Committee for Risk Assessment

RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at EU level of

Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T

EC Number: -
CAS Number: -

CLH-O-0000007108-75-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

18 March 2022

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

International Chemical Identification:

Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T

EC Number: -

CAS Number: -

Index Number: tba

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Version number: 2.0 **Date: March 2021**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON MULTI-WALLED CARBON TUBES (SYNTHETIC GRAPHITE IN TUBULAR SHAPE) WITH A GEOMETRIC TUBE DIAMETER RANGE ≥ 30 NM TO < 3 μM AND A LENGTH ≥ 5 μM AND ASPECT RATIO ≥ 3:1, INCLUDING MULTI-WALLED CARBON NANOTUBES, MWC(N)T

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SUMMARY AND RATIONALE OF THE CLH PROPOSAL

1. The present CLH proposal comprises Multi-Walled Carbon Tubes (MWCT) and Multi-Walled Carbon Nanotubes (MWCNT) as well as bundles with a tube length ≥ 5 µm and a diameter range ≥ 30 nm to < 3 µm.
2. If differentiation to other MWCNT is necessary for clarification, terms such as "high diameter MWCNT "(> 30 nm)," low diameter MWCNT" (≤ 30 nm), or "rigid WHO fibre-like MWCNT" are used in this proposal. Next to this, throughout the document the geometric diameter is meant, when no other specific diameter is mentioned.
3. The proposal comprises MWCTs that fulfil the EU recommendation for a definition of nanomaterial and that therefore, in line with the EU definition, have one external dimension (the diameter) in the size range 1-100 nm. However, the applicability domain of the CLH proposal is extended to MWCTs with a diameter up to 3 µm, as it is expected according to the fibre pathogenicity paradigm that MWCT with diameters fitting into the respirable range will possess similar fibre-like properties. However, it is not known if MWCT beyond a diameter range of > 200 nm (regarding the constituing particle) are manufactured.
4. Accordingly, all studies which positively tested MWCT with a (mean) diameter > 100 nm were included in the dossier.
5. The diameter is deemed critical as it determines the rigidity of the carbon tubes and thus their fibre-like morphology. At present, there is no exact measurand available for the parameter "rigidity". As soon as a respective analytical tool becomes available, the identifier needs to be adapted accordingly. A cut-off value of 30 nm was set for the lower boundary diameter based on available data related to tumour induction.
6. The proposal is based on the observation that fibres of high diameter MWCT (≥ 30 nm) have equivalent potential with asbestos and asbestiform fibres and thus is consistent with the "fibre pathogenicity paradigm" (Donaldson et al., 2010, 2013). It considers the triad; length, diameter and biopersistence as necessary and sufficient criteria for describing the carcinogenic potential of fibre-like materials. The available data considered for this CLH proposal is in good agreement with this paradigm.
7. Low diameter MWCNT (< 30 nm) are not subject to the proposed classification, as it is assumed that due to a more tangled morphology, the fibre pathogenicity paradigm does not apply (to date negative evidence with regard to fibre-related mesotheliomatogenesis is limited to MWCNT with 15 nm mean diameter).
8. The focus of this proposal is on carcinogenicity. Data on mutagenicity and repeated dose toxicity were also evaluated, both to support classification for carcinogenicity and separate consideration of classification for the other two endpoints. It was concluded that data is sufficient for classification with regard to carcinogenicity and specific target organ toxicity after repeated exposure but not for germ cell mutagenicity.
9. The CLH proposal is consistent with existing harmonized classifications for e.g. asbestos (Carc. 1A, H350; STOT RE1, H372**; none for Muta), refractory ceramic fibres (Carc. 1B, H350i; none for STOT RE or Muta) and mineral wools (Carc. 2, H351; none for STOT RE or Muta):

Carcinogenicity

Classification as Carc. 1B is based on sufficient evidence from animal studies on several different types of MWCT with fibre dimensions (≥ 5 μm in length and a diameter range ≥ 30 nm to < 3 μm), collectively including:

- Significantly increased incidence of lung tumours in rats following long-term inhalation.
- Experimental mesothelioma formation in rats and (mutant) mice following intraperitoneal injection.
- Preneoplastic changes (fibrosis) of lung and pleura tissue and tumour types strikingly similar to those after asbestos exposure.
- Pleura drift and retention following inhalation exposure.
- Promotion of lung tumour development after short term inhalation exposure of initiated mice.
- Mesothelioma formation after intratracheal instillation.
- Pathogenicity progress after inhalation and intratracheal instillation similar to asbestos.

The classification for carcinogenicity is restricted to the inhalation route as according to the present knowledge the inhalation route is the only relevant route. It is highly improbable that exposure by the dermal or even oral route would lead to a carcinogenic response taking into account that long-term deposition of MWCT in the tissues, as can occur in lung, is a prerequisite for carcinogenicity. According to present knowledge, there is no evidence that other carcinogenic fibres meeting the WHO definition have carcinogenic properties after oral or dermal exposure.

Specific Target Organ Toxicity – Repeated Exposure (STOT RE 1)

- The classification proposal is based on the available data and a weight of evidence approach for rigid WHO fibre-like MWCT.
- A key 90d-inhalation study derived a LOAEC of 0.0002 mg/L for MWNT-7, a prototype MWCNT used in a number of repeat-dose and carcinogenicity studies.
- Chronic retention of inhaled fibre-like MWCNT in the gas-exchanging region of the lung due to disturbed clearance by alveolar macrophages (“frustrated phagocytosis”).
- Irreversible, severe lung damage following subacute or subchronic inhalation including persistent inflammation, granulomatosis, as well as epithelial hyperplasia and bronchiolo-alveolar multifocal/diffuse fibrosis, similar to asbestosis.

Germ Cell Mutagenicity: No classification

- Mutagenicity data was evaluated to support carcinogenicity classification.
- The available genotoxicity database is inconsistent and insufficient for classification. This could be due to heterogeneity in test material properties, absence of standardisation in sample preparations, as well as lack of reaching the target tissue (*in vivo*) and cellular uptake (*in vitro*), thus tending to lead to false negative results.

- Indirect genotoxicity due to oxidative stress and persistent inflammation (secondary) or because of ROS/RNS or metal contaminant release from MWCT/MWCNT resulting in DNA lesions (primary) may play a mechanistic role.
- Some evidence exists that inhalation exposure of thin (≤ 15 nm) MWCNT induce local mutagenic (aneugenic and clastogenic) effects in the lung: Aneugenic effects in the micronucleus test and specific investigations which observed disturbed mitotic spindle formation suggest a direct genotoxic potential. To date it is unclear if MWCT/MWCNT of larger diameter would similarly interfere with microtubules and/or spindle formation (microtubule diameter is about 25 nm).

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 μm and a length ≥ 5 μm and aspect ratio > 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T
Other names (usual name, trade name, abbreviation)	MWCNT
ISO common name (if available and appropriate)	Not available
EC number (if available and appropriate)	
EC name (if available and appropriate)	Not available
CAS number (if available)	Not available
Other identity code (if available)	Not available
Molecular formula	Not available
Structural formula	Not available
SMILES notation (if available)	Not available
Molecular weight or molecular weight range	Not available
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	*Not relevant*
Description of the manufacturing process and identity of the source (for UVCB substances only)	*The substance is not an UCVB substance*
Degree of purity (%) (if relevant for the entry in Annex VI)	*Not assigned*

The key factors for fibre toxicity are dose, dimensions (length and diameter) and durability. It is generally accepted that respirable high aspect ratio particles (fibres) pose an additional hazard beyond that produced by conventional more spherical non-fibrous compact particles. A high aspect ratio is defined by the WHO as a

ratio of fibre length to diameter ≥ 3. With regard to length and diameter, WHO fibre dimensions set length ≥5 µm and diameter < 3 µm (WHO, 1985). Fibres with a diameter above 3 µm are not considered respirable (Harrison, 2005; Lippmann, 2014). Next to the composition of the substance its fibre-like geometry is of high relevance.

Therefore and to describe the substance as good as possible, the main geometry parameters are summarized here:

Diameter: ≥ 30 nm - < 3 µm;

Length: ≥ 5 µm

This CLH proposal comprises Multi-Walled Carbon Nanotubes falling under the nanomaterial definition recommendation of the Commission (2011/696/EU). However, with regards to the proposed entry the applicability domain is extended to cover the whole hazardous spectrum (up to 3 µm) with regard to diameter. The extension is required because there is evidence that MWCT > 100 nm in diameter (~ 150 nm) may induce mesothelioma. According to the fibre pathogenicity paradigm it is anticipated that fibre-dependent toxicity (and carcinogenicity) is assumed even for MWCT with larger diameters than those tested. However, it is currently not known if MWCT beyond a diameter range of > 200 nm (regarding the constituing particle) are or will be manufactured.

The scope of this CLH proposal is limited to synthetic graphite in tubular shape, multi-walled within a specific dimensional range. Therefore, this CLH proposal excludes other similar (nano)carbonaceous materials, such as carbon (nano)fibres, graphene, SWCNT, etc.

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)
Synthetic graphite in tubular shape, multi-walled	not assigned		

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
metal oxides used as catalyst (typically Fe, Co, Ni, Zn, Mg, etc., used as catalysts)	Typically 0-5			

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
none					

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5: Proposed harmonised classification and labelling according to the CLP criteria

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No entry										
Dossier submitters proposal	tba	Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 μm and a length ≥ 5 μm and aspect ratio > 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T	n/a	n/a	Carc. 1B STOT RE 1	H350i H372 (lung)	GHS08 Dgr	H350i H372 (lung)			
Resulting Annex VI entry if agreed by RAC and COM					Carc. 1B STOT RE 1	H350i H372 (lung)	GHS08 Dgr	H350i H372 (lung)			

Table 5a: Summary of classification proposal

	Carcinogenicity	Mutagenicity	STOT-RE
MWCT With the following dimensional specifications: • diameter ≥ 30 nm to < 3 µm • length: ≥ 5 µm	Cat. 1B[1]	_	Cat. 1[2]

[1)] Based on i) lung adenoma and carcinoma development following chronic inhalation in rat (including lung carcinoma/adenoma promotion after subacute inhalation in mouse), ii) malignant mesothelioma development following transtracheal administration in rat, and iii) malignant mesothelioma development following intraperitoneal administration in rat or (mutant) mouse.

