UNIVERSITY OF COPENHAGEN

Genotoxicity of multi-walled carbon nanotube reference materials in mammalian cells and animals

Peter Møller[1], Regitze Sølling Wils[1,2], Emilio Di Ianni[2], Claudia Andrea Torero Gutierrez[1,2], Martin Roursgaard[1] and Nicklas Raun Jacobsen[2]

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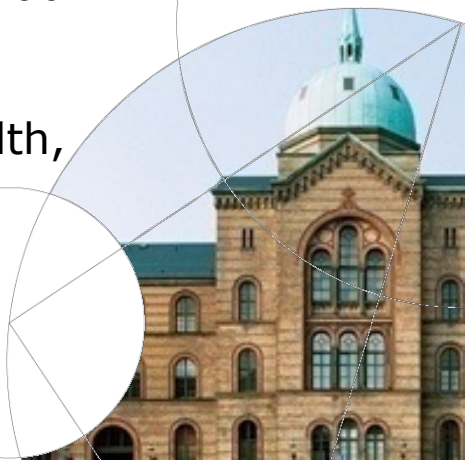
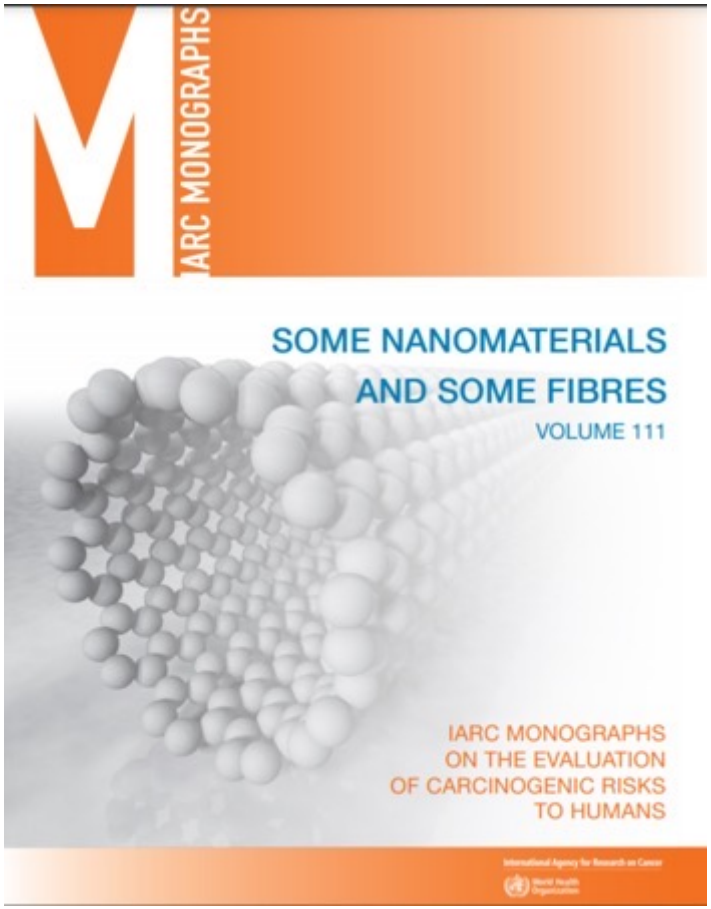


Lyon, 30 September – 7 October 2014



MWCNT-7: Group 2B
(possibly carcinogenic)
"Long, thick, straight"

Other MWCNTs: Group 3
(not classifiable)

SWCNTs: Group 3

Table 6. Overall summary of studies of lung and pleural responses to specific types of carbon nanotube or nanofiber

Type and source of carbon nanotube or nanofiber	Dimension (mid-point)		Lung Effects (rat, mouse)				
	Diameter (nm)	Length (μm)	Biopersistent, interstitial retention	Bronchiol-alveolar hyperplasia	Genotoxicity	Fibrosis	Persistent inflammation
Multi-walled Carbon Nanotube (MWCNT)							
Baytubes	10	0.2	Pauluhn 2010			Pauluhn 2010	Pauluhn 2010
MWCNT-7 (Mitsui)	50	4	Mercer 2011, 2013; Xu 2012	Porter 2013; Sargent 2014		Porter 2010, 2012; Kasai 2015	Porter 2010, 2013; Aiso 2010; Mercer 2011, 2013; Kasai 2015
MWCNT-N (Nikkiso)	nr	3.02	Xu 2012				
Nikkiso	63	1.1	Oyabu 2011				Morimoto 2012
HELIX	20	20	Ryman-Rasmussen 2009b				
Nanocyl	10	5	Muller 2005	Ma-Hock 2009	Muller 2008 (G)	Neumann 2013	Ma-Hock 2009; Muller 2008 (G)
Arkema	13	2.5			Cao 2014		
CM-95/100	4	0.5		Yu 2013	Kim 2014		Kim 2014
Mitsui	40-50	13		Mühlfeld 2012		Mühlfeld 2012	
Nanolab	15	1-5		Mühlfeld 2012		Mühlfeld 2012	
Single-walled Carbon Nanotube (SWCNT)							
Hi							
supergrowth							

Green: positive association, Red: no association

Kuempel et al., Crit Rev Toxicol 47:1-58, 2017

Advisory Group recommendations on priorities for the IARC Monographs

An Advisory Group of 29 scientists from 18 countries met in March, 2019, to recommend priorities for the Group meeting convened in 2014.[3] The expertise of the Advisory Group covered multiple disciplines, helped to reveal coverage and gaps in the extent of evidence across data streams supporting decisions

