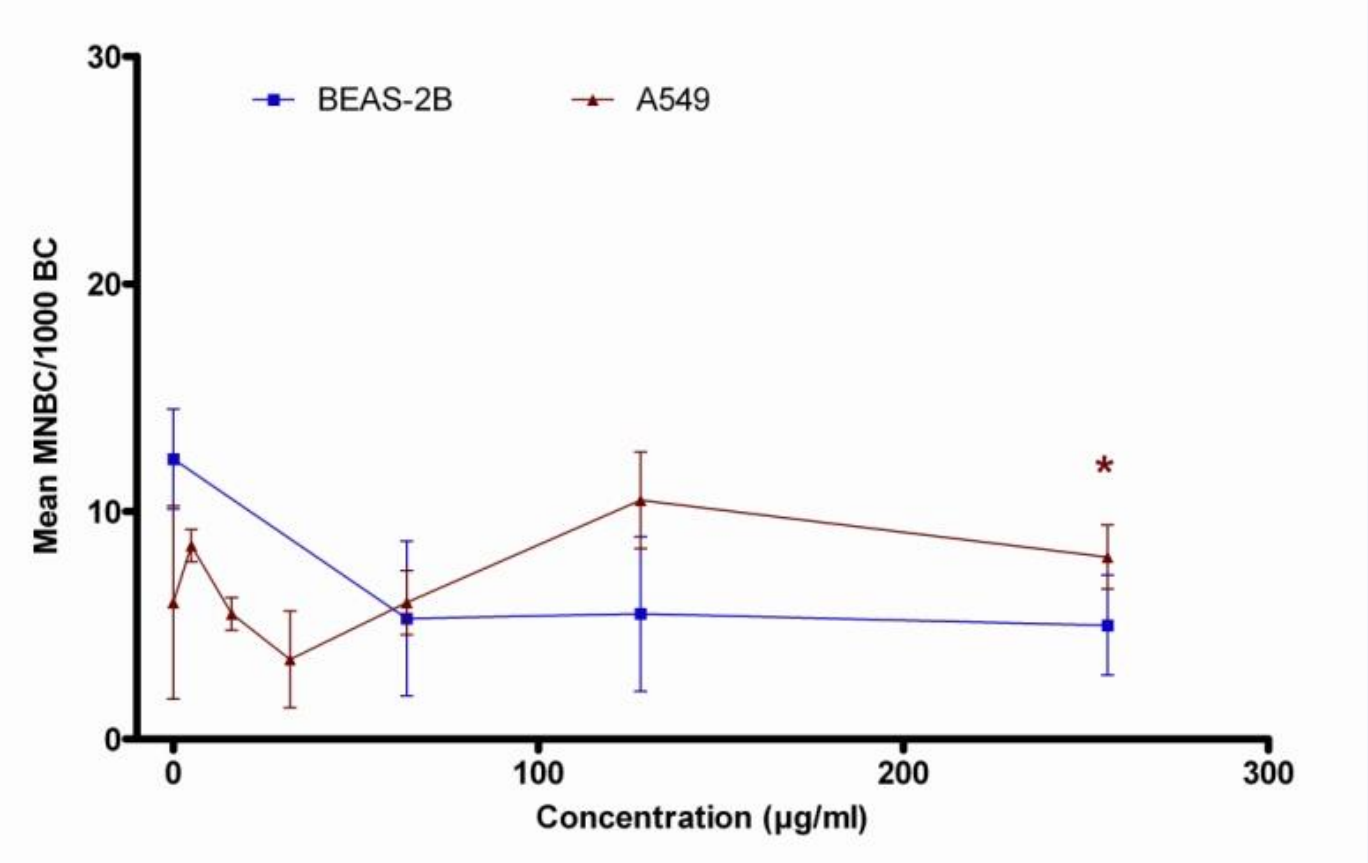


No Significant decreases of CBPI

Results

In vitro testing of TiO_2

1. MN assay in pulmonary cells

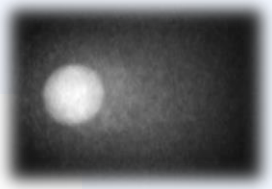


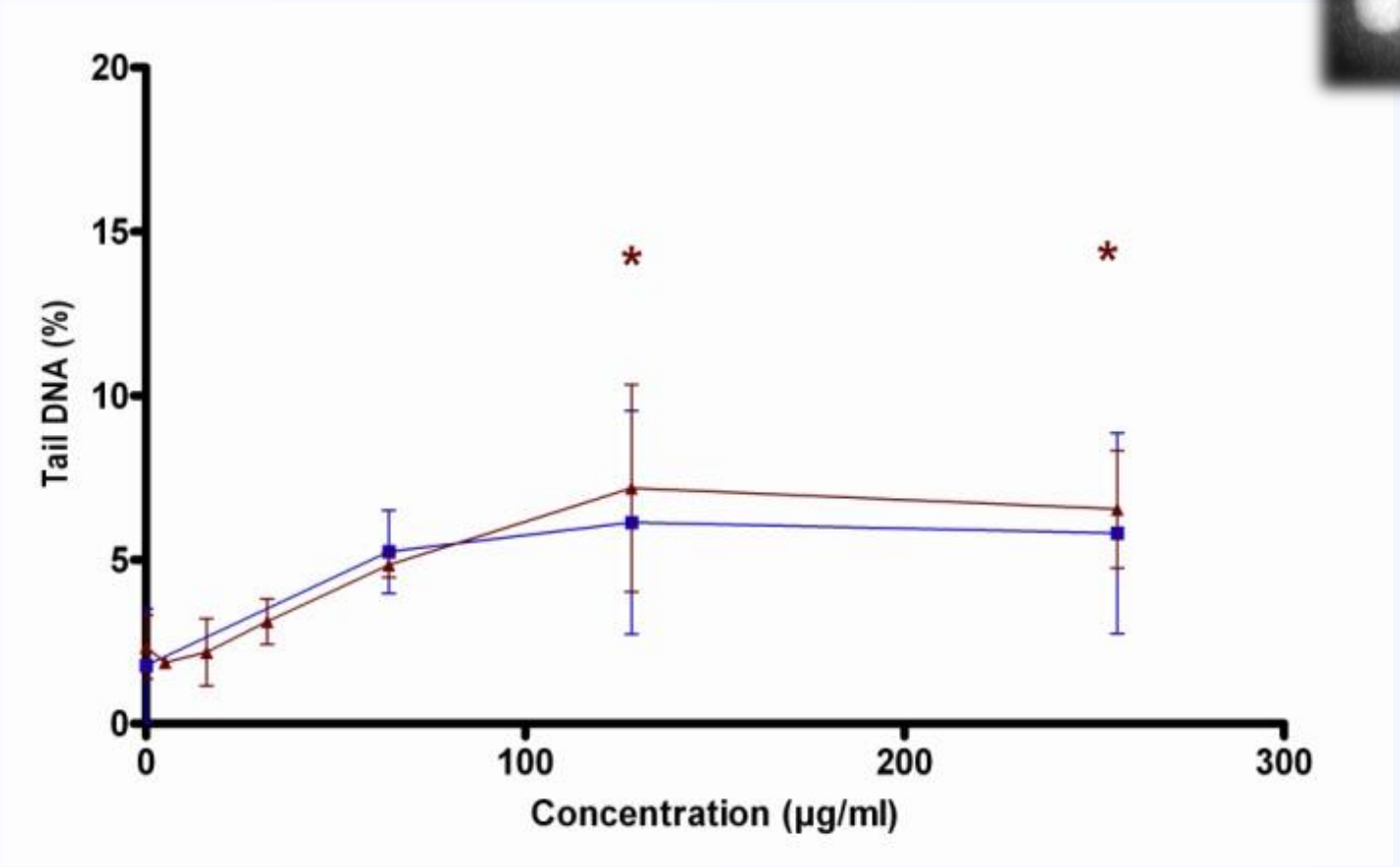
Significant increase in the micronucleus frequency in A549 cells exposed to 256 µg/ml.

Results

In vitro testing of TiO_2 NM-102

2. Comet assay in pulmonary cells





Significant (low) increase in the level of DNA breaks in A549 cells exposed to 128 and 256 µg/ml ; no significant oxidative DNA stress (FPG-modified comet assay)

Remarks

- **NMs obtained under GLP and international benchmarks; good physico-chemical characterization; variability associated to experimental conditions minimized:**

  - **Differential genotoxicity for closely related NMs observed in human lymphocytes** - importance of investigating the toxic potential of each NM individually, instead of assuming a common mechanism and similar genotoxic effects for a set of similar NMs.