[2)] Based on dust inhalation guidance value of C ≤ 0.02 mg/L/6h/day (90 d inhalation study in rat)

Table 6: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)		
Oxidising gases		
Gases under pressure		
Flammable liquids		
Flammable solids		
Self-reactive substances		
Pyrophoric liquids		
Pyrophoric solids		
Self-heating substances		
Substances which in contact with water emit flammable gases		
Oxidising liquids		
Oxidising solids		
Organic peroxides		
Corrosive to metals		
Acute toxicity via oral route		
Acute toxicity via dermal route		
Acute toxicity via inhalation route		
Skin corrosion/irritation		
Serious eye damage/eye irritation		
Respiratory sensitisation		
Skin sensitisation		

Hazard class	Reason for no classification	Within the scope of public consultation
Germ cell mutagenicity	hazard class not assessed in this dossier (evaluation as genotoxic carcinogen only)	No
Carcinogenicity	**Classification proposed**	**Yes**
Reproductive toxicity	hazard class not assessed in this dossier	No
Specific target organ toxicity-single exposure		
Specific target organ toxicity-repeated exposure	**Classification proposed**	**Yes**
Aspiration hazard	hazard class not assessed in this dossier	No
Hazardous to the aquatic environment		
Hazardous to the ozone layer		

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

No harmonised C&L available.

RAC general comment

General considerations related to definition

Fibre paradigm

The Dossier submitter's (DS) proposal covers Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T. Thus, the scope is limited to synthetic graphite in tubular shape, multi-walled within the defined dimensional range and excludes other similar carbonaceous materials, such as carbon (nano)fibres, graphene, single walled carbon nanotubes (SWCNT) etc.

The DS proposal is largely based on the so called "fibre paradigm" that has evolved during several decades of studies on the pathogenicity of asbestos and other fibres (Stanton *et al* 1981, Donaldson *et al* 2010, 2011, 2013). The features of this paradigm and their relevance to the proposal are described briefly below.

The fibre paradigm can be seen as a time-tested structure: toxicity model that identifies the features related to fibre pathogenicity. The features identified in the fibre paradigm include:

- Sufficient length of the fibre.
- Thinness, allowing for a small enough aerodynamic diameter enabling deposition beyond ciliated airways.
- Biopersistence of the fibre (including retaining the fibre shape) allowing for prolonged effects of the deposited fibres and the accumulation of the fibres.

The WHO (1985) criteria for the fibre dimensions for which the paradigm is applicable to were originally applied as counting rules of asbestos fibres for occupational hygiene measurements in the 1960s. These criteria are also used by the DS for MWC(N)Ts. The dimensions are: length > 5 µm, diameter < 3µm, aspect ratio 3:1 as noted in the proposal.

The proposal deviates from the fibre paradigm by adding a requirement for rigidity. Fibre diameter is considered as a proxy for this feature; the thicker the fibre, the more likely it is to be rigid. The lower limit of 30 nm for the fibre diameter is based on studies on carbon nanotubes that seem to indicate that thinner fibres, presumably because of their flexibility, do not cause mesotheliomas after intraperitoneal (IP) injection, suggesting that pathogenetic mechanisms related to the fibre shape do not play a role in the pathogenicity of these fibres. The fibre paradigm in original form does not include this feature, probably because asbestos and other fibres where it has been applied are inherently rigid.

The fibre paradigm is not affected by the chemical composition of the fibre unless this affects biopersistence and thus the chemical composition is in general not as important. In addition, MWC(N)Ts all have basically the same chemical composition, being synthetic graphite in tubular form. The dimensions of MWC(N)Ts vary between each producer and they can also vary between production batches, meaning that the fibres produced are not uniform but represent a range of lengths and diameters. Therefore, RAC agrees with the DS and considers it unnecessary to evaluate fibre types in other respects than their dimensions. However, a decrease in biopersistence affects the pathogenicity of fibres. Decreasing biopersistence in fibres has been the main approach for achieving "safety by design" in fibre materials, notably man-made vitreous fibres (MMVF). It is not clear if this approach can be applied to MWC(N)Ts. The DS had considered introduction of exemption criteria similar to Note Q for the classification of mineral wool. This note requires either *in vivo* biopersistence testing or intraperitoneal injection, or long-term inhalation testing. Since any pristine MWCNT is known to be quite bioresistant (see section "Toxicokinetics", below), this is an ambiguous criterion when proving fibre pathogenicity. Instead, the IP injection test is deemed highly informative but would likely need long-term follow-up. Therefore, the DS decided not to propose including Note Q but notes that in the event that industry provides scientific evidence, a negative IP test may be reconsidered as a valid exemption criterion. RAC acknowledges that IP and biopersistence testing are generally considered as possible tests to evaluate fibres in relation to their ability to cause pathogenicity by a fibre related mechanism. Negative results can be considered to show that the fibre paradigm (and therefore classification based on this mechanism) may not be applicable. However, it does not rule out effects, including carcinogenicity caused by other mechanisms.

The fibre paradigm is applicable to all pathologies resulting from the exposure to the appropriate fibres. For asbestos the main pathologies are lung cancer, mesothelioma, and lung fibrosis, all of these have been caused by MWC(N)Ts in animal experiments. Lung cancer and lung fibrosis can be caused by other mechanisms than fibre related ones while mesotheliomas are quite specific for asbestos exposure in humans and can be caused by other fibres in animal models. As mentioned above, an IP test is commonly used to evaluate fibres in relation to the to their ability to cause pathogenicity, specifically by a fibre related mechanism. The DS pays special attention to studies with mesotheliomas, most of these studies consists of intraperitoneal injection of MWC(N)Ts; studies where pulmonary exposure of MWC(N)Ts cause mesothelioma in animal experiments are rare.

Definition of the "substance" covered in the proposal

The definition of the substance and the dimensions were one of the main points commented on during the consultation of the CLH report: according to industry, the CLH proposal should be targeted only to MWCNT-7 type tubes since the majority of data comes from this type and they consider that extending to other types of MWC(N)Ts is not appropriate mainly due to lack of data. Two commenting Member States, on the other hand considered the possible extension of the scope to MWCNTs with a diameter of <30 nm. When considering the dimension issue, it is important to note that:

1) It is not possible to give a scientifically based lower limit for the "rigid" MWCNTs or MWCNTs causing asbestos like effects (incl. cancer) and there are data suggesting that also MWCNTs with a smaller diameter may have carcinogenic properties. The cut-off limit of 30 nm chosen by the DS is based on the available data on mesothelioma induction: the lowest diameter of MWCNTs that has been observed to cause mesothelioma is currently 37 nm (Rittinghausen *et al*., 2014). This issue will be discussed further later in the RAC opinion.
2) The range does not fulfil the EU recommendation for a definition of nanomaterial and which states that one external dimension (in this case the diameter) should be in the size range of 1-100 nm. The DS has extended the applicability domain of the CLH proposal to MWC(N)Ts with a diameter up to 3 µm, as it is expected, according to the fibre pathogenicity paradigm, that MWC(N)T with diameters fitting within the respirable range will possess similar fibre-like properties. In addition, there is evidence that MWC(N)T ~ 150 nm may induce mesothelioma. It is, however, not known if MWC(N)T beyond a diameter range of > 200 nm (in the constituent particles) are manufactured.
3) The diameter was used in the CLH proposal as a pragmatic surrogate for fibre rigidity since precise and reproducible method to express rigidity is not available and rigidity has been suggested to be one critical point in lung and pleural pathogenicity of these types of fibres.

Another specific issue raised in comments received during consultation of the CLH report was related to the classification threshold for the concentration of fibres fulfilling critical dimensions in a mixture. However, these concentrations are defined in Annex VI of the CLP regulation (EG) 1272/2008, which gives general concentration limits for different hazard classes. Thus, if the substance is present on its own, as a constituent/impurity or in a mixture at above the generic concentration limit defined in the CLP regulation for the specified hazard the substance or the mixture needs to be classified. In this case, it means that if these MWC(N)Ts are classified for carcinogenicity in category 1B, substances with 0.1% or more (w/w) of these MWC(N)Ts need to be classified for carcinogenicity in category 1B.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level. This holds true for the endpoint carcinogenicity.

Reason for a need for action at Community level concerning STOT RE:

- Disagreement by DS with current self-classification
- There is a lack of self-classification on STOT RE.
- Requirement for harmonised classification by other legislation or process.

5 IDENTIFIED USES

In general, Multi-Walled Carbon Nanotubes (MWCNT) possess several properties which make them useful for industrial applications in new or enhanced materials and products (e.g. tensile strength, electrical and thermal conductivity). Their potential application, however, is linked to their specific properties and thus varies with the type of MWCNT and quality of the MWCNT material. Jensen et al. (2015) reported the most important applications to be in various types of composites, coatings and energy. According to the authors current and potentially near market applications of MWCNT include antistatic and electro-paintable thermoplastics, anti-fouling coatings, batteries (Li-ion), textiles, structural composites (e.g. for windmill blades and high performance sporting goods) and possibly printed electronics (conductive inks) and conductive coatings for displays and touch screens. For CNT the authors list additional current and potentially near market applications including thin heat mats, gas- and biosensors, and high-durability epoxy-paints.

6 DATA SOURCES

Data searches encompassed medical and toxicological databases such as PubMed, ToxNet, Embase and scientific literature databases such as, Scopus, ScienceDirect, Wiley Online Library, and Web of Science[1]. The CLH report also included results from a report of the WPMN Sponsoring Programme (OECD)[2] and the European project NANOGENOTOX[3]

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Solid		
Melting/freezing point			Not available//Not applicable
Boiling point			
Relative density			
Vapour pressure			
Surface tension			
Water solubility			
Partition coefficient n-octanol/water			

[1] https://www.embase.com/#search
https://toxnet.nlm.nih.gov/newtoxnet/toxline.htm
https://www.sciencedirect.com/
https://www.scopus.com/search/form.uri?display=basic
https://www.ncbi.nlm.nih.gov/pubmed/advanced
https://onlinelibrary.wiley.com/search/advanced
http://apps.webofknowledge.com/WOS_GeneralSearch_input.do?product=WOS&search_mode=GeneralSearch&SID=C6ohzsqLSqjFpTHOBkq&preferencesSaved=

[2] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)20&doclanguage=en

[3] https://www.anses.fr/en/system/files/ANSES-Ft-Nanogenotox_FinalReport.pdf

Property	Value	Reference	Comment (e.g. measured or estimated)
Flash point			
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry			
Stability in organic solvents and identity of relevant degradation products			
Dissociation constant			
Viscosity			

Since the substance under evaluation is not registered yet, there are no reliable data on physico-chemical properties available. However, taking into account the information publically available the substance is a powder, having a high thermal stability and being not soluble in water.

