Grant agreement number 2009 21 01

WP5

In vitro methods for genotoxicity

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WP5 - Specific objectives

To generate *in vitro* genotoxicity data on nanomaterials

- Production of *in vitro* genotoxicity data on NMs using **standard tests and modified assays** utilizing specific cell models

To perform a round robin test on *in vitro* testing of NMs

- Based on *in vitro* genotoxicity and physical/chemical characterisation data obtained, a ring test on selected MNs will be carried out using **the most promising *in vitro* assays**

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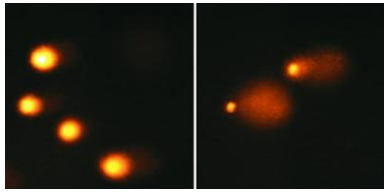
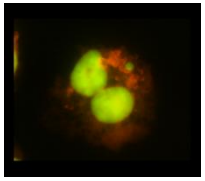
Genotoxicity endpoints chosen



- DNA damage
  - Alkaline comet assay
  - FpG-modified as voluntary assay in a few labs



- Micronuclei
  - Cytokinesis block micronucleus assay
  - Micronucleus assay without Cyt-B (16 HBE)
- Mutations
  - Mouse lymphoma assay (in one lab) – mutation assay

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Cell systems chosen

- Human pulmonary cells
  - Bronchial epithelial cells: BEAS 2B, 16 HBE
  - Alveolar cells: A549
- Human intestinal cells
  - Caco-2
- Human dermal cells
  - Keratinocytes: NHEK (+ HaKaT)
  - Reconstructed full thickness skin models (TiO_2 and ZnO only)
- Lymphatic cells
  - Human primary lymphocytes (MN only)
  - Mouse lymphoma L5178Y TK +/- cells (mutations)

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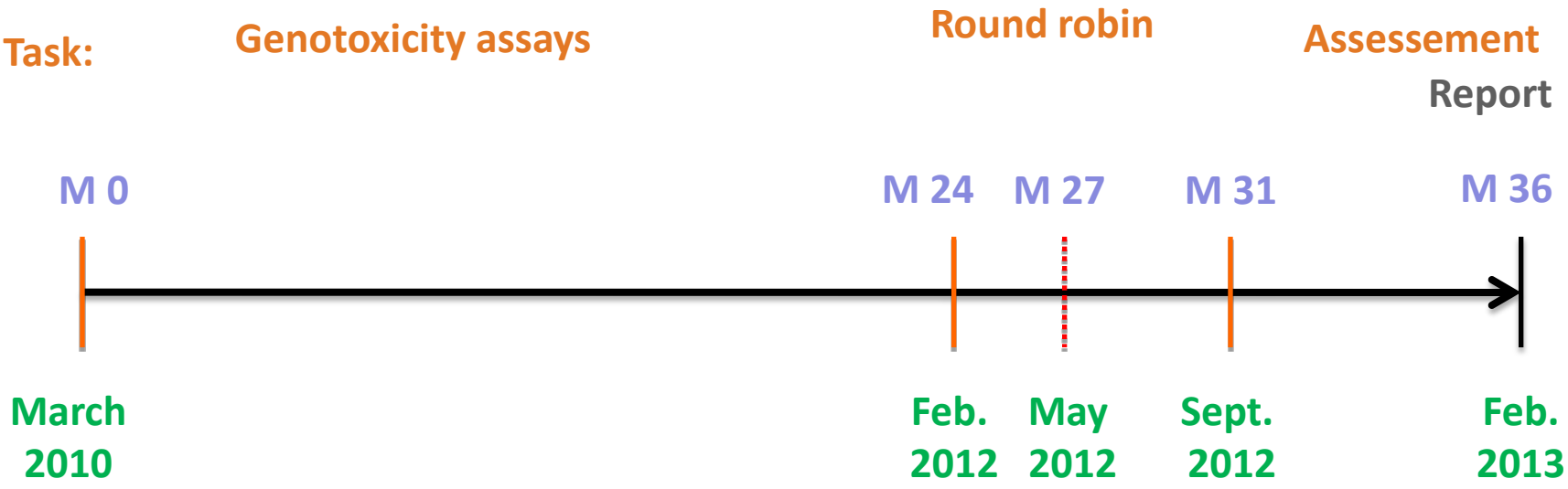
schedule and nanomaterials