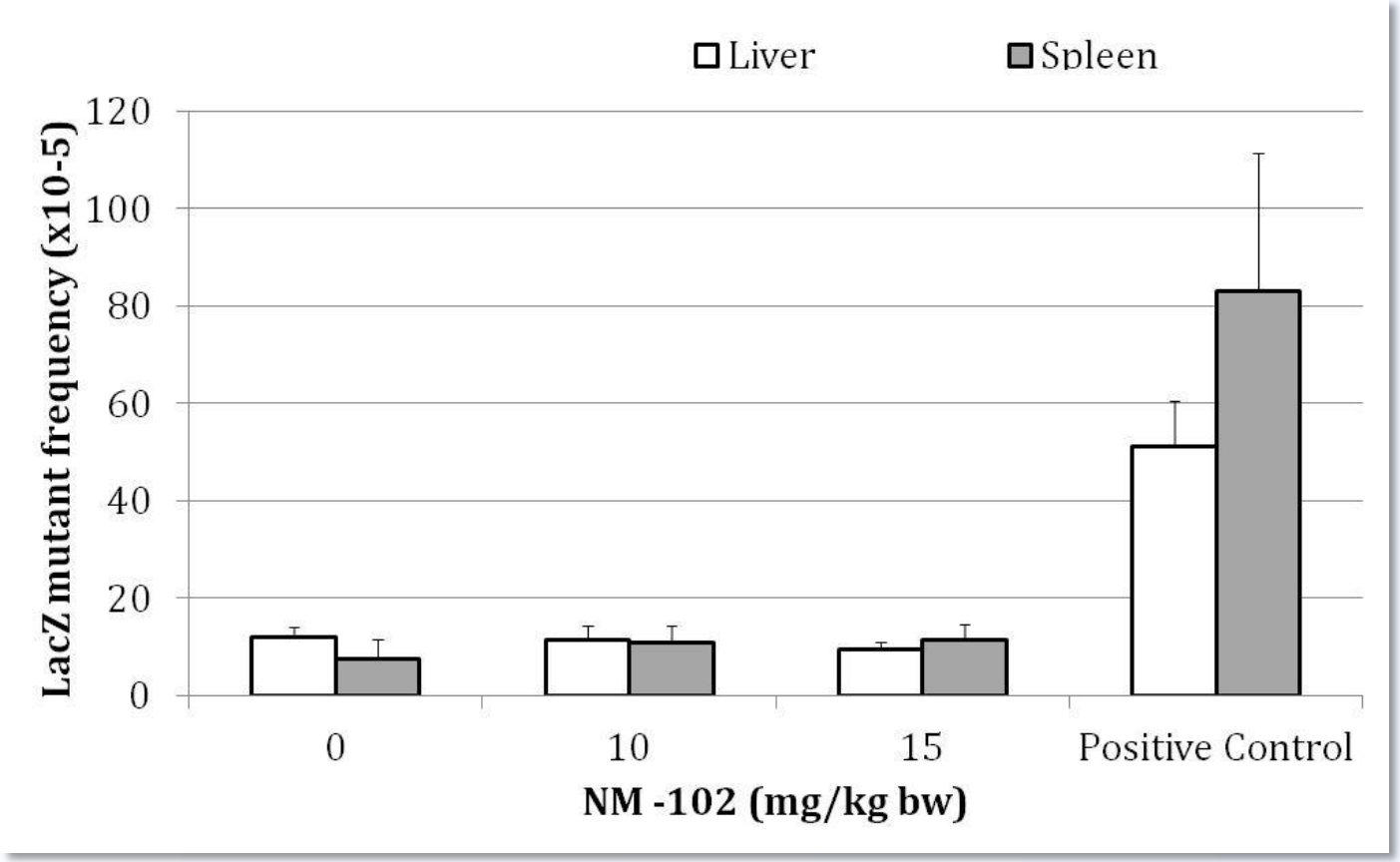
  - **Standard genotoxicity tests are useful, and can be applied, for the safety evaluation of nanomaterials –** provided that standardized protocols for NM preparation are used, the physicochemical characteristics of NMs are considered.