8 EVALUATION OF PHYSICAL HAZARDS

Not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies[4] (see also Annex I, 2.1.1.-2.1.12

Method	Results	Remarks	Reference
Studies in rats			
Inhalation OECD TG 413 F344 DuCrlCrlj rats, m/f Whole-body exposure to 0.2, 1 or 5 mg/m^3 cyclone-sieve generated MWCNT aerosol for 6 h/day, 5 days/week for 13 weeks (n=10/group) Doses corresponded to number concentrations of about 115000, 5770000, and 2933000 cpm (i. e. "particles" > 0.3 µm)	Lung burden increased dose-dependently: 3.23, 21.2 and 120.3 µg/left lung in males (lung burden in females was ca. 1.5 times lower because of physiologically lower breathing rate). The total lung burden was calculated to be 54-81 % of the simulated lung burden estimated by the Multiple-Path Particle Dosimetry (MPPD) model. MWCNT in the lung were primarily detected within alveolar macrophages but few single non-phagocytosed fibres were also found in the bronchiolar and alveolar spaces. Notably, two types of alveolar macrophages were predominant in the alveoli: alveolar macrophages which phagocytosed many MWCNTs and alveolar macrophages with foamy cytoplasm which phagocytosed only a few long MWCNT fibres. MWCNT deposition in bronchus-associated lymphoid tissue (BALT) and mediastinal lymph nodes was found in all MWCNT-exposed rats. In the highest dose group, occasionally MWCNT could be observed in the visceral subpleural areas and in the parietal pleura at the diaphragm (other organs were not explored). MWCNTs were also deposited in the nasal cavity (respiratory epithelium) of all MWCNT-exposed rats, primarily in non-ciliated respiratory epithelia (mucosa) and the lamina propria of the nasal cavity. MWCNT deposition and retention increased in a concentration- and time-dependent manner.	Test material: MWNT-7 (Hodogaya, Lot No. 071223, 080126) Mean D: 90.7 nm Mean L: 5.7 µm (48.7 % > 5 µm) Purity: > 99.5 % MMAD = 1.4-1.6 µm (~80 % mass as inhalable fraction)	Kasai et al. (2015)
Transtracheal intrapulmonary spraying No standard test guideline followed F344 rats, m	The longer, needle-like (MWCNT-L) but not the shorter, "cotton candy"-type (MWCNT-S) MWCNT translocated into the pleural cavity and deposited primarily at the parietal pleura. MWCNT-S were found phagocytosed in alveolar macrophages close to the visceral pleura.	Test material: 1. MWCNT-L D: 150 nm L. 8 µm Mean length: 7.34 µm (needle-shaped)	Xu et al. (2014)

[4] Most studies also reported toxic effects, which, however, are discussed separately.

Method	Results	Remarks	Reference
Exposure to 1.625 mg MWCNT/rat (total amount) over a period of 24 weeks (n=6/group)	MWCNT-L were found in extrapulmonary organs, such as lymph nodes (mediastinal, submandibular and mesentery), few tubes were also in the liver, kidney, spleen and brain. MWCNT-S were not detected in extrapulmonary organs.	2. MWCNT-S D: 15 nm L: 3 µm (cotton candy like aggregates)	
Transtracheal intrapulmonary spraying No standard test guideline followed F344 rats, m Exposure to 1.25 mg MWCNT/rat (total amount) over a period of 9 days. (n=6/group)	Like crocidolite, both MWCNT types translocated into the pleural cavity when administered into the rat lung. Here they were mainly found within pleural macrophages. Only few MWCNT and crocidolite fibres were observed penetrating through the visceral pleura. In addition, MWCNT and crocidolite frequently deposited in the mediastinal lymph nodes (mostly found phagocytosed by macrophages). Few fibres were also detected in liver sinusoid cells, blood vessel wall cells in the brain, renal tubular cells and spleen sinus and macrophages without reporting how they could get there. The authors assumed that the lymphatic flow is a relevant route of extrapulmonary transport of fibres. The absence of fibres at the parietal pleura was assumed to be due to the short exposure period.	Test material: 1. MWCNT-M (= MWNT-7, Mitsui) Median length: 4.47 µm Average length: 5.11 µm 3.82 x 10^{11} fibres/g 2. MWCNT-N (Nikkiso) Median length: 3.02 µm Average length: 3.64 µm 3.46 x 10^{11} fibres/g Positive control: Crocidolite (1.25 mg/rat)	Xu et al. (2012)
Intratracheal instillation No standard test guideline followed F344/DuCrlCrlj rats, m Exposure to 0, 40 or 160 µg/rat (n=8/group, m) Rats were sacrificed on day 1, 7, 28 or 91 following instillation	MWCNT migrated to the right and left posterior mediastinal lymph nodes and – to a lesser extent – to the parathymic lymph node. The deposition of MWCNT in these lymph nodes increased gradually and dose-dependently during the postexposure period. At 91 days postexposure, aggregated nodal macrophages laden with MWCNT were observed, which the authors speculated to progress into microgranulomas.	Test material: MWNT-7 (Mitsui) Fibres of 35.5 or 53 nm diameter	Aiso et al. (2011)
Studies in mice			
Pharyngeal aspiration No standard test guideline followed BalB/c mice, f	Translocation of MWCNT to distant organs after pulmonary exposure investigated by using in situ radiolabelling, combining tissue radioimaging of organ tissue sections to ex vivo analysis of MWCNTs by electron microscopy. 10 % of the calculated applied dose in the lung (10 µg) was retained in the lung after 12 months.	Test material: Synthesised ^{14}C-MWCNT L: 500 nm – 12 µm (mean: 3.9 µm)	Czarny et al. (2014)

Method	Results	Remarks	Reference
Single dose exposure (20 µg) of pristine ^{14}C-MWCNT (285 x 10^3 Bq) (n=4) Analysis 1 and 7 days, 1, 3, 6, 9 and 12 months after lung exposure.	Whereas most of the deposited dose was assumed to be cleared early via the mucociliary escalator, concomitant re-location to distant organs was observed. In particular, 200 ng MWCNT in the spleen and in liver 75 ng MWCNT was detected after 12 months postexposure. Little radioactivity was detected in the heart and none in brain and thymus after 360 days. TEM analysis detected MWCNT of different lengths and diameters in peripheral organs such as spleen and bone marrow (MWCNT of 40 nm diameter in spleen, one 4 µm long fibre was found in liver extracts), accumulating over the whole period of the study, from day 1 to month 12, without decreasing, indicating high biopersistence.	D: 10-150 nm (mean: 40 nm) (measured in dispersion medium; only few agglomerates formed)	
Inhalation No standard test guideline followed C57BL/6J mice, m Whole-body exposure of 5 mg/m^3) for 12 days (n= 7-9/group) Postexposure: 1, 14, 84, 168, and 336 days	The lung burden and changes in the compartmentalisation of MWCNT within the lung during almost one year of postexposure (p.e.) was investigated. By using sensitive optical techniques, MWCNT could be detected in liver, kidney, heart, and brain already 1 day p.e. Opposed to the initial lung burden mainly consisting of agglomerates, MWCNT found in extracellular organs were individual tubes ('singlets') of about 7-8 µm in length or small assembled structures containing few tubes. These accumulated over the 336-day follow-up period. In the tracheobronchial lymph nodes, which contained the highest share of translocated MWCNT, the initial presence of singlets disappeared in favour of multiple fibre-containing foci by day 336 p.e. Expressed as percentage of deposited lung burden (28.1 µg/lung), tracheobronchial lymph nodes contained 1.08 and 7.34 % at day 1 and day 336 p.e., respectively. The percentages in liver (the target organ containing second-highest amounts of MWCNT) were 0.002 and 0.027 % 1 and 336 days p.e., respectively. In general, the increase over time in extrapulmonary organs was 6-7-fold (excluding the chest wall, where MWCNT burden did not change significantly). The authors assumed that the tracheobronchial lymphatic is a major route for systemic delivery of inhaled MWCNT.	Test material: MWNT-7 (Hodogaya, Lot No. 061220-31) L: 4.3 µm (mean) Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm. 1.32 % metal contamination (1.06 % iron)	Mercer et al. (2013a)