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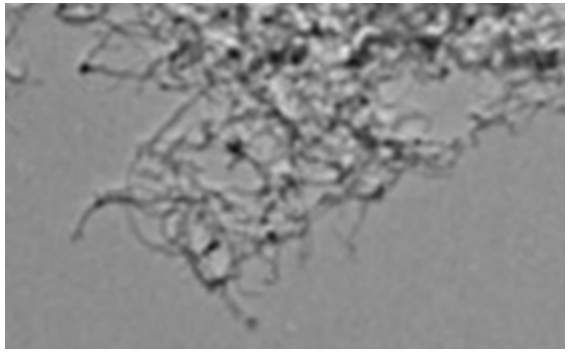
Agents previously evaluated by IARC Monographs†	
Automotive gasoline (leaded and unleaded), carbaryl, malaria	New human cancer, bioassay, and mechanistic evidence to warrant re-evaluation of the classification
Acrylamide, acrylonitrile, some anthracyclines, coal dust, combustion of biomass,* domestic talc products, firefighting exposure, metallic nickel, some pyrethroids (ie, permethrin, cypermethrin, deltamethrin)	*New* human cancer and mechanistic evidence to warrant re-evaluation of the classification
Aniline, acrolein, methyl eugenol and isoeugenol*, multi-walled carbon nanotubes*, non-ionising radiation (radiofrequency)*, some perfluorinated compounds (eg, perfluorooctanoic acid)	New bioassay and mechanistic evidence to warrant re-evaluation of the classification
Ostrogen:oestradiol and oestrogen-progestogens‡, hydrochlorothiazide, Merkel cell polyomavirus, perchloroethylene, very hot foods and beverages	New human cancer evidence to warrant re-evaluation of the classification
1,1,1-trichloroethane, weapons-grade alloy (tungsten, nickel, and cobalt)	New bioassay evidence to warrant re-evaluation of the classification
Acetaldehyde, bisphenol A*, cobalt and cobalt compounds, crotonaldehyde, cyclopeptide cyanotoxins, fumonisin B_1, inorganic lead compounds, isoprene, o-anisidine	New mechanistic evidence to warrant re-evaluation of the classification

Evidence of human exposure was identified for all agents. *Advised to conduct in latter half of 5-year period. †See current International Agency for Research on Cancer (IARC) list of classifications, volumes 1–123. ‡Group 1 carcinogen; new evidence of cancer in humans indicates possible causal associations for additional tumour sites (see Section 3 of Preamble to the IARC Monographs[1]).

Table 1: **Agents recommended for evaluation by the IARC Monographs with high priority**

L-MWNT-1020: *"short, thin and curled"*

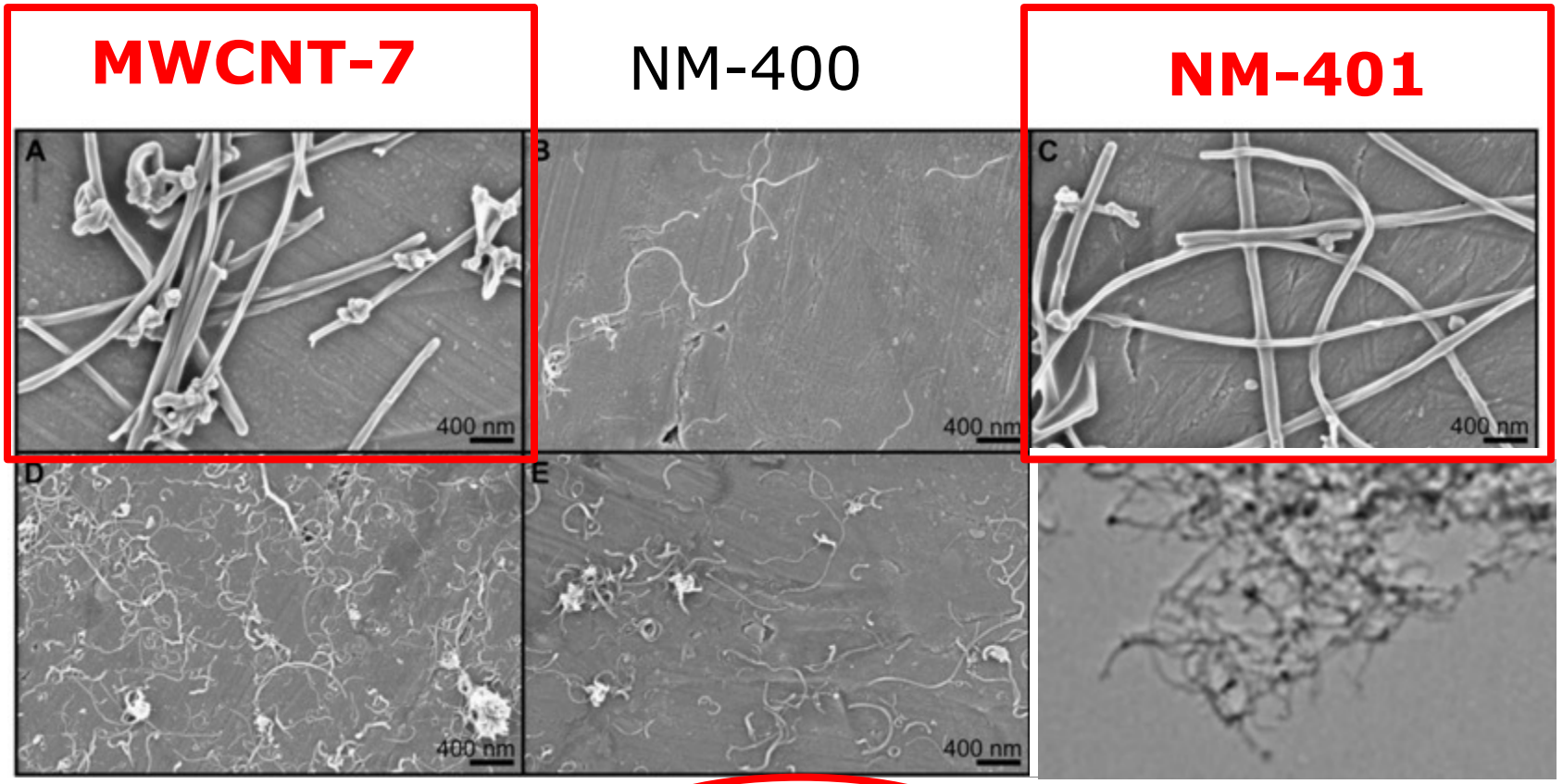


(From NTP Tox 94, 2019; modified)