- All TiO_2, SAS, MWCNTs of the project
- Zinc oxide (ZnO) NM-110 as potential nanoparticle positive control

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Harmonisation of protocols

- General test principles agreed upon for both endpoints based on OECD- TG 487

- Protocols for each cell line and endpoint

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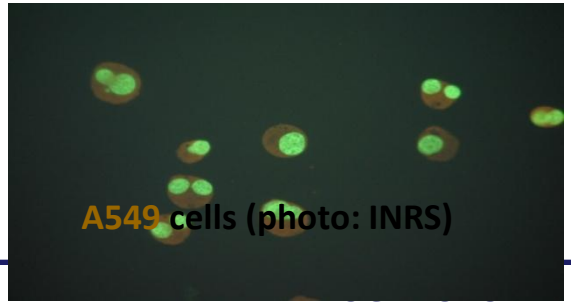
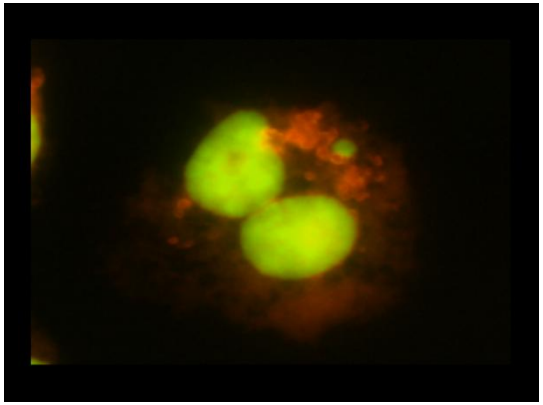
Micronucleus assay *in vitro*

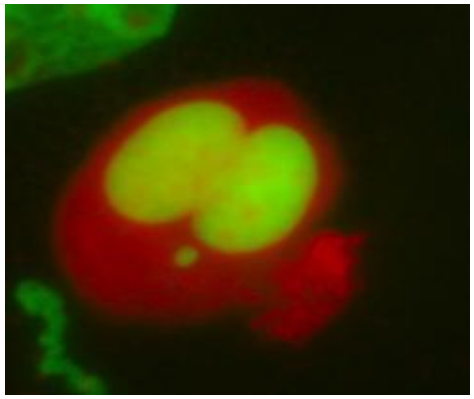
- Treatment time 1.5-2.0 x cell cycle (Caco-2: 24 h)
- Cyt-B added after 6 h of treatment (Caco-2: after 24 h of treatment; 16 HBE: no Cyt-B)
- Duplicate cultures minimum, ≥2 slides
- 2000 cells/dose



A549 cells (photo: INRS)



BEAS 2B cell (photo: FIOH)



Caco-2 cell (photo: Anses)

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Comet assay *in vitro*

- Treatment time 3 h and 24 h
- Duplicate cultures minimum, ≥2 slides
- %DNA in tail the main parameter
- 200 cells minimum per dose

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in vitro mutation assay

- Mouse lymphoma L5178YTK+/- cells
- Performed by only one lab

Usual protocol from this lab with nangenotox dispersion

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Criteria used for outcome

POSITIVE	WEAK POSITIVE	NEGATIVE	NO DATA
+	(+)	–	ND
▪Significant dose-dependent increase, ≥2 significant doses ▪Dose-dependent increase and statistically significant at high dose	▪No significant dose-dependent increase, 1 significant dose		

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TiO_2 - micronucleus assay *in vitro*