Method	Results	Remarks	Reference
Inhalation No standard test guideline followed C57BL/6J mice, m Whole-body exposure of 5 mg/m^3) for 12 days (n= 7-9/group) Postexposure: 1, 14, 84, 168, and 336 days	Total lung burdens successively but incompletely decreased from 28 to 18 µg from day 1 to day 336 postexposure. Initially, 84 % of the total lung burden was found in the alveolar region (including 1.2 % in subpleural tissue), 16 % in the airways. Clearance reduced the alveolar macrophage burden of MWCNTs by 35 percent between 1 and 168 days post-exposure, while the content of MWCNTs in the alveolar tissue increased by 63 percent. At 336 days, 95.8 % of the initial lung burden remained in the alveolar region (including 4.8 % in subpleural tissue), whereas 4.2 % was found in the airways. Within the alveolar region, the burden contained in alveolar macrophages was initially 3-fold that of the alveolar tissue, including subpleural alveolar tissue (56 % vs. 20 %). However, over time it declined due to clearance by 35 % between 1 and 336 days postexposure, while the alveolar tissue burden increased by 36 %. Large MWCNT structures containing greater than 4 fibres were 53.6 % of the initial lung burden and accounted for the majority of the decline with clearance, while lung burden of singlet MWCNT was essentially unchanged.	Test material: MWNT-7 (Hodogaya, Lot No. 061220-31) L: 4.3 µm (mean) Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm. 1.32 % metal contamination (1.06 % iron)	Mercer et al. (2013)
Inhalation No standard test guideline followed C57BL/6J mice, m Whole-body exposure of 10 mg/m^3) for 2, 4, 8, or 12 days (n= 7-9/group)	After 4 days of exposure, 76 % of CNT lung burden was localised in the alveolar region, 14.6 % of which was distributed in the airspace, 11.9 % in the alveolar (including subpleural) tissue and 49.2 % in alveolar macrophages. MWCNT were also found in tracheobronchiolar lymphocytes and were shown to reach the pleural wall, which they occasionally penetrated. Lung burden was calculated to be equivalent to humans in occupational settings.	Test material: MWNT-7 (Hodogaya, Lot No. 061220-31) Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm. 1.32 % metal contamination (1.06 % iron)	Porter et al. (2013)
Intrapleural injection Exposure to a single dose of 5 ^{111}In-radiolabeled MWCNT µg/mouse (n= 4-5/group)	Indirect evidence was presented that passage through parietal stoma to mediastinal lymph nodes is dependent on MWCNT length. Thus, short fibres are able to be cleared via the lymphatics, whereas long fibres are retained at the parietal pleura. The length-dependent pleura passage block was confirmed by nickel wire administration of defined lengths.	Test material: - Short straight MWCNT: D: 20-30 nm, L: 0.5-2 µm [Nanostructured & Amorphous Materials, Inc.]	Murphy et al. (2011)

Method	Results	Remarks	Reference
Analysis 1-24h after injection by Single-Photon Emission Computed Tomography (SPECT/CT) imaging		"Length controls": - Nickel nanowires of 200 nm diameter as short (4.3 µm) and long (24 µm) fractions	
Pharyngeal aspiration No standard test guideline followed C57BL/6J mice, m Single dose exposures (10, 20, 40 or 80µg) (n=4/group) Examinations 1, 7, 28, or 56 days (d) post-exposure (p. e.).	The aspirated dose was widely distributed throughout the lungs and rapidly incorporated into the alveolar walls and alveolar cells. Within one hour after aspiration, fibres were found engulfed by type II alveolar epithelial cells and alveolar macrophages. At later time points MWCNT were generally no longer present on the surface of epithelial cells but had been transported within the alveolar interstitium and/or interstitial cells as well as within macrophages in the interstitium (as single fibres up to larger clusters). Incomplete phagocytosis by macrophages, lasting for weeks, was also observed.	Test material: MWNT-7 (Mitsui, Lot No. 05072001K28) D: 49 nm L: 3.86 µm (median) 0.78 % metal contamination (0.31 % iron)	Porter et al. (2010)
Pharyngeal aspiration No standard test guideline followed C57BL/6J mice, m Single dose exposures (10, 20, 40 or 80µg) (n=4/group) Examinations 1, 7, 28, or 56 days (d) post-exposure (p. e.).	At 1 day 18 %, 81.6 % and 0.6 % of the MWCNT lung burden was in the airway, the alveolar, and the subpleural regions, respectively. There was an initial high density of penetrations into the subpleural tissue and the intrapleural space one day following aspiration, which appeared to decrease due to clearance by alveolar macrophages and/or lymphatics by day 7. However, the density of penetrations increased to steady state levels in the subpleural tissue and intrapleural from day 28 - 56. 56 days after exposure to 80 µg MWCNT, it became evident that MWCNT can reach the pleura, as fibres were detected in the subpleural lymphatics. 1 in every 400 fibre penetrations was found either in the subpleural tissue (i.e. the alveolar epithelium adjacent to the pleura) or in the intrapleural space, defined as visceral pleural surface.	Test material: MWNT-7 (Mitsui, Lot No. 05072001K28) D: 49 nm L: 3.86 µm (median) 0.78 % metal contamination (0.31 % iron)	Mercer et al. (2010)
Inhalation No standard test guideline followed C57BL6 mice, m Nose only exposure to 1 or 30 mg/m^3 for 6 h (n=10/group)	MWCNT were detected in the subpleura one day after inhalation exposure to 30 mg/m^3 for 6h. Mononuclear cell aggregates on the pleural surface increased in number and size after 1 day and nanotube-containing macrophages were observed within these foci. Most of the inhaled nanotubes appeared to be cleared, though some remained in the subpleural wall for at least 14 weeks.	Test material: MWCNT (Helix Material Solutions) L: 0.3-50 µm D: 30-50 nm (average) Purity: > 94 % (mixture of agglomerated and individual nanotubes)	Ryman-Rasmussen et al. (2009)

Method	Results	Remarks	Reference
Mice were sacrificed 1 day, 2 weeks, 6 weeks or 14 weeks post-exposure			

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Pristine MWCNT

Toxicokinetic studies investigating pristine, i.e. not functionalized or modified, fibre-like MWCNT aimed at elucidating modes of toxic and carcinogenic action following inhalation. Accordingly, these studies focussed on the impact of physicochemical and morphological properties of MWCNT deposited in the respiratory tract on lung burden, retention and clearance, translocation to the pleura and to distant organs as well as their compartmentalisation (including intracellular localisation). There is no information available for MWCNT from standard guideline studies such as OECD TG 417. Rather, relevant information is retrieved from a number of experimental and toxicological studies. The findings of these studies are summarized in Table 8 and discussed below.

Frequent, particularly important findings related to the carcinogenic mode of action are that inhaled pristine fibre-like MWCNT are largely retained in the deep lung and that these inhaled fibres will re-locate from the alveolar region into the alveolar interstitium over time, also translocating to lung-associated lymph nodes and reaching as well as penetrating the pleura. The majority of toxicokinetic studies used a specific type of MWCNT by the Japanese company Mitsui, known as MWNT-7. As this material was also frequently used in toxicity studies (see below), it is viewed as sort of prototype for fibre-like MWCNT of high diameter (diameter specifications ranges by different investigators vary considerably, mostly between 30-90 nm).

It was demonstrated that well-dispersed inhaled MWNT-7 tubes are largely respirable and rapidly deposit in the alveoli. The majority of the total lung burden of inhaled MWNT-7 was still extractable one year after exposing mice to MWNT-7 at a dose resulting in an initial lung burden equivalent to occupationally relevant human burdens (28 µg at day 1 vs. 18 µg at day 336). This demonstrates that clearance via the mucociliary escalator is ineffective and other clearance routes and compartments are to be considered. Over time, individual MWCNT which are not cleared by the mucociliary escalator (or become walled-off in granulomas) re-distributed to the alveolar interstitium (Mercer et al., 2013).

Inhaled MWNT-7 tubes that deposited in the lung also translocated to extrapulmonary tissue proximal to the pleura (lung-associated lymph nodes, diaphragm, chest wall), as well as to distant organs such as liver, kidney, heart and brain where they can accumulate over time. Overall, 1.1 % of the lung burden translocated 1 day after 12 days of exposure and the extrapulmonary burden increased to 7.3 % after 336 days, almost all of it initially translocated to the tracheobronchial lymph nodes. The high lymphatic burdens observed 1 day postexposure and at 336 days post-exposure indicate that the transport of MWCNT through the lymphatics and ultimately into the venous circulation may be a major route for systemic delivery of MWCNT. The calculated distant organ accumulation of fibres was considered to be possibly too low to elicit fibrotic effects (Mercer et al., 2013a). Translocation to other extrapulmonary organs and accumulation was also reported in tracing experiments with ^{14}C-MWCNT of slightly different size (10-150 nm (mean: 40 nm) compared to MWNT-7 (Czarny et al., 2014). However, this study did not find MWCNT in brain and heart even one year after administration, which could have been due to different test materials used but also to different administration methods (bolus exposure by pharyngeal aspiration vs. continuous exposure by inhalation).

Pleural migration and penetration after inhalation, a hallmark of fibre pathogenicity, also applies to rigid MWCNT. This has been repeatedly demonstrated with MWNT-7 (Kasai et al., 2015, Porter et al., 2013, Mercer et al., 2013, 2013a) but has also been shown for other lung-exposed MWCNT with diameters that limit their flexibility (Ryman-Rasmussen et al., 2009, Murphy et al., 2011, Xu et al., 2012, 2014). Mercer et al. (2010) counted pleural penetrations of MWCNT following exposure of mice to 10 - 80 µg MWNT-7 using pharyngeal

aspiration. Numbers increased during postexposure and at day 56 postexposure ~ 12,000 penetrations/lung into subpleural tissue (i.e. alveolar epithelium immediately below the visceral pleura), and ~ 6,000 penetrations/lung into the intrapleural space were counted at the highest dose.

In contrast, thin, tangled low-diameter MWCNT (< 15 nm in diameter), which spontaneously form low density coiled agglomerates, have not been reported migrating to the pleura and/or producing pleural injury following pulmonary exposure (Ma-Hock et al., 2009, Pothmann et al., 2015). However, Pauluhn (2010) observed thickening of the visceral pleura after inhalation of tangled Baytubes at high concentrations.

Measuring the rigidity of MWCNT is a challenging task and still a matter of research. However, for routine use, the outer tube diameter appears to be an appropriate proxy for rigidity. In general the greater the number of walls the MWCNT tube is made up, the larger its diameter is and the less flexible it becomes (taking into account that the tube diameter of MWCNT may be influenced by size of the particles used as catalyst for their synthesis). The above findings on the fibre-like kinetic behaviour are consistent with this relationship.

In addition to diameter, the tube length is a critical determinant of lung and pleura retention. Frequently, single MWCNT reaching the pleura post exposure (often in already fibrotic tissue) were longer than 4 µm. Studies using as test materials silver and nickel nanowires of defined lengths demonstrated that lengths of 5 µm but not 4 µm were inflammogenic when directly injected in the pleura (Murphy et al. 2011). This was explained by the inability of materials with a length of at least 5 µm to passage through the stomata[5] in the parietal pleura (resulting in inflammation and eventually in mesotheliomagenesis), whereas shorter materials are removed from the pleural space via the stomata, facilitated by lymphatic drainage (Schinwald et al., 2012, Donaldson et al., 2013). It is noted that fibre length-dependent stomata passage has not yet been directly demonstrated for MWCNT.