Inhalation

- 2 weeks
- 13 weeks
- 2 years

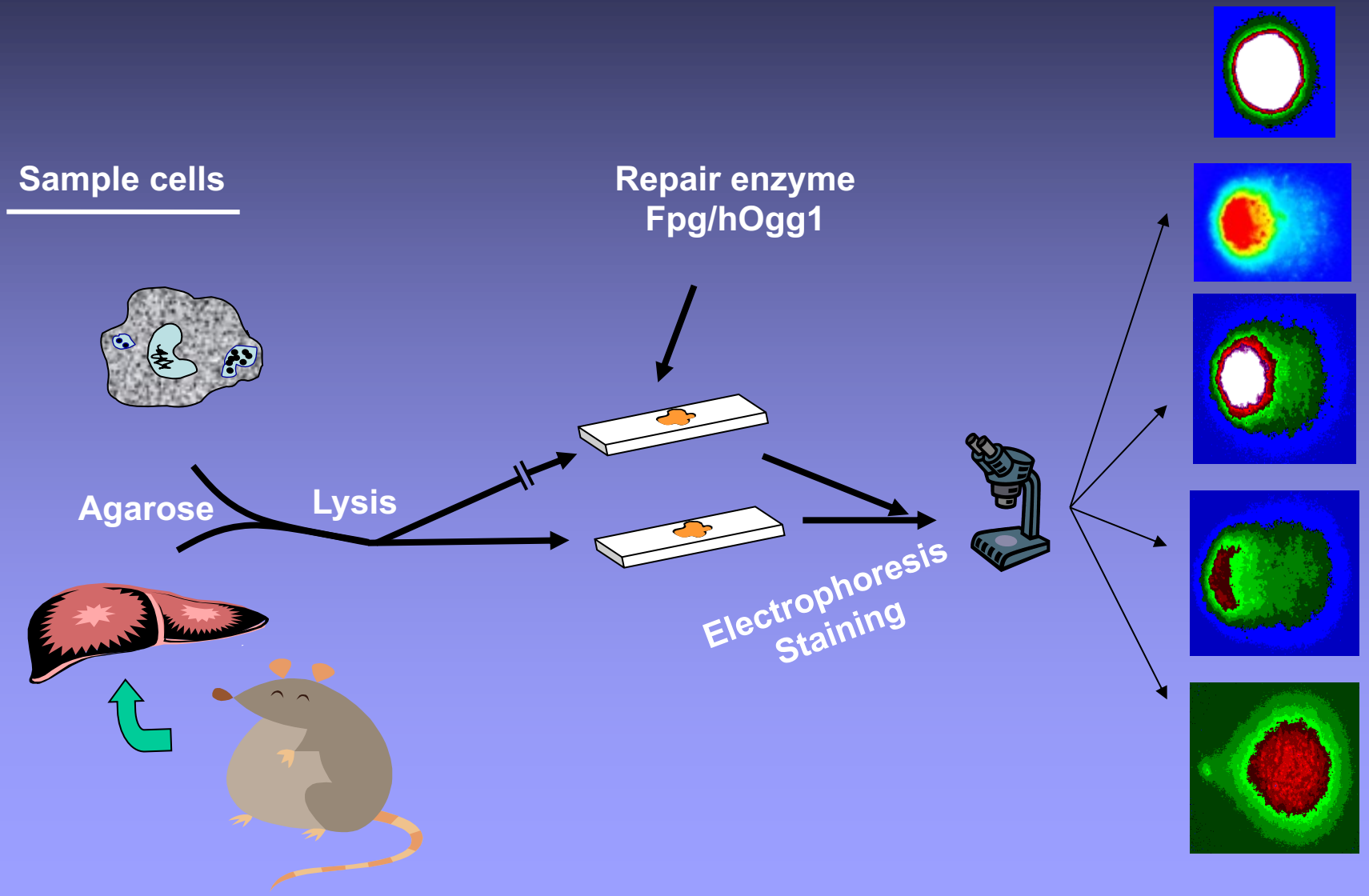
Short, thin and curled *vs.* **long, thick and straight**



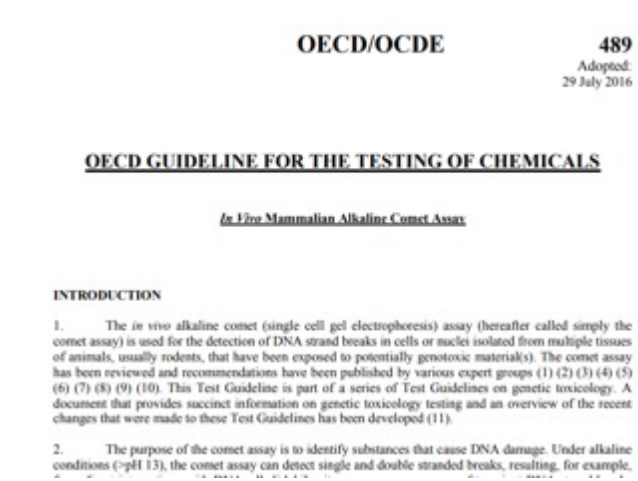
**Note*: different resolution for L-MWNT-1020

(From NTP Tox 94, 2019; modified)

Measurement of DNA damage (comet assay)



OECD Guideline test (animal tissues) - protocols



OECD/OCDE

489

Adopted:
29 July 2016

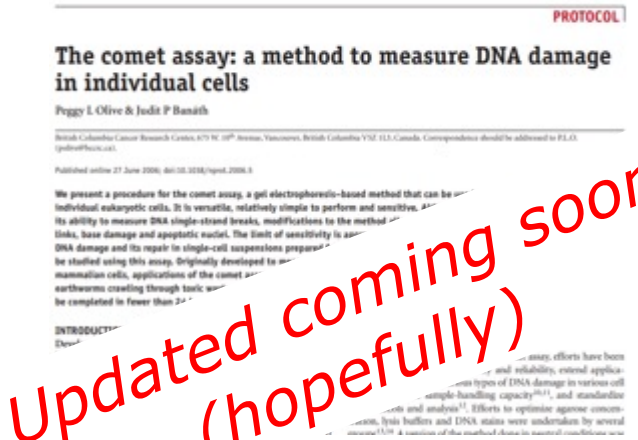
OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vivo Mammalian Alkaline Comet Assay

INTRODUCTION

1. The *in vivo* alkaline comet (single cell gel electrophoresis) assay (hereafter called simply the comet assay) is used for the detection of DNA strand breaks in cells or nuclei isolated from multiple tissues of animals, usually rodents, that have been exposed to potentially genotoxic material(s). The comet assay has been reviewed and recommendations have been published by various expert groups (1) (2) (3) (4) (5) (6) (7) (8) (9) (10). This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (11).