- **Predictivity of the *in vitro* genotoxicity assays for *in vivo* situation with NMs?**

Results

In vivo testing of TiO_2 NM102

1. Frequency of mutations in the *LacZ* gene recovered from liver and spleen



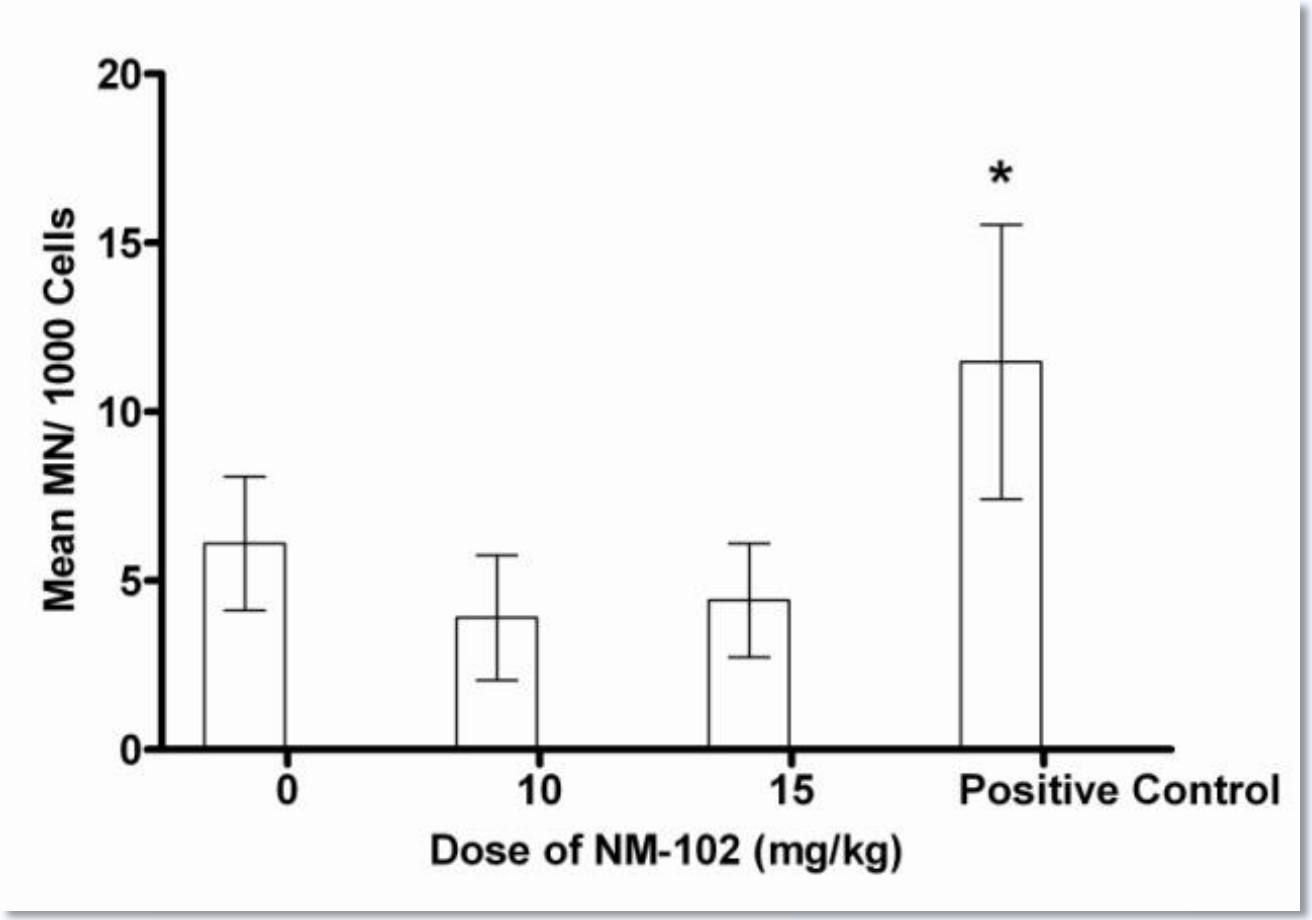
No mutagenic effects in liver or spleen , 28 days after exposure

Louro et al., EnvironMol Mut (2014)