Stoma retention supports the WHO fibre definition (WHO, 1985), which generically sets a critical threshold fibre length of 5 µm. It is noteworthy that this critical length was originally derived from the phenomenon of "frustrated phagocytosis" (Donaldson et al., 2010), i.e. the inability of macrophages to completely engulf rigid fibres considerably longer than 5 µm thus fostering pulmonary retention and inflammation. In fact, the above distribution studies with MWNT-7 and similar fibre-like MWCNT provided evidence for both, pleura retention and frustrated phagocytosis, proving the applicability of the WHO fibre principle on rigid MWCNT with regard to length and biopersistence as it was formulated earlier for asbestos and other man-made mineral fibres.

Despite illustrative microphotographs showing single MWCNT fibres penetrating the visceral pleura after inhalation (e.g. Mercer et al., 2013, 2013a), it is not quite clear how they get there and if cellular transport is required. As long as tracing experiments are missing, at least two routes need to be taken into account: migration to the sub-pleura after translocation to alveolar interstitium and/or deposition from subpleural lymphatic when cleared via LALN. Both routes may also involve cellular (macrophage) transport and release. In addition, the degree of tube dispersion and the number of individual fibres, respectively, may strongly affect the uptake, distribution and penetration behaviour of the MWCNT.

Since most of these studies also reported toxicological findings, evidence was presented that MWCNT deposition and retention increased in a concentration- and time-dependent manner, and was related to toxic responses. Thus inhaled MWNT-7 exposure resulted in rapid development of pulmonary fibrosis and persisting granulomatous inflammation but also to pleural fibrosis and mesothelial proliferation (see sections 10.9 and 10.12).

The Dossier Submitter concludes that there is sufficient evidence proving that rigid poorly soluble MWCNT with fibre-like morphology are retained in the lung and may reach the pleura and even distant organs after inhalation in a dose-dependent manner. Because of their fibre dimension-driven biopersistence and translocation, inhaled non-cleared MWCNT trigger inflammatory tissue injury and neoplastic processes both in the lung and the pleura. The available kinetic data strongly suggest an asbestos-like mode of action and support proof-of-principle for carcinogenicity (i.e. mesothelioma) in studies with rigid MWCNT in which the test material has been applied by intraperitoneal injection.

[5]Stomata are pore-like structures which overlie a specialised network of submesothelial lymphatic channels with openings of 2-12 µm.

Other carbon nanotubes

Available toxicokinetic information has been summarised in the present proposal in order to support considerations on the carcinogenic mode of action of fibre-like MWCNT, including its deposition and retention in the lung, its interaction with target and immune cells, as well as its occurrence at the pleura following inhalation as a prerequisite for mesotheliomagenesis.

Studies that investigated all kinetic aspects of MWCNT (absorption, distribution, metabolism and excretion) are rare. A number of investigations is available that characterised the pharmacokinetic behaviour of carbon nanotubes (CNT) for drug delivery purposes (reviewed e.g. in Ali-Boucetta and Kostarelos, 2013 and Ye et al., 2013). However, these CNT (both SWCNT and MWCNT) are usually functionalised to increase their hydrophilic properties, thereby modifying their kinetic behaviour substantially.

Regarding intracellular uptake, CNT can be internalised passively or actively, requiring an energy-driven transport. Passive internalisation of SWCNT and MWCNT of sub-micron length was demonstrated in the presence of metabolic inhibitors and cell lines which lack active transport systems (Ye et al., 2013). The MWCNT with a needle-like structure may be taken up passively but also injure cells by direct piercing (Nagai et al., 2011). Active internalisation may involve different endocytic mechanisms, phagocytosis, clathrin-mediated endocytosis (Maruyama et al., 2015), caveolae-mediated endocytosis and may be receptor-mediated (Wang et al. 2016). Contribution of mesothelial cell uptake and injury is further discussed in section 10.9. Phagocytosis is the principal mechanism by specialised cells (macrophages, monocytes, neutrophils). This process is size-dependent as CNT constructs longer than 400 nm underwent phagocytosis, whereas very long, inflexible fibre-like MWCNT cause incomplete or "frustrated" phagocytosis (Donaldson et al., 2013). Apart from uptake by professional phagocytes, the major endocytic mechanism appears to be clathrin-mediated endocytosis (however, the mechanism for lung epithelial cells is not well studied). This process is also size dependent. For SWCNT (DNA-wrapped), maximum endocytosis was modelled for a radius of about 25 nm and a threshold length of < 200 nm (Jin et al., 2009).

Pristine CNT are highly stable and biopersistent. Inhaled CNT might be cleaved and degraded in the lung tissue, possibly catalysed by myeloperoxidase from neutrophils and macrophages, although there is only in vitro evidence for this enzyme-mediated degradation (Kagan et al., 2010). It should also be considered, that functionalised CNT may undergo defunctionalisation in vivo as has been observed for intravenously injected polyethylene glycol-conjugated SWCNT in liver (Ye et al., 2013).

Partially because of their insolubility, inhaled non-functionalized CNT deposited in the deep lung are largely retained if not removed by the mucociliary escalator. A low percentage may translocate to other organs, where they may slowly accumulate under conditions of continuous exposure (see below). There is no data on the excretion via urine or bile.

RAC evaluation of the toxicokinetic data

Summary of the Dossier Submitter's proposal

The DS provided summaries of a number of toxicokinetic studies with MWCNT, a short overall summary and a statement on the overall relevance of this information for the proposed classification. According to the DS, the available studies show that pristine fibre-like MWCNT (mainly MWCNT-7) are retained in the deep lung and slowly relocate into e.g. the alveolar interstitium, lung-associated lymph nodes, parietal pleura and distant organs such as liver and kidney. Systemic exposure occurs mainly via the lymphatic system. MWCNT disposition and retention was concentration and time dependent and was related to the toxic response. Pleural migration and penetration have been shown for rigid MWCNT

in several studies whereas thin tangled low-diameter MWCNT forming coiled agglomerates were not reported as migrating to the pleura. However, effects on visceral pleura were observed after inhalation of tangled Baytubes.

The DS stated that measuring the rigidity of MWCNT is a matter needing further research but proposed to use the outer diameter as a proxy because in general, the greater the number of walls which make up the MWNCT tube, the larger its diameter is and the less flexible it becomes. The diameter also depends on the size of the particle used as catalyst for the synthesis. The relationship between diameter and relocation to the pleura was confirmed, according to the DS, by the available toxicokinetic studies.

Furthermore, the tube length was considered critical as studies with other types of fibre have shown that fibres longer than 5 µm can reach and remain in the pleura but fibres of 4 µm can reach the pleura but are removed via the stomata in the parietal pleura. This aligns their toxicity with the WHO fibre definition.

Overall, the DS concluded that there is sufficient evidence proving that rigid poorly soluble MWCNT with fibre like morphology are retained in the lung and may reach the pleura and more distant organs after inhalation in a dose-dependent manner. This triggers inflammatory injury in the tissue and neoplastic processes in both the lung and the pleura comparable to other carcinogenic fibres, such as asbestos.

For other carbon nanotubes, the DS provided a summary of the available data on the non-functionalised form with emphasis on the biopersistence, intracellular uptake and distribution.

Comments received during consultation

No specific comments regarding the toxicokinetic properties of MWCNT were provided during the public consultation. However, a discussion emerged regarding the influence of the form (straight versus tangled and rigid versus flexible) on the proposed mode of action which in part depends upon the toxicokinetic behaviour.

One additional study containing toxicokinetic information was provided in the consultation, namely Saleh *et al*. (2020) which was taken into account.

Additional key elements

One additional study regarding toxicokinetics was provided in the consultation. The toxicokinetic parts of this study are summarised below.

Rats were exposed to two types of MWCNT once weekly for seven weeks via intra-tracheal pulmonary spraying at two dose levels (total amount of 0.5 and 1.0 mg per rat) with an observation period of two years (Saleh *et al*., 2020). The first type of MWCNT consisted of straight fibres with a diameter of approximately 150 nm and the second of tangled fibres with a diameter of 7.4 nm. Both types of fibres were present at 52 and 104 weeks with approximately 3 fold higher concentrations for the tangled fibres and with only a minimal reduction from week 52 to week 104. The results expressed as µg fibre per g of lung showed high biopersistence of both types of fibres in the range of 10 to 40 % of the total

applied amount of fibre based on 1.3 gram of lung per male animal (rapporteurs' calculation).

Assessment

The proposed classification for carcinogenicity is based on the fibre paradigm as described above. It is assumed that a sufficient dose of biopersistent fibres with a certain diameter and length can induce lung cancer and the formation of mesothelioma. However, fibres may induce carcinogenicity in the lung via other mechanisms as shown for short tangled MWCNT (Saleh *et al*., 2020). As toxicokinetic data can be used to assess whether specific forms of MWCNT fulfil some of these properties, the focus of the RAC assessment was primarily on whether:

- the fibres can reach the alveoli
- the fibres are biopersistent
- the fibres can translocate to the pleura including whether the fibres were observed visceral, parietal or in the pleural cavity.

Therefore, the table below was made describing the tested MWCNT and whether one or more of the toxicokinetic properties, with focus on distribution, was shown. However, it should be noted that not all potential properties were determined in all studies.