2. The purpose of the comet assay is to identify substances that cause DNA damage. Under alkaline conditions (>pH 13), the comet assay can detect single and double stranded breaks, resulting, for example,

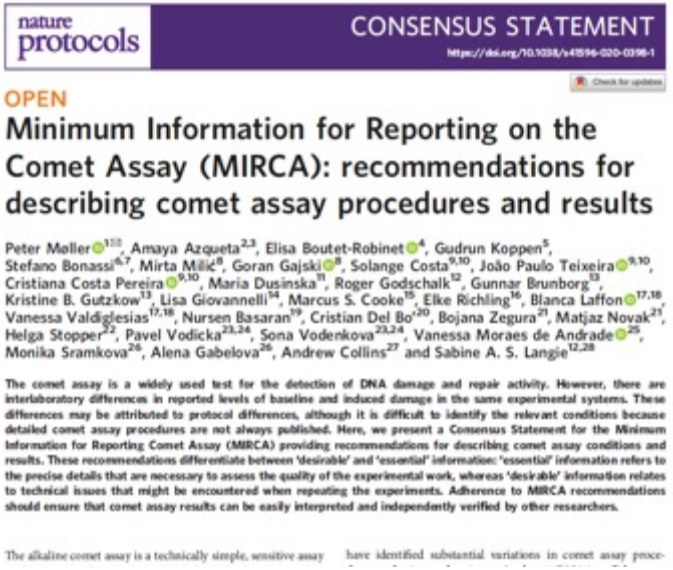


PROTOCOL

The comet assay: a method to measure DNA damage in individual cells

Peggy L Olive & Judit P Banáth

Updated coming soon (hopefully)



nature protocols

CONSENSUS STATEMENT

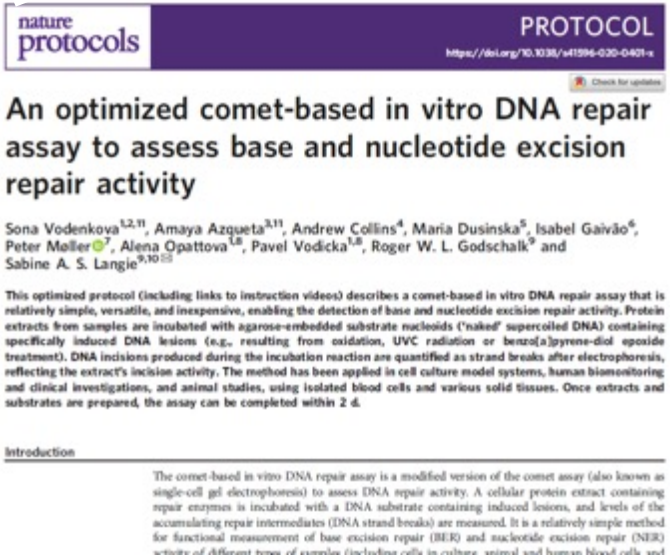
OPEN

Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results

Peter Møller, Amaya Azqueta, Elisa Boutet-Robinet, Gudrun Koppen, Stefano Bonassi, Mirta Milić, Goran Gajski, Solange Costa, João Paulo Teixeira, Cristiana Costa Pereira, Maria Dusinska, Roger Godschalk, Gunnar Brunborg, Kristine B. Gutzkow, Lisa Giovannelli, Marcus S. Cooke, Elke Richling, Blanca Laffon, Vanessa Valdiglesias, Nursen Basaran, Cristian Del Bo', Bojana Zegura, Matjaz Novak, Helga Stopper, Pavel Vodicka, Sona Vodenkova, Vanessa Moraes de Andrade, Monika Sramkova, Alena Gabelova, Andrew Collins and Sabine A. S. Langie

The comet assay is a widely used test for the detection of DNA damage and repair activity. However, there are interlaboratory differences in reported levels of baseline and induced damage in the same experimental systems. These differences may be attributed to protocol differences, although it is difficult to identify the relevant conditions because detailed comet assay procedures are not always published. Here, we present a Consensus Statement for the Minimum Information for Reporting Comet Assay (MIRCA) providing recommendations for describing comet assay conditions and results. These recommendations differentiate between 'desirable' and 'essential' information: 'essential' information refers to the precise details that are necessary to assess the quality of the experimental work, whereas 'desirable' information relates to technical issues that might be encountered when repeating the experiments. Adherence to MIRCA recommendations should ensure that comet assay results can be easily interpreted and independently verified by other researchers.

The alkaline comet assay is a technically simple, sensitive assay have identified substantial variations in comet assay proce-



nature protocols

PROTOCOL

An optimized comet-based in vitro DNA repair assay to assess base and nucleotide excision repair activity

Sona Vodenkova, Amaya Azqueta, Andrew Collins, Maria Dusinska, Isabel Gaivão, Peter Møller, Alena Opattova, Pavel Vodicka, Roger W. L. Godschalk and Sabine A. S. Langie

This optimized protocol (including links to instruction videos) describes a comet-based in vitro DNA repair assay that is relatively simple, versatile, and inexpensive, enabling the detection of base and nucleotide excision repair activity. Protein extracts from samples are incubated with agarose-embedded substrate nucleoids ('naked' supercoiled DNA) containing specifically induced DNA lesions (e.g., resulting from oxidation, UVC radiation or benzo[a]pyrene-diol epoxide treatment). DNA incisions produced during the incubation reaction are quantified as strand breaks after electrophoresis, reflecting the extract's incision activity. The method has been applied in cell culture model systems, human biomonitoring and clinical investigations, and animal studies, using isolated blood cells and various solid tissues. Once extracts and substrates are prepared, the assay can be completed within 2 d.