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Organ of origin	Lung			Intestine	Blood	Skin	
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Lymphocytes	Keratino-cytes	Recon-structed skin model
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2	Primary	NHEK	
TiO_2							
NM-102	–	–	–	–	(+)	+	ND
NM-103	–	–	–	–	+	+	ND
NM-104	–	–	–	–	+	+	ND
NM-105	–	–	–	–	–	+	ND

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TiO_2 - comet assay *in vitro* (3 h)

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Organ of origin	Lung			Intestine	Skin
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Keratinocytes
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2	NHEK
TiO_2					
NM-102	+	–	+	–	(+)
NM-103	–	–	–	–	(+)
NM-104	–	–	–	–	(+)
NM-105	–	–	+	–	(+)

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TiO_2 - comet assay *in vitro* (24 h)

Organ of origin	Lung			Intestine	Skin
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Keratinocytes
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2	NHEK
TiO_2					
NM-102	+	–	–	+	(+)
NM-103	–	–	–	(+)	(+)
NM-104	–	–	–	–	(+)
NM-105	–	–	–	+/+	(+)

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TiO_2 - comet assay *in vitro*, 3D skin model

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NM	Reconstructed skin model
TiO_2	
NM-102	–
NM-103	–
NM-104	–
NM-105	–

No penetration of TiO_2 through the stratum corneum of reconstructed human full thickness skin models **even after a 72-h exposure by TEM**.

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TiO_2 - *in vitro* mutation assay

Mouse lymphoma L5178YTK+/- cells

NM	L5178Y$^{TK+/-}$
TiO_2	
NM-102	–
NM-103	–
NM-104	–
NM-105	–

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TiO_2 - Conclusions

- Micronucleus assay :
  - positive for each TiO_2 in NHEK; some positive in primary lymphocytes
  - negative for all TiO_2: in other type of cells
- Comet assay :
  - mostly positive in intestinal Caco-2 cells (24 h, not 3 h)
  - often positive for NM-102 (pure anatase) than other forms of TiO_2
  - negative for in reconstructed 3D skin model for TiO_2
- Mutation assay
  - negative for all

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SAS - micronucleus assay *in vitro*

Organ of origin	Lung			Intestine	Blood
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Lymphocytes
Cell line or type	BEAS 2B[a]	16 HBE[b]	A549[c]	Caco-2[d]	Primary[e]
SAS					
NM-200	–	–	–/–	+/–	–
NM-201	–	–	+/+	+/–	–
NM-202	–	–	+/+	+/–	–
NM-203	(+)	–	–/(+)	+/–	–

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SAS - comet assay *in vitro* (3 h)

Organ of origin	Lung			Intestine
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2
SAS				
NM-200	+	+	(+)	+
NM-201	(+)	–	+	–
NM-202	+	–	+	(+)
NM-203	+	–	–	+

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SAS - comet assay *in vitro* (24 h)

Organ of origin	Lung			Intestine
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2
SAS				
NM-200	–	–	–	+
NM-201	–	–	(+)	(+)
NM-202	–	–	(+)	(+)
NM-203	(+)	–	+	+

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SAS - comet assay FpG *in vitro* (3 h)

Organ of origin	Lung			Intestinal
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2
SAS				
NM-200	**(+)**	**–**	**–**	**(+)**
NM-201	**–**	**–**	**–**	**–**
NM-202	**+**	**–**	**+**	**+**
NM-203	**+**	**–**	**–**	**+**

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SAS - comet assay FpG *in vitro* (24 h)

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Organ of origin	Lung		Intestine
Tissue	**Bronchial epithelial**	**Alveolar epithelial**	**Colon epithelial**
Cell line or type	16 HBE	A549	Caco-2
SAS			
NM-200	–	–	+
NM-201	–	(+)	+
NM-202	–	–	–
NM-203	–	+	(+)

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SAS - *in vitro* mutation assay