***Table**: The types of fibres that were tested in the available toxicokinetic studies*

Name fibre	**Length**	**Diameter**	**MMAD**	**Form: straight or tangled**	**Distribution**	**Study**
MWCNT-7	Mean: 5.7 µm (48.7% > 5 µm)	Mean: 90.7 nm	1.4-1.6 µm	straight	alveolar visceral subpleura parietal pleura nasal cavity	Kasai *et al*., 2015
MWCNT-L	Mean: 7.34 µm	150 nm		needle shaped	alveolar visceral pleura pleural cavity parietal pleura extrapulmonary	Xu *et al*., 2014
MWCNT-S	3 µm	15 nm		cotton candy like aggregates	alveolar	Xu *et al*., 2014
MWCNT-M (=MWCNT-7)	median: 4.47 µm mean: 5.11 µm				alveolar pleural cavity lymph nodes	Xu *et al*., 2012
MWCNT-N	median: 3.02 µm mean: 3.64 µm				alveolar pleural cavity lymph nodes	Xu *et al*., 2012
MWCNT-7		35.5 or 53 nm			alveolar	Aiso *et al*., 2011

^{14}C-MWCNT	mean: 3.9 µm (0.5 – 12 µm)	mean: 40 nm (10-150 nm)			Biopersistent Alveolar spleen and liver	Czarny *et al*., 2014
MWCNT-7	mean: 4.3 µm		1.5 µm		Biopersistent alveolar visceral pleura pleural cavity extrapulmonary (liver, lymph nodes)	Mercer *et al*., 2013a Mercer *et al*., 2013
MWCNT-7			1.5 µm		alveolar pleural	Porter *et al*., 2013
short straight MWCNT	0.5 – 2 µm	20-30 nm		straight	Cleared via lymphatics after interpleural administration	Murphy *et al*., 2011
long straight 2	max: 56 µm	165±5 nm		straight	Retained in parietal pleura after interpleural administration	
MWCNT-7	median: 3.86 µm	49±13 nm			alveolar pleural	Porter *et al*., 2010
MWCNT-7	median: 3.86 µm	49±13 nm			Biopersistent alveolar visceral pleura	Mercer *et al*., 2010
MWCNT	0.3-50 µm	30-50 nm (average)			Biopersistance alveoli pleural	Ryman-Rasmussen *et al*., 2009
MWCNT-A MWCNT-B	6.39±3.07 µm 1.04±0.71 µm	150±43 nm (213 walls) 7.4±2.7 nm (6-7 walls)		straight tangled	Biopersistance alveoli Biopersistance alveoli	Saleh *et al*., 2020

MWCNT-7 fibres were shown to reach the alveoli of rats after repeated inhalation exposure and were mainly present within the macrophages. In the highest dose group, occasionally MWCNT could be observed in the visceral subpleural areas and in the parietal pleura at the diaphragm (Kasai *et al*., 2015).

The longer, needle-like MWCNT type (MWCNT-L) but not the shorter, "cotton candy"-type (MWCNT-S) was found in the pleural cavity and deposited primarily at the parietal pleura

after repeated transtracheal exposure of rats during 24 weeks. MWCNT-S were found phagocytosed in alveolar macrophages close to the visceral pleura (Xu *et al*., 2014).

Both needle like MWCNTs and crocidolite asbestos translocated into the pleural cavity when administered into the rat lung after repeated transtracheal intrapulmonary spraying during 9 days. However, no fibres were observed in the parietal pleura. Only few MWCNT were observed penetrating through the visceral pleura (Xu *et al*., 2012).

MWCNT migrated to the right and left posterior mediastinal lymph nodes and – to a lesser extent – to the parathymic lymph node in rats after a single intratracheal installation. The deposition of MWCNT in these lymph nodes increased gradually and dose-dependently during the postexposure period (Aiso, 2011).

Twelve months after a single exposure of mice via pharyngeal aspiration to radioactive MWCNT, 10% of the estimated dose was retained in the lung. Also in other organs such as the spleen and the liver radioactivity and fibres were observed. The results indicate high biopersistance (Czerny *et al*., 2014).

Repeated whole body inhalation exposure of mice with a post-exposure period of almost one year showed high retention of MWCNT fibres in organs outside the lung with the highest concentration in the tracheobronchial lymph nodes (Mercer *et al*., 2013a). Fibres were also observed in the pleural lavage. In the same study, the total lung burden decreased by 36% over almost one year. During this period there was a shift of distribution of the MWCNT fibres from the alveolar macrophages towards the alveolar tissue (Mercer *et al*., 2013). MWCNT were also observed in the sub-pleural tissue region.

Repeated whole body inhalation exposure of mice with a post-exposure period of 4 days showed that these fibres could reach the pleural wall which they occasionally penetrated (Porter *et al*., 2013).

After a single intrapleural injection of MWCNT in mice, there was indirect evidence that passage through parietal stomata to mediastinal lymph nodes is dependent on MWCNT length. Thus short, tangled fibres were able to be cleared via the lymphatics, whereas long fibres are retained at the parietal pleura. The length-dependent pleural passage block was confirmed by nickel wire administration of defined lengths (Murphy *et al*., 2011).

Single pharyngeal aspiration of MWCNT in mice with post-exposure durations of up to 56 days showed that some fibres could reach the pleura (Porter *et al*., 2010).

Single pharyngeal aspiration of MWCNT in mice with post-exposure durations of up to 56 days showed that MWCNT can reach the pleura, as fibres were observed in the intrapleural space (visceral pleural surface) and the subpleural lymphatics at all observation times up to day 56 (Mercer *et al*., 2010).

Single inhalation exposure of mice (6 hours, 30 mg/m^3) resulted in the observation of MWCNT engulfed by macrophages and in mesenchymal cells in the subpleural region and MWCNT containing macrophages in mononuclear cell aggregates on the pleural surface on day 1 after exposure. These declined afterwards but some remained up to 14 weeks after exposure (last measurement). No such effects were observed after exposure to the low dose of 1 mg/m^3 (Ryman-Rasmussen *et al*., 2009).

Exposure of rats to straight and tangled MWCNT via intra-tracheal intra-pulmonary spraying once a week for 7 weeks at two different dose levels with a 2 year post-exposure follow up resulted in clear biopersistence of both type of materials with a higher

biopersistence for the tangled material in lung and mediastinal lymph node (Saleh *et al*., 2020).

Overall, there is evidence from the available toxicokinetic information that MWCNT which reach the alveoli, are taken up by macrophages and mesenchymal cells, are highly biopersistent and can translocate to the sub-pleural areas and into the pleural space and are retained there. However, it is unclear whether these toxicokinetic properties are applicable to all forms of MWCNT. In some of the studies, not all physical properties of the tested MWCNT were known. No information on the flexibility of the tested MWCNTs was available.

All tested MWCNT reached the alveoli. The diameter of rigid straight fibres is considered determinative for the ability of fibres to reach the alveoli as these fibres align with the airstream. Therefore, the diameter of the rigid straight MWCNT is also the diameter of the fibre particle. However, for tangled MWCNT, the diameter of the tube is much less than the diameter of the particles. Therefore, to measure the respirability of tangled rigid fibres, the Mass Median Aerodynamic Diameter (MMAD) is likely to be a better parameter than the diameter, which may overestimate the respirability. However, this is likely to become a relevant issue in classification only with thicker fibres (just below 3 µm in diameter) and is considered hypothethical, as fibres with a diameter just below 3 µm are not put on the market. For these pragmatic reasons, the proposed upper diameter of 3 µm is retained. The lower diameter of 30 nm cannot be based on the ability to reach the alveoli as there is evidence that MWCNT with smaller diameter can reach the alveoli (tangled 7.4 nm).

Information on the ability to reach the pleural cavity was available for straight MWCNT with a diameter of 30 - 150 nm (Ryman-Rasmussen *et al*., 2009 and Xu *et al*., 2014) but was shown not to apply to cotton-candy like MWCNT with a diameter of 15 nm (Xu *et al*., 2014).

Information on biopersistence is available for a range of diameters (7.4 – 150 nm) and for both straight and tangled MWCNTs (Saleh *et al*., 2020). No reduction in biopersistence is expected for fibres with a larger diameter.

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

Hazard class not assessed in this dossier

10.2 Acute toxicity - dermal route

Hazard class not assessed in this dossier

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier

10.4 Skin corrosion/irritation

Hazard class not assessed in this dossier

10.5 Serious eye damage/eye irritation

Hazard class not assessed in this dossier

10.6 Respiratory sensitisation

Hazard class not assessed in this dossier

10.7 Skin sensitisation

Hazard class not assessed in this dossier

10.8 Germ cell mutagenicity

The present dossier does not intend to assess MWCT/MWCNT for classification as human germ cell mutagen. Rather, the available genotoxicity data is evaluated in support of carcinogenicity classification of MWCNT with fibre dimensions as described in section 1.1 and to inform on the mode of action and pathology.

The database below - which is not exhaustive - includes few studies on MWCNT with non-fibre characteristics as well, thus enlightening on commonalities and differences between rigid fibrous and non-rigid fibre-like MWCNT.