Introduction

The comet-based in vitro DNA repair assay is a modified version of the comet assay (also known as single-cell gel electrophoresis) to assess DNA repair activity. A cellular protein extract containing repair enzymes is incubated with a DNA substrate containing induced lesions, and levels of the accumulating repair intermediates (DNA strand breaks) are measured. It is a relatively simple method for functional measurement of base excision repair (BER) and nucleotide excision repair (NER)

Associations (in this talk) are summarized from these papers:

Wils 2021 ”Inflammatory response, reactive oxygen species production and DNA damage in mice after intrapleural exposure to carbon nanotubes” Toxicol Sci 183: 184-194

Wils 2021 ”Reactive oxygen species production, genotoxicity and telomere length in FE1-Muta™Mouse lung epithelial cells exposed to carbon nanotubes” Nanotoxicology 15:661-672

Di Ianni 2021 ”In vitro-in vivo correlations of pulmonary inflammogenicity and genotoxicity of MWCNT” Part Fibre Toxicol 18:25

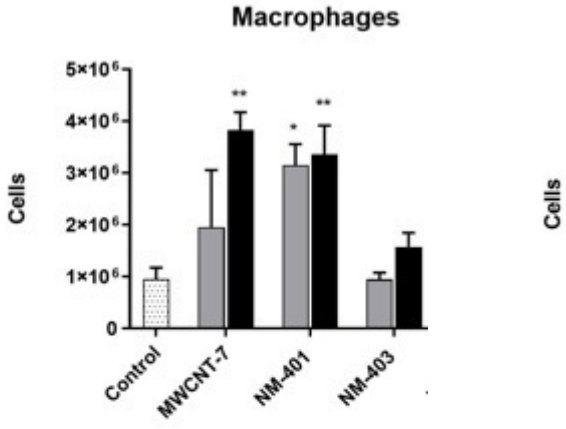
Møller 2021 ”Genotoxicity of multi-walled carbon nanotube reference materials in mammalian cells and animals” Mutat Res Rev 788:109393

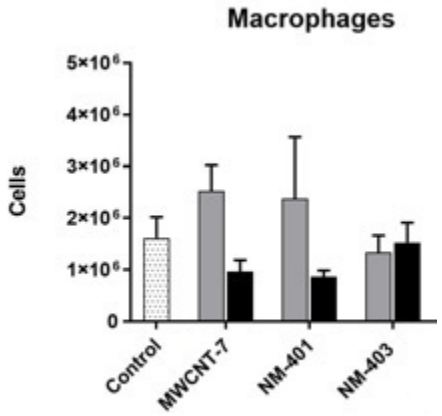
In this talk: genotoxicity = comet assay

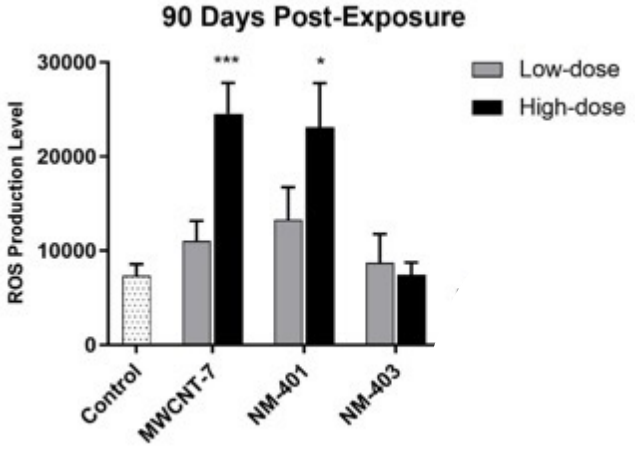
Inflammation and ROS production in pleura lavage cells after exposure to intra-pleural injection in mice