Results

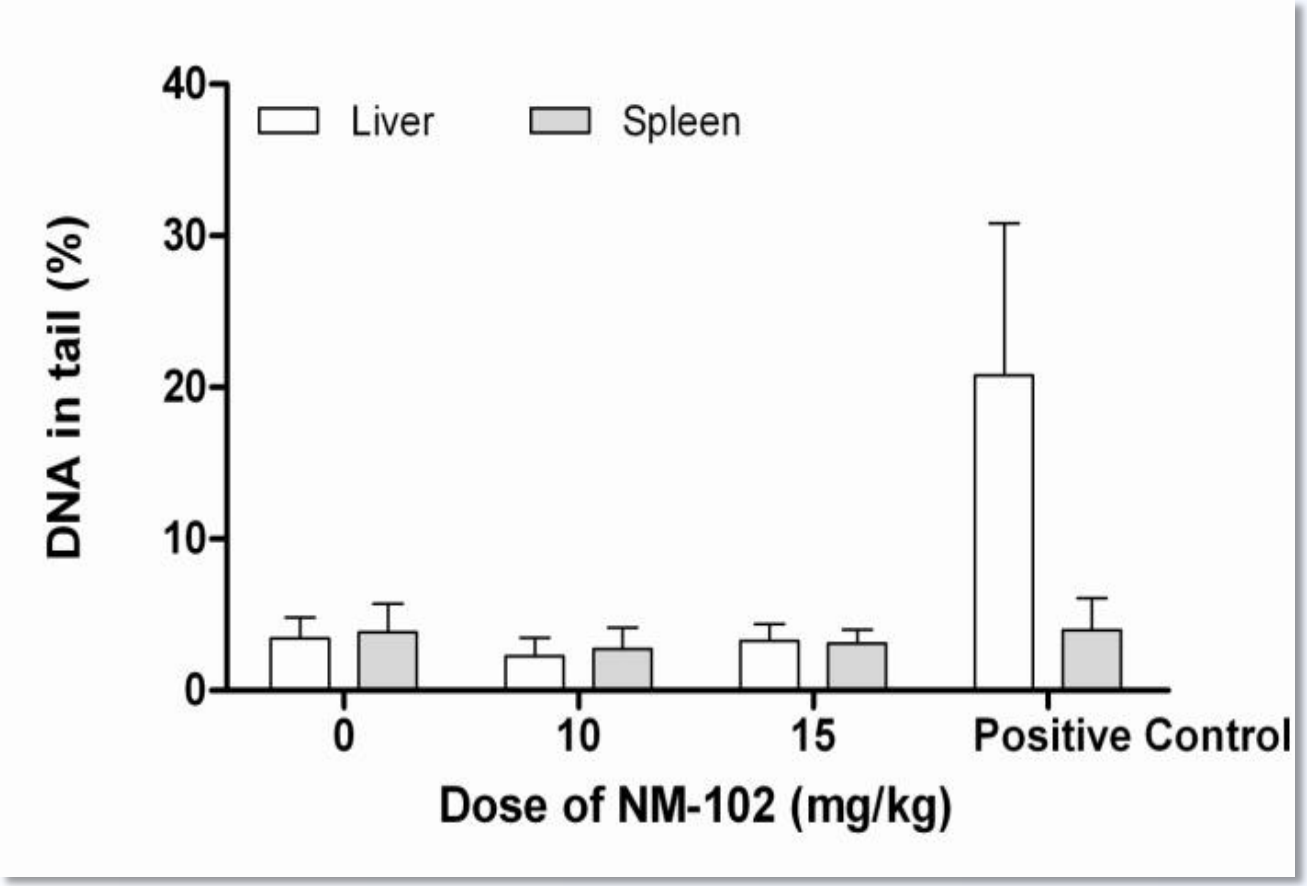
In vivo testing of TiO_2

2. MN in mouse blood immature erythrocytes



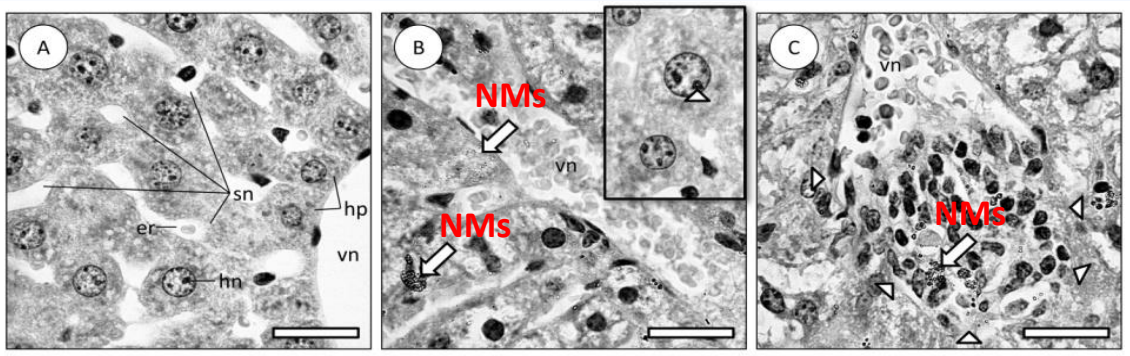
No induction of micronuclei

3. Comet assay in liver and spleen cells

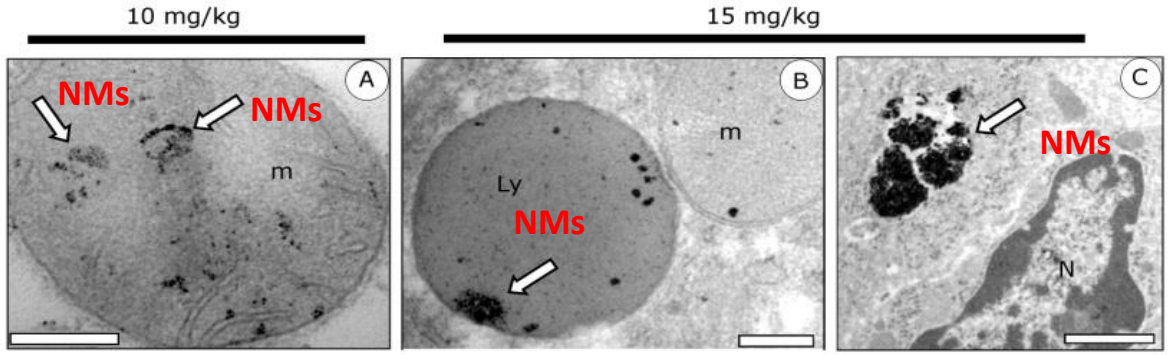


No induction of DNA damage

4. Cellular effects in liver cells



Histological analysis of mouse liver



TEM analysis of mouse liver

Persistence of NM in mouse liver and mild inflammatory effects

Louro et al., EnvironMol Mut (2014)

TiO2 NMs – summary of results

NMs	Experimental system	Genotoxicity (MN assay, comet assay or somatic gene mutaions)	Cytotoxicity/ inflammation
NM-102 NM-103 NM-104 NM-105	Primary lymphocytes	MN assay: Positive, without a dose-response relationship (NM-103, 104) Equivocal (NM-102) Negative (NM-105)	No cell cycle disturbance (CBPI)
NM-102	Alveolar cells type-II (A549)	Equivocal (low +) - comet assay Equivocal – MN assay	Negative
	Lung epithelial cell line (BEAS 2B)	Negative	Negative
	In vivo, Transgenic mice harbouring *lacZ*	Negative: Comet assay MN assay Somatic mutations in liver	Moderate inflammatory effect in liver NPs accumulation in liver cells

Final remarks

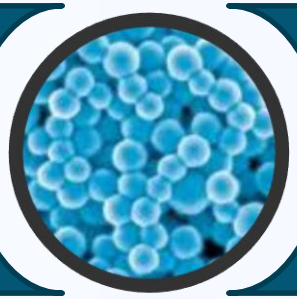
- **No systemic mutagenic effects were disclosed for NM-102 in blood, liver and spleen cells of transgenic mice, under the tested conditions**

- **Histological and TEM analyses confirmed the persistence of TiO_2 in liver and showed a moderate inflammatory effect**

- **The integration of the *in vitro* and *in vivo* data strengthens the weight of evidence of an absence of NM-102 primary genotoxicity, although the possibility of a secondary genotoxic effect driven by an inflammatory response within a longer time window or at different doses cannot be excluded.**

HAZARD ASSESSMENT OF NANOMATERIALS (NMs)