A. In vitro database

Table 9: Summary table of mutagenicity/genotoxicity tests in vitro

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Bacterial gene mutation test (± S9 mix) OECD TG 471	1. MWNT-7 (Mitsui) L: not provided D: 70 nm (containing 3600 ppm Fe, 14 ppm Cr, 6 ppm Bi, 4 ppm Ni)	Dosing: 0, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/plate (n=3)	Negative MWNT-7 and N-MWCNT were not mutagenic in the Ames test.	Ema et al. (2012)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Bacterial strains: - Salmonella typhimurium strains TA98, TA100, TA1535, TA1357 - Escherichia coli mutant WP2uvrA	2. N-MWCNT (Nikkiso) L: not provided (cf. Ema et al. 2013) D: 44 nm (containing 176 ppm calcium, 80 ppm aluminium, 53 ppm iron, 16 ppm cadmium and 0.5 ppm lithium) Well dispersed MWCNT	No inhibition of growth was observed in a preliminary cytotoxicity study, at concentrations ranging from 0.05 to 100 µg/plate of MWCNT (± S9 mix) Positive controls: sodium azide (2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, 2-aminoanthoracene and 9-aminoacridine hydrochloride	However, bacterial uptake was not demonstrated	
Gene mutation test (*HPRT* locus) No standard test guideline followed Chinese hamster lung cells (CHL/IU)	MWNT-7 (Mitsui, Lot No. 061220) L: 5.0 ± 4.5 µm (38.9 % > 5 µm) D: 88 ± 5 nm (av.: 57 nm) Well dispersed isolated fibres Chrysotile A asbestos fibres were concomitantly tested	Dosing: 6.3 to 100 µg/mL (48 h of exposure) Chrysotyle: 1.56 to 25 µg/mL for 48 h) Positive control: Ethyl methanesulfonate at 200 µg/mL Mutation frequency rate was expressed as the scored number of 6-thioguanine-resistant cells per 10^6 cells corrected by cell viability. Cytotoxicity induced by MWNT-7 depended on solvent used and ultrasonication duration to reduce agglomeration but was generally lower than that of chrysotyle.	Negative No gene mutations were induced in the *HPRT* locus by either MWNT-7 or chrysotyle A. Cell viabilities decreased to 21 and 17 %, respectively, in a dose-dependent manner. CHL/IU cells were demonstrated to incompletely internalise MWCNT (as well as chrysotile).	Asakura et al. (2010)
Chromosome aberration test (± S9 mix) OECD TG 473 Chinese hamster lung cells (CHL/IU)	1. MWNT-7 (Mitsui) L: not provided D: 70 nm (containing 3600 ppm Fe, 14 ppm Cr, 6 ppm Bi, 4 ppm Ni) 2. N-MWCNT (Nikkiso) L: not provided (cf. Ema et al. 2013)	Dosing: 0, 12.5, 25, 50, and 100 µg/mL for N-MWCNT and 6.25, 12.5, 25, 50, and 100 µg/mL for MWNT-7 Exposure time: 6 h and 24 h	(Positive) Neither MWNT-7 nor N-MWCNT induced structural chromosomal aberrations.	Ema et al. (2012)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
	D: 44 nm (containing 176 ppm calcium, 80 ppm aluminium, 53 ppm iron, 16 ppm cadmium and 0.5 ppm lithium) Well dispersed MWCNT	Positive controls: mitomycin c (0.06, 0.12 µg/mL) and benz[A]pyrene (30 µg/mL) No inhibition of growth was observed in a preliminary cytotoxicity study, at concentrations ranging from 0.05 to 100 µg/plate of MWCNT (± S9 mix).	However, MWNT-7 strongly increased the incidence of numerical chromosomal aberrations at 100 µg/mL after 6 and 24 h exposure in the absence of metabolic activation. Numerical aberrations were slightly increased also in case of N-MWCNT at the highest concentration. Positive controls induced marked structural mutations under the conditions used.	
Chromosome aberration test No standard test guideline followed Chinese hamster lung cells (CHL/IU)	MWNT-7 (Mitsui, Lot No. 061220) L: 5.0 ± 4.5 µm (38.9 % > 5 µm) D: 88 ± 5 nm (av.: 57 nm) Well dispersed isolated fibres Chrysotile A asbestos fibres were concomitantly tested	Dosing: MWNT-7: 0.078 to 80 µg/mL for 24 and 48 h Chrysotyle A: 0.8 to 20 µg/mL for 24 and 48 h Positive control: Mitomycin C at 0.04 µg/mL. Cytotoxicity induced by MWNT-7 depended on solvent used and ultrasonication duration to reduce agglomeration but was generally lower than that of chrysotyle.	(Positive) MWNT-7 induced polyploidy without structural chromosome aberration, similar to chrysotile. CHL/IU cells were demonstrated to incompletely internalise MWCNT (as well as chrysotile).	Asakura et al. (2010)
Micronucleus test (Cytokinesis block) OECD TG 487 BEAS 2B (transformed human bronchial epithelial cells)	Test material: 1. “MWCNT-S” (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = “NRCWE-6”) L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.)	Dosing: MWCNT-S: 0, 2.5, 5, 10 and 20 µg/cm^2, corresponding to 12.5, 25, 50 and 100 µg/mL MWCNT-T: 0, 5, 10, 50 and 100 µg/cm^2, corresponding to 25, 50, 250 and 500 µg/mL	Negative Neither straight nor tangled MWCNT induced micronuclei under the conditions applied. The highest dose of MWCNT-S tested was 20 mg/cm^2 since at higher doses the analysis was hampered by the presence of the fibres.	Catalán et al., 2016

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
	Straight MWCNT (= MWNT-7) 2. "MWCNT-T" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) Tangled MWCNT	Exposure time: 4, 24 and 48 h; addition of cytochalasin B (Cyt B) after 6 h (Extended exposure period should have allowed access of TS to chromatin; delayed Cyt B addition ensured no interference with cellular uptake). Cytotoxicity: MWCNT-S produced a dose-dependent decrease in BEAS-2B cell number after 4, 24 and 48h). MWCNT-T induced a slight dose-dependent decrease in the cell number at 4 h and 24 h, but not at 48 h. Top doses were adjusted accordingly. Positive controls: mitomycin C		
Micronucleus test (Cytokinesis-blocked) No standard test guideline followed. BEAS 2B (transformed human bronchial epithelial cells) A549 (human lung adenocarcinoma epithelial cell line)	1. NM-400 (= Baytubes; tangled) L: 726.3 ± 1.8 nm D: 10.8 ± 1.3 nm 2. NM-401 (JRC; physico-chemically similar to MWNT-7 but somewhat more flexible) L: 3366.4 ± 1.9 nm D: 62.8 ± 1.4 nm 3. NM-402 (JRC; tangled?) L: 1141.3 ± 2.0 nm D: 10.7 ± 1.3 nm 4. NM-403 (JRC; tangled?) L: 394.3 ± 1.6 nm D: 11.1 ± 1.5 nm	Dosing: 0, 16, 32, 64, 128 µg/cm^2 Exposure time: 3 and 48 h; addition of Cyt B after 6 h Positive control: 0.15 µg/mL mitomycin Cytotoxicity: (Replication index [RI]): A549 cells: NM-401 at 32 and 128 µg/cm^2 only. BEAS-2B cells: NM-401 at 64 µg/cm^2. NM-400 and NM-402 actually increased the RI, whereas NM-403 did not affect RI.	Positive (depending on MWCNT and cell type) Only the two longest MWCNT - NM-401 and NM-402 - produced statistically significant increases in the micronuclei frequencies in A549 cells but not in BEAS 2 B cells, comparatively to the controls. [Diagrams revealed high limits of variation and micronuclei induction at each concentration level, increases becoming more significant at concentrations > 32 and < 64 µg/cm^2 only]	Louro et al. (2016)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Micronucleus test (Cytokinesis block) OECD TG 487 Human peripheral lymphocytes	1. NM-400 (= Baytubes; tangled) L: 726.3 ± 1.8 nm D: 10.8 ± 1.3 nm 2. NM-401 (JRC; physico-chemically similar to MWNT-7 but somewhat more flexible) L: 3366.4 ± 1.9 nm D: 62.8 ± 1.4 nm 3. NM-402 (JRC; tangled?) L: 1141.3 ± 2.0 nm D: 10.7 ± 1.3 nm 4. NM-403 (JRC; tangled?) L: 394.3 ± 1.6 nm D: 11.1 ± 1.5 nm 5. NRCWE-006 (= MWNT-7) L: 4423.6 ± 2.3 nm D: 69.4 ± 1.4 nm 5. NRCWE-007 (= Cheap Tubes) L: 368.7 ± 2.0 nm D: 15.3 ± 1.5 nm	Dosing: 0 – 256 µg/mL Exposure time: 30 h Cyt B addition after 6h. (Extended exposure period should have allowed access of TS to chromatin; delayed Cyt B addition ensured no interference with cellular uptake). None of the MWCNT were found to be strongly cytotoxic, thus the highest concentration was limited by TS dispersibility. Positive control: mitomycin c (0.075 and 0.167 µg/mL) Agglomeration of the materials was reduced by a standard dispersion protocol (including 16 min. of probe sonication in 0.05 wt% BSA/water). Top dose tested was 256 µg/mL since higher concentrations affected dispersibility.	Positive Significant increases in micronuclei frequencies were detected at 15 µg/mL for NM-402, at 2.5 and 15 µg/mL for NRCWE-006, and for NM-403 at all doses except at 125 µg/mL. The remaining MWCNT did not induce micronuclei. Although NM-403 and NRCWE-006 displayed positive results, no dose-effect relationship was found.	Tavares et al. (2014)
Micronucleus test No standard test guideline followed A 549 cells (human lung carcinoma)	MWNT-7 (Mitsui) (iron contamination: 3500 ppm) L: 2 µm (mean; > 70 % 1-4 µm) D: 90 nm (mean; > 80 % 70-120 nm	Dosing: 0, 20, 100, 200 µg/mL Exposure time: 6 h Cells were harvested 42 h later. No information on cytotoxicity provided. A positive control was not run. No Cyt B block applied.	Positive Significant and dose-dependent increase in the number of micronucleated cells (frequency 8.6 % at the highest dose compared to 1.2 % in control).	Kato et al. (2013)
Micronucleus test No standard test guideline followed	1. MWNT-7 (Mitsui, Lot No. 061220) L: 5.0 ± 4.5 µm (38.9 % > 5 µm)	Dosing: 0.02 to 5 µg/mL Exposure time: 48 h	Negative	Asakura et al. (2010)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Chinese hamster lung cells (CHL/IU)	D: 88 ± 5 nm (av.: 57 nm) Chrysotile A (UICC) (34.9 % 0.5 to 1.0 µm, 15.2 % 2-5 µm in length) served as positive control fibre	Cytotoxicity induced by MWNT-7 depended on solvent used and ultrasonication duration to reduce agglomeration but was generally lower than that of chrysotyle.	Neither MWNT-7 nor chrysotile clearly induced micronuclei formation. However, both materials significantly increased the number of bi-and multinucleated cells,	
Comet assay (alkaline single cell gel electrophoresis) No standard test guideline followed. BEAS 2B (transformed human bronchial epithelial cells)	1. "MWCNT-S" (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = "NRCWE-6") L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.) Straight MWCNT (= MWNT-7) 2. "MWCNT-T" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) Tangled MWCNT	Dosing: MWCNT-S: 0, 5, 10, 50 and 100 µg/cm^2, corresponding to 19, 38, 190 and 380 µg/mL) for 24 h. MWCNT-T: 0, 5, 10, 50, 100 and 200 µg/cm^2, corresponding to 0, 19, 38, 190, 380 and 760 µg/mL) for 24 h MWCNT-S produced a dose-dependent decrease in BEAS-2B cell number after all treatment times). MWCNT-T induced a slight dose-dependent decrease in the cell number at 4 h and 24 h, but not at 48 h. Top doses were adjusted accordingly.	Positive MWCNT-S induced DNA strand breaks in BEAS-2B cells at low doses (5 and 10 µg/cm^2), whereas MWCNT-T strand breakage increased only at 200 µg/cm^2.	Catalán et al., 2016
Comet assay (alkaline single cell gel electrophoresis) and FPG-modified comet assay (DNA repair endonuclease treatment to detect oxidative damage) No standard test guideline followed.	1. NM-400 (= Baytubes; tangled) L: 726.3 ± 1.8 nm D: 10.8 ± 1.3 nm 2. NM-401 (JRC; physico-chemically similar to MWNT-7 but somewhat more flexible) L: 3366.4 ± 1.9 nm D: 62.8 ± 1.4 nm 3. NM-402 (JRC; tangled?) L: 1141.3 ± 2.0 nm D: 10.7 ± 1.3 nm	Dosing: 0, 16, 32, 64, 128 µg/cm^2 Exposure time: 3 h and 24 h Positive controls: 0.75 mM of ethyl methanesulfonate or 10 mM of hydrogen peroxide. Cytotoxic effects investigated in accompanying MN assay (Replication index [RI]): A549 cells: only NM-401 at 32 and 128 µg/cm^2.	Negative None of the MCNT types caused a significant alteration in the percentage of DNA in tail in either cell type, irrespective of exposure time or FPG modification of the comet assay.	Louro et al. (2016)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
BEAS 2B (transformed human bronchial epithelial cells) A549 (human lung adenocarcinoma epithelial cell line)	4. NM-403 (JRC; tangled?) L: 394.3 ± 1.6 nm D: 11.1 ± 1.5 nm	BEAS-2B cells: NM-401 at 64 µg/cm^2. NM-400 and NM-402 actually increased the RI, whereas NM-403 did not affect RI.	Authors attributed negative findings (which partially contradicted positive findings in other studies using the same cell types) by the shorter length of MWCNT but also considered variability in preparation, media and performance of the assay.	
Comet assay (alkaline single cell gel electrophoresis) No standard test guideline followed. Transformed rat peritoneal mesothelial cells	„NT-50“ (Showa Denko) Fibres of high crystallinity, highly aggregated D: 52.4 nm, L: 4.6 µm [The test material was identical to "NT50b" in the study by Nagai et al. (2011), which proved to induce mesothelioma at a high incidence in rats following i.p. administration but failed to be internalised by MeT5A or HPMC mesothelial cells or MWCK II epithelial cells (however, NT50b was phagocytosed by RAW264.7 macrophages)] .	Dosing: Not reported Exposure time: Not reported Cytotoxicity: No information	Negative (pristine MWCNT) Positive (coated MWCNT) Whereas no significant increase in tail length or tail moment was observed with pristine NT50, coating with haemoglobin (Hb) or transferrin (Tf) significantly increased these DNA strand break parameters in mesothelial cells without concomitant increase in cytotoxicity or apoptosis. It was hypothesised that Hb and Tf coating enabled receptor-mediated endocytosis that resulted in intracellular iron overload which in turn induced high intracellular ROS levels leading to oxidative DNA damage.	Wang et al. (2016)
Comet assay (alkaline single cell gel electrophoresis) and	Commercial MWCNT, pristine and functionalised (Heji, China): 1. pristine MWCNT: D: 32 ± 2 nm	Dosing: 5, 10, 20 and 40 mg/mL for MWCNT and 1, 5, 10, 20 and 40 µg/mL for MWCNT-OH	Positive	Ursini et al. (2012)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
FPG-modified comet assay (DNA repair endonuclease treatment to detect oxidative damage) No standard test guideline followed. A549 (human lung adenocarcinoma epithelial cell line)	L: 0.07 - 7.80 µm Aggregate size: 8.1 ± 2 µm Zeta potential: -9.8 mV 2. functionalised MWCNT: D: 18 ± 1 nm L: 0.02 – 1.70 µm Aggregate size: 6.6 ± 1.3 µm Zeta potential: -10.1 mV Purity (both) : up to 97.37 % Impurities: Cl: 0.20 % Fe: 0.55 % Ni: 1.86 % S: 0.02 % The functionalized nanotubes have –OH > 5 wt.%. "Bamboo-like" nanotubes w/o a defined inner channel and not well defined outer diameter which changes abruptly along the tube.	Exposure times: 2,4 and 24 h No positive control Cell viability after 24 h by corrected MTT assay: MWCNT: 64 % at 5µg/mL and MWCNT-OH: 58 % at 40 µg/mL	A concentration-dependent increase of direct DNA damage (tail moment), significant at 40 µg/mL of MWCNTs and beginning from 5 µg/mL of MWCNT-OH was detected at all exposure times. Oxidative DNA damage by FPG modified comet assay was not observed. Analyses of cell membrane damage (LDH) and apoptosis suggested different uptake mechanisms for pristine vs. functionalised (more soluble) MWCNT.	
SCE test (sister chromatid exchange) No standard test guideline followed. Chinese hamster ovary (CHO) AA8 cells	MWNT-7 (Mitsui) (iron contamination: 3500 ppm) L: 2 µm (mean; > 70 % 1-4 µm) D: 90 nm (mean; > 80 % 70-120 nm	Dosing: 0, 0.1, 1.0, 2.0 µg/mL Exposure time: 1 h No information on cytotoxicity provided.	Positive An SCE frequency approximately three times the control level was observed in cultures treated with 1.0 mg/mL MWCNT. The increase was statistically significant at 0.1 mg/mL or higher concentrations.	Kato et al. (2013)