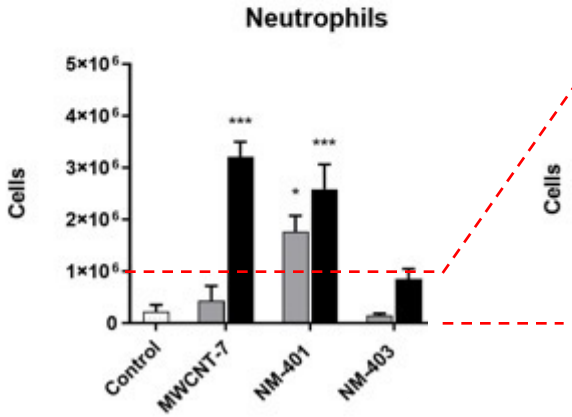
1-day post-exposure

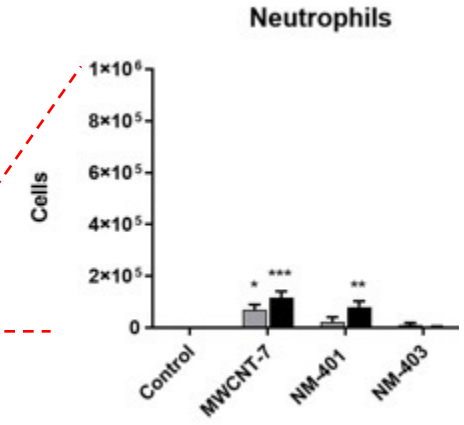
90-days post-exposure

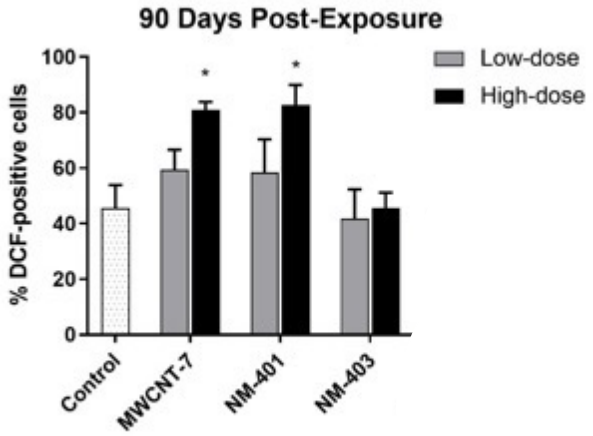






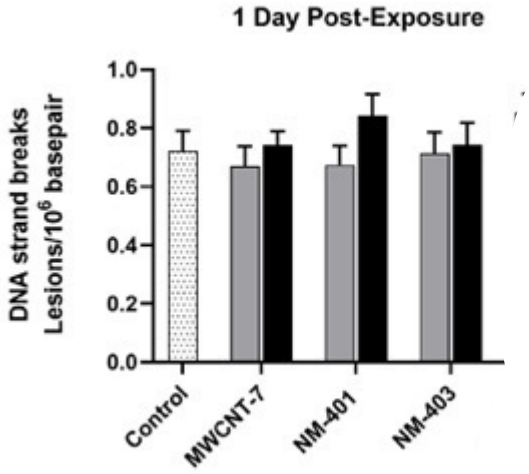


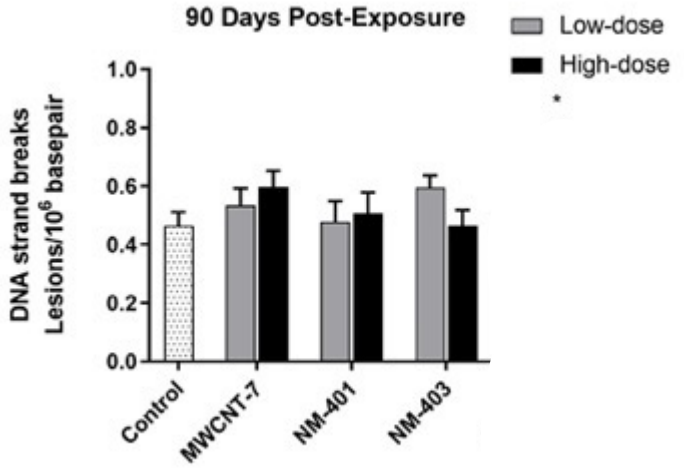


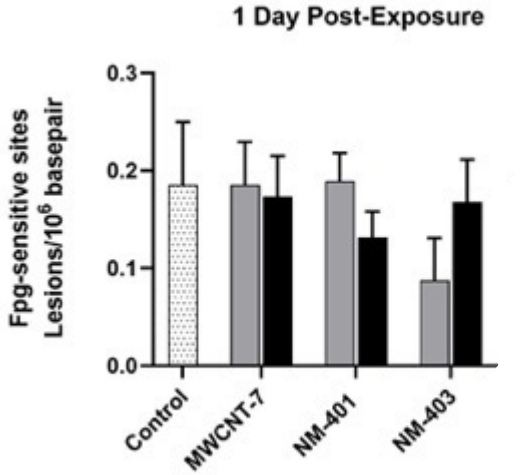


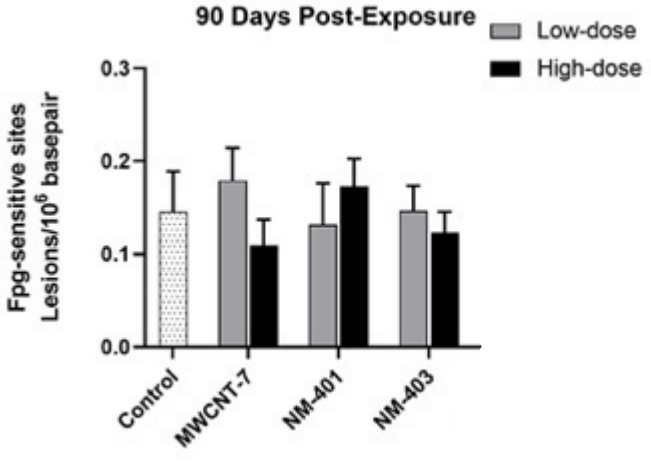
Low dose = 0.2 µg; high dose = 5 µg

DNA damage in mesothelial cells after exposure to intra-pleural injection in mice