Genotoxicity *in vitro*



Genotoxicity *in vivo*

Toxicokinetics

NANOREG

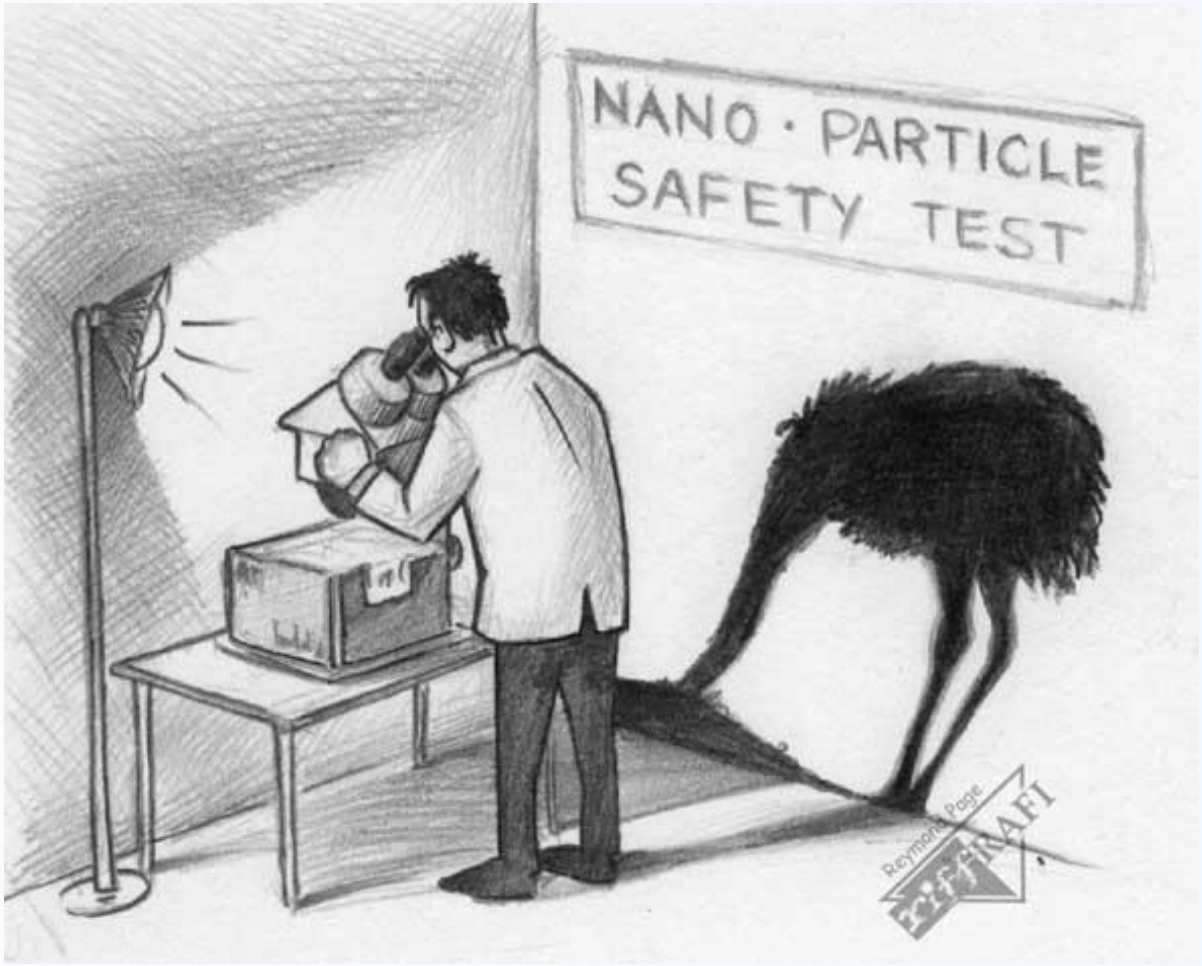
PHYSICOCHEMICAL PROPERTIES

STRUCTURE-ACTIVITY RELATIONSHIP (SAR)

ROBUST METHODOLOGIES TO CHARACTERIZE THE GENOTOXIC AND POTENTIAL CARCINOGENIC EFFECTS

INNOVATIVE APPROACHES:
ALTERNATIVE *IN VITRO* TESTS (e.g.,cell transformation assay)
3D/ORGANOTYPIC CELL SYSTEMS
NEW EXPOSURE METHODS (e.g. ALI to "mimetize" inhalation)
DOSING (real exosure scenario)

Thank you for your attention!



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