Mouse lymphoma L5178YTK+/- cells

NM	L5178Y$^{TK+/-}$
SAS	
NM-200	–
NM-201	–
NM-202	–
NM-203	–

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SAS - Conclusions

- Micronucleus assay :
  - Initial positive results for each SAS in intestinal Caco-2 cells could not be ascertained in a new experiment
  - positive data for some SAS with alveolar A549 cells
  - mostly negative in other cells

- Comet assay :
  - mostly positive in bronchial BEAS 2B cells in 3-h exposure
  - Positive for NM-200 in all cell lines with 3-h exposure

- Mutation assay
  - negative for all

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MWCNTs - micronucleus assay *in vitro*

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Organ of origin	Lung			Intestine	Blood
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Lymphocytes
Cell line or type	BEAS 2B[a]	16 HBE[b]	A549[c]	Caco-2[d]	Primary[e]
MWCNT					
NM-400	(+)	–	(+)	(+)	–
NM-401	+	–	–	+	–
NM-402	+	–	+	+	(+)
NM-403	+	–	+	(+)	+
NRCWE-006	+	–	+	–	+
NRCWE-007	+	–	+	+	–

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MWCNTs – comet assay *in vitro* (3 h)

Organ of origin	Lung			Intestine
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2
MWCNT				
NM-400	–	–	–	–
NM-401	–	–	–	–
NM-402	–	–	–	–
NM-403	–	–	–	–
NRCWE-006	–	–	–	–
NRCWE-007	–	–	–	–

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MWCNTs – comet assay *in vitro* (24 h)

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Organ of origin	Lung			Intestine
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2
MWCNT				
NM-400	–	–	–	–
NM-401	–	–	–	–
NM-402	–	–	–	–
NM-403	–	–	–	–
NRCWE-006	–	–	–	–
NRCWE-007	–	–	–	–

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MWCNTs - comet assay FpG *in vitro* (3 h)

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Organ of origin	Lung	Intestinal
Tissue	Bronchial epithelial	Colon epithelial
Cell line or type	BEAS 2B	Caco-2
MWCNT		
NM-400	–	–
NM-401	–	–
NM-402	–	–
NM-403	–	–
NRCWE-006	–	–
NRCWE-007	–	–

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MWCNTs - comet assay FpG *in vitro* (24 h)

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Organ of origin	Lung	Intestinal
Tissue	Bronchial epithelial	Colon epithelial
Cell line or type	BEAS 2B	Caco-2
MWCNT		
NM-400	–	–
NM-401	–	–
NM-402	–	–
NM-403	–	–
NRCWE-006	–	–
NRCWE-007	–	–

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MWCNTs - *in vitro* mutation assay

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Mouse lymphoma L5178YTK+/- cells

NM	L5178Y$^{TK+/-}$
MWCNT	
NM-400	–
NM-401	–
NM-402	–
NM-403	–
NRCWE-006	–
NRCWE-007	–

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MWCNTs - Conclusions

- Micronucleus assay :
  - mostly positive in bronchial BEAS 2B, alveolar A549 and intestinal Caco-2 cells
  - negative in bronchial 16HBE cells
- Comet assay :
  - negative for all
- Mutation assay
  - negative for all

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Further conclusions

- Bronchial epithelial 16HBE cells (**Cyt-B not used**) gave only negative results in the micronucleus assay
- The mouse lymphoma mutation assay was negative for all NMs
- Zn0 was not a suitable nanoparticulate positive control : cell lines showed great differences in sensitivity to ZnO

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Round robin

- 3 NMs
  - TiO_2 NM-102
  - SiO_2 NM-203
  - MWCNTs NM-403
- 2 cell lines
  - BEAS 2B
  - Caco-2
- 2 genotoxicity assays
  - Comet assay
  - Cytokinesis block micronucleus assay