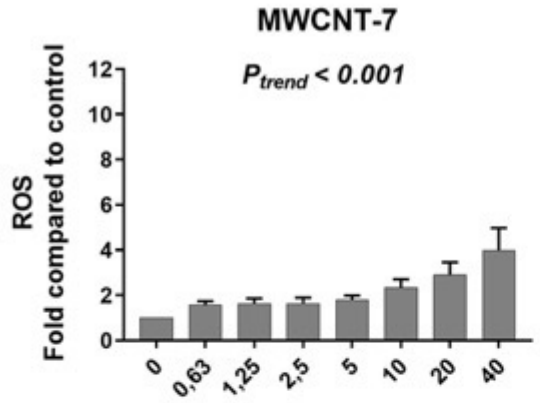


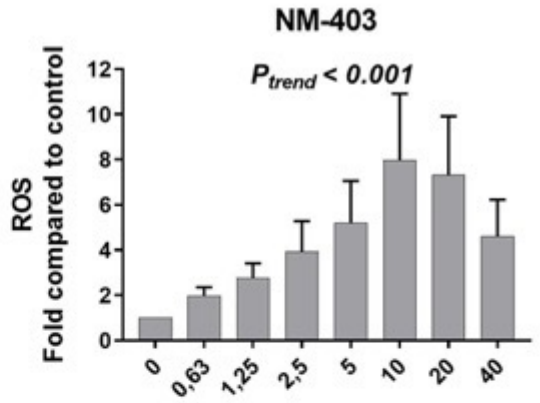


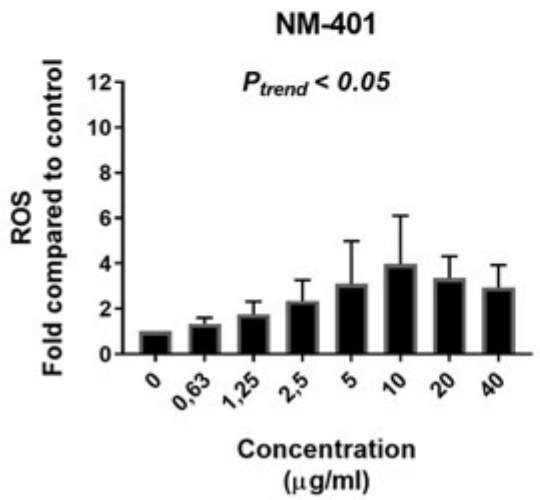
Low dose = 0.2 µg; high dose = 5 µg

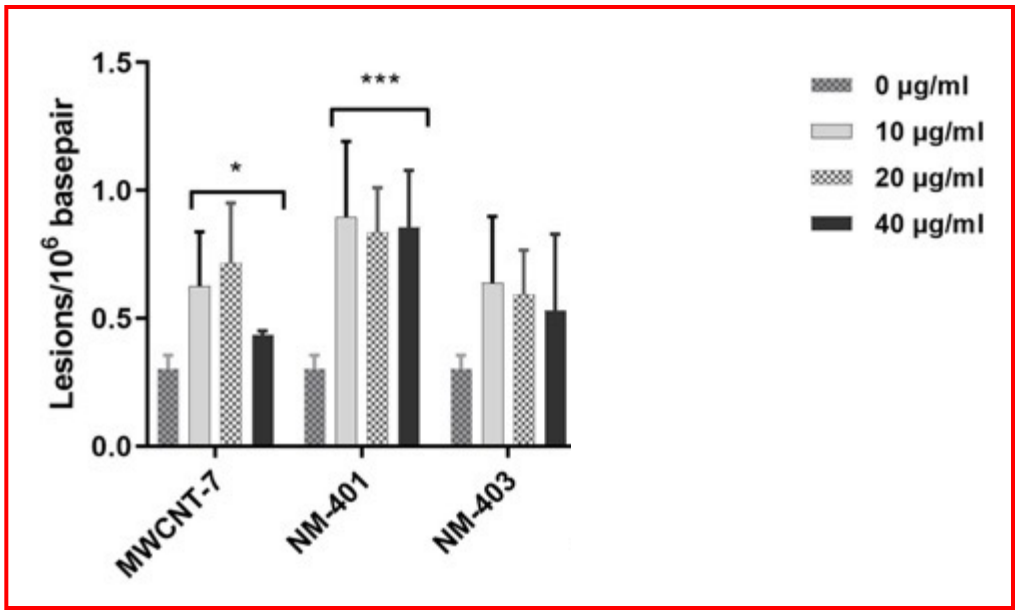
Fpg-sensitive sites = oxidatively damaged DNA

MML lung epithelial cells: intra-cellular ROS production and DNA strand breaks (comet assay): 3 h exposure



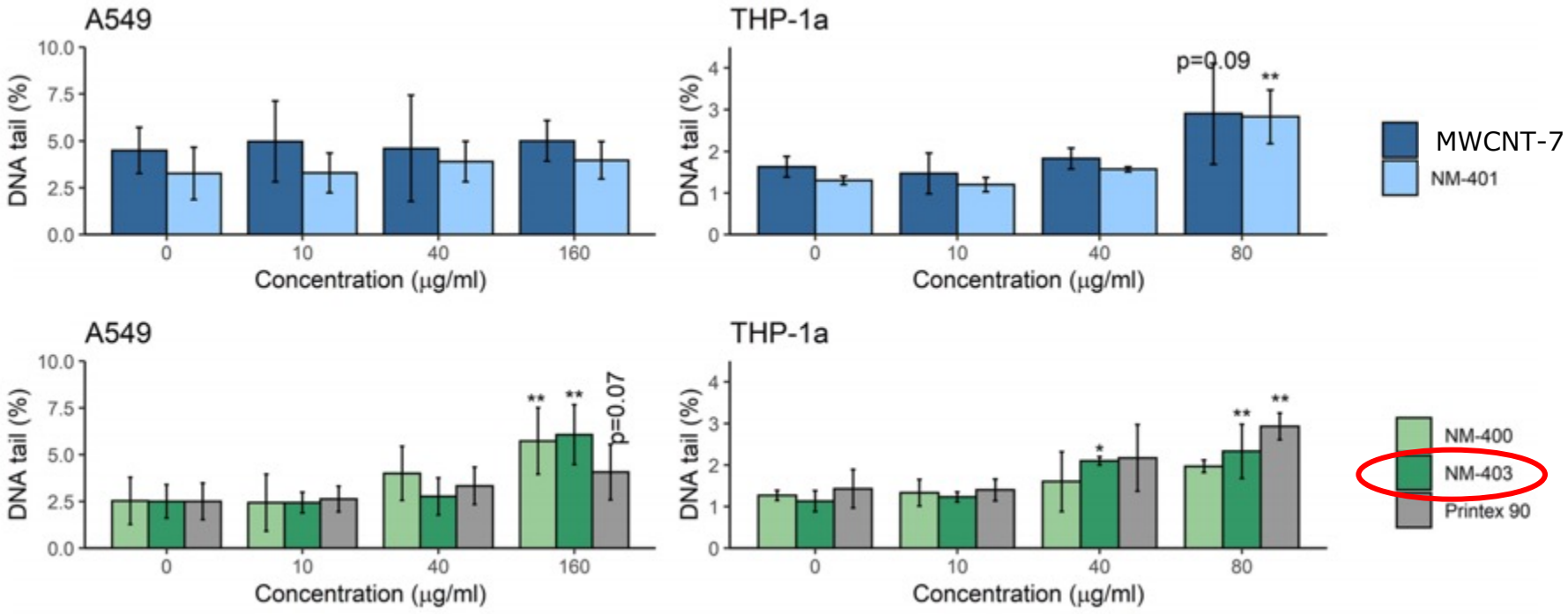






Wils et al., Nanotoxicology 2021

A549 or activated THP-1a cells: DNA strand breaks (comet assay): 24 h exposure



Di Ianni et al., Part Fibre Toxicol 18:25, 2021

Mutation Research-Reviews in Mutation Research 788 (2021) 108393

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Review

Genotoxicity of multi-walled carbon nanotube reference materials in mammalian cells and animals