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Round robin results, TiO_2

Cell line Partner No.	TiO_2 NM-102	
	Comet assay	Micronucleus assay
Caco-2 cells	+	–
1	–	–
5	+	
6	–	+
8	–	–
9		
13	+	
BEAS 2B cells	+	–
3	+	+
4	–	–
7	+	(+)
10	+	–
11	+	–
15	+	–

1st phase results

Round-robin results

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Round robin results, SAS

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Cell line Partner No.	SAS NM-203 Comet assay	 Micronucleus assay
Caco-2 cells	**+**	**+/–**
1	–	+
5	+	+
6	–	–
8	+	–
9		–
13	–	+
BEAS 2B cells	**(+)**	**(+)**
3	–	+
4	+	–
7	+	+
10	+	–
11	–	+
15	–	–

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Round robin results, MWCNTs

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Cell line Partner No.	MWCNTs NM-403	
	Comet assay	Micronucleus assay
Caco-2 cells	–	(+)
1	–	+
5	+	+
6	–	+
8	–	–
9		–
13	–	(+)
BEAS 2B cells	–	+
3	+	+
4	–	–
7	+	–
10	–	–
11	–	–
15	+	–

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Round robin results, ZnO

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Cell line Partner No.	ZnO **NM-110**	
	Comet assay	Micronucleus assay
Caco-2 cells		
1	–	+
5	+	+
6	–	+
8	–	+
9		+
13	+	+
BEAS 2B cells		
3	+	+
4	+	–
7	+	+
10	–	+
11	–	–
15	+	–

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WP5 Deliverable

publically available soon

***In vitro* genotoxicity testing strategy for nanomaterials including database**

- Part 1: summary of data by NM, cell system, and endpoint
- Round robin: summary of data by NM, cell system, and endpoint
- *In vitro* x *in vivo* association
- Association with physico-chemical characteristics
- Testing strategy

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General conclusions

- Success in preparing dispersion is expected to affect agglomerate size → sedimentation → cell exposure → cytotoxicity → choice of doses → genotoxicity
- Cell lines that take up MNs can be used for their genotoxicity testing
- BEAS 2B cells appear to perform somewhat better than Caco-2 cells
- Full thickness 3D skin models are not recommended for MNs hazard assessment of genotoxicity
- Many MNs show slight genotoxic activity *in vitro*, possibly due to indirect mechanisms not yet fully understood
- As the effect is weak, it is not easily reproducible
- Possible low-dose effects

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Suggestions for the future research

- Cellular uptake could possibly serve as a measure of dose for comparison among experiments and test systems → techniques should be developed to allow this
- Further understanding of low-dose effects
- Defining dose range to be tested – measures besides cytotoxicity:
  - Stop at doses showing no more uptake?
  - Stop at doses where cytotoxicity levels (due to saturation of MN uptake or increase of agglomerate size so that uptake is compromised)?
  - MNs of low toxicity: how to distinguish real cytotoxicity from secondary toxicity related to, eg, MN compromising culture conditions?
- Does the dispersent used (eg BSA) influence MN genotoxicity?
- Partly soluble MN:
  - Importance of Trojan horse effect
  - Influence of medium and other culture conditions on test outcome with partly soluble MNs: ratio of dispersion in medium vs dispersion inside the cell
- Genotoxic mechanisms of MNs *in vitro* and *in vivo* - to better understand test outcome

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WP5 participants

FIOH	Finland	WP5 leader
Anses	France	
WIS-ISP (IPH)	Belgium	
IMB-BAS	Bulgaria	
BfR	Germany	
NRCWE	Denmark	
IPL	France	
UAB	Spain	
INRS	France	
RIVM	Netherlands	
NIOM	Poland	
INSA	Portugal	

- 12 participants from 10 countries

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NANOGENOTOX

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WP5 comments of external experts

Laetitia Gonzalez, Micheline Kirsch-Volders

Vrije Universiteit Brussel

David Kirkland

Kirkland Consulting

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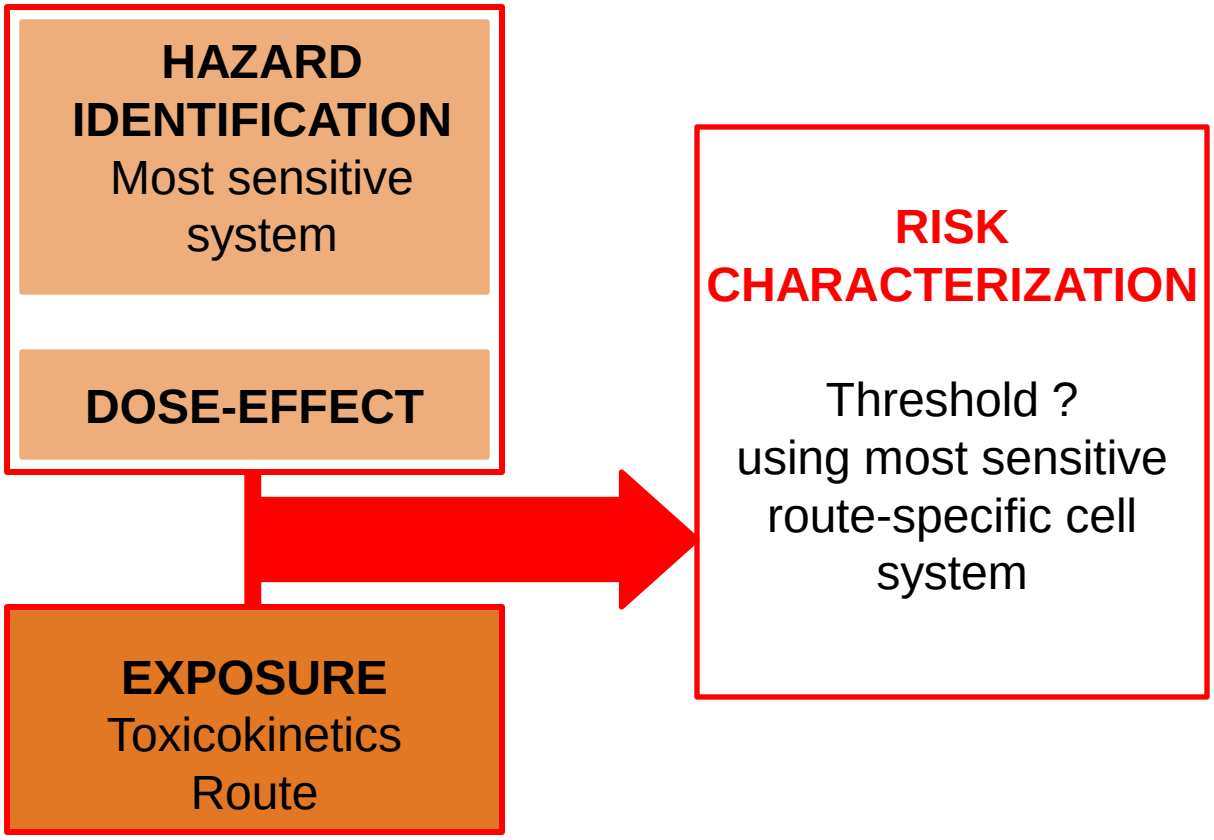
Hazard versus risk assessment



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Strengths

- NM choice and characterization
- Use of comet assay (DNA strand breaks and alkali-labile sites)
- Use of OECD validated MN assay (chr breakage and loss)
- Cell types representing different tissues

Weaknesses

- Interaction between NM and assay?
- Acceptability criteria and historical controls
- Control data
- Experience with cell types in different labs/ Training

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Recommendations for future research

- Define a NM-adapted protocol for the OECD guidelines
- Perform large interlaboratory exercise for reproducibility of genotoxic effects in cell types/lines used (genetic background) → recommendation of cell types/lines for hazard and/or risk assessment
- Develop new test methods to assess NM-specific modes of action

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