Peter Møller [a,*], Regitze Sølling Wils [a,b], Emilio Di Ianni [b], Claudia Andrea Torero Gutierrez [a,b], Martin Roursgaard [a], Nicklas Raun Jacobsen [b]

[a] Department of Public Health, Section of Environmental Health, University of Copenhagen, Øster Farimagsgade 5A, DK-1014, Copenhagen K, Denmark
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ARTICLE INFO

Keywords:
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Comet assay
DNA damage
Micronucleus assay
Mutations

ABSTRACT

Carbon nanotubes (CNTs) were the first nanomaterials to be evaluated by the International Agency for Research on Cancer (IARC). The categorization as possibly carcinogenic agent to humans was only applicable to multi-walled carbon nanotubes called MWCNT-7. Other types of CNTs were not classifiable because of missing data and it was not possible to pinpoint unique CNT characteristics that cause cancer. Importantly, the European Commission's Joint Research Centre (JRC) has established a repository of industrially manufactured nanomaterials that encompasses at least four well-characterized MWCNTs called NM-400 to NM-403 (original JRC code). This review summarizes the genotoxic effects of these JRC materials and MWCNT-7. The review consists of 36 publications with results on cell culture experiments (22 publications), animal models (9 publications) or both (5 publications). As compared to the publications in the IARC monograph on CNTs, the current database represents a significant increase as there is only an overlap of 8 publications. However, the results come mainly from cell cultures and/or measurements of DNA strand breaks by the comet assay and the micronucleus assay (82 out

Outcomes (in the database) – NB: more than one outcome per article

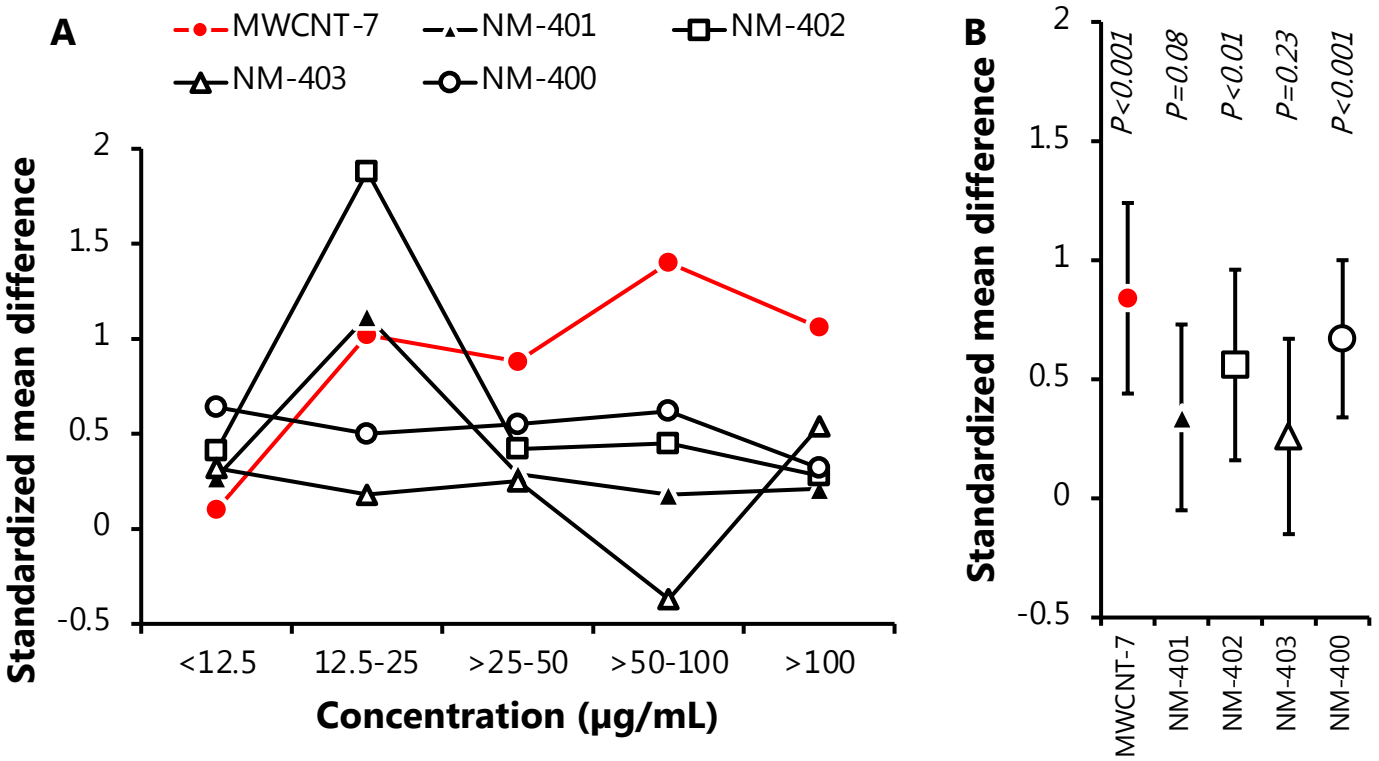
Type	Cells	Animals
MWCNT-7	22	12
NM-401	10	5
NM-402	8	5
NM-403	10	3
NM-400	16	6

Type	Cells	Animals
Strand breaks	30	19
Fpg/8-oxodG	8	3
γH2AX	1	1
Micronuclei	19	4
Mutations	3	4
CA/SCE	5	0

Mainly comet assay

Meta-analysis (concentration-response relationship)

DNA strand breaks (comet assay) – in vitro experiments



Based on concentration-response relationships in 13 publications
Review Manager 5.4 (mean and 95% CI)

Summary and knowledge gaps – research needs

- Among the CNTs, MWCNT-7 is most consistently shown to be genotoxic (based mainly comet assay endpoints)

Knowledge gaps

- Oxidatively damaged DNA (using reliable assays)
- Irreversible genotoxic changes (especially other materials than MWCNT-7).
- Exposure in animal models, including measurements of genotoxicity in lung tissue, pleural mesothelial cells and other extra-pulmonary tissues.
- MWCNT exposure in humans should be investigated.
- Investigate causes of heterogeneity in genotoxic outcomes by the same MWCNTs (ring-trials).