Further *in vitro* information

A number of in vitro studies are summarised below, which, however, mostly investigated the genotoxicity of MWCNT with deviating tube dimensions compared to the length and diameter requirements of this CLH

proposal. Long, low-diameter MWCNT (≤ 15 nm) in particular usually agglomerate strongly, resulting in particle-like structures of more or less densely tangled nanotubes, if not rigorously dispersed.

For instance, agglomerated, particle-shaped Baytubes with a suspected tube diameter of 14 nm (Wirnitzer et al., 2009) and long (~ 20 µm) and thin (10-15 nm) dispersed CM-95 fibres (Kim et al. 2011) were negatively tested in the Ames test and in the chromosome aberration test. In contrast, MWCNT with a diameter of 10-30 nm and length range between 0.5 and 50 µm (Sigma) induced chromosomal aberrations in RAW 264.7 mouse macrophages, including defects, irregular condensation and aneuploidy (DiGiorgio et al., 2011). The study also reported a positive comet assay but only at a lower concentration, whereas micronuclei were induced in a dose-dependent manner. Catalàn et al. (2012) observed structural chromosomal aberrations in human lymphocytes induced by short MWCNT (1-2 µm) and a diameter of 10-30 nm, but only after completion of two cell cycles. Using the same test material, Lindberg et al. (2013) exposed human epithelial (MeT-5A) and bronchial epithelial (BEAS 2B). In MeT-5A cells but not in BEAS-2 cells MWCNT caused a dose-dependent induction of (slight) DNA damage as proved by the comet assay. No clear effects were found with regard to micronuclei or DNA adducts. Administration of up to 100 µg/mL of pristine of 3 different types of functionalized, tangled Nanocyl types of an average diameter of 15 nm and variable in length (< 1 -1.5 µm) did not result in micronuclei formation in Balb/3T3 mouse fibroblasts but induced morphological transformation in the cells (Ponti et al., 2013).

The EU project NANOGENOTOX tested a number of well-characterised nanomaterials (Table 9a) following standardised sample preparation protocols in a variety of genotoxicity tests in vitro and *in vivo*. The results produced were not without ambiguity across participating laboratories (NANOGENOTOX, 2013), demonstrating that even slight modifications in test conditions and handling may influence the test outcome. Another important finding of the project was the clear cell type differences in vitro assays, as exemplified for the micronucleus test in Table 9b. No further details are presented from the project, as major outputs are published and considered elsewhere (*in vitro* genotoxicity: Tavares et al., 2014, Louro et al., 2016, Catalán et al., 2016; in vivo: Poulsen et al. 2015).

Table 9a. MWCNT samples as used in the NANOGENOTOX project

Sample code	Commercial name (Supplier)	Average TEM particle size	Average BET & SAXS SSA	TGA mass-loss	Main elemental impurities	Average DLS Zeta-size
NM-400	Baytubes (Bayer)	D=14 nm L<1 µm	254 m²/g	84 wt%	Al, Fe, Co, Zn, Na	55 nm
NM-401	JRC (IO-LE-TECNanomaterials, CP0006-SG) (physicochemically similar to MWNT-7 but somewhat more flexible)*	D=64 nm L<5 µm	18 m²/g	82 wt%	Fe, Zn, Na	710 nm
NM-402	JRC?	D=13 nm L<5 µm	2526 m²/g	89 wt%	Fe, Al, Na	NA
NM-403	JRC?	D=12 nm L<0.5 µm	135 m²/g	97 wt%	Co, Mn, Mg, Al, Na	NA
NRCWE-006	MWNT-7 (Mitsui)	D=74 nm L<10 µm	26 m²/g	82 wt%	Co, Fe, Mg, Al, Na	682 nm
NRCWE-007	MWCNT 8-15 OD (Cheap Tube)	D=17 nm L<0.5 µm	96 m²/g	94 wt%	Ni, Fe, Cr, Co, Na	223 nm

TEM: transmission electron microscopy; BET: Brunauer–Emmett–Teller; SSA: specific surface area; DLS: Dynamic Light Scattering; SAXS: Small-angle X-ray scattering; TGA: Thermogravimetric Analysis (Source: NANOGENOTOX Final Report, March 2013)

Table 9b. Micronucleus testing in vitro results of different MWCNT in NANOGENOTOX (Source: NANOGENOTOX Presentation at Final Conference, February 2013)

Organ of origin	**Lung**			**Intestine**	**Blood**
Tissue	Bronchial epithelial		Alveolar epithelial	Colon epithelial	Lymphocytes
Cell line or type	BEAS 2B	16 HBE	A549	Caco-2	Primary
NM-400	(+)	-	(+)	(+)	-
NM-401	+	-	-	+	-
NM-402	+	-	+	+	(+)
NM-403	+	-	+	(+)	+
NRCWE-006	+	-	+	-	+
NRCWE-007	+	-	+	+	-

***In vivo* database**

Table 10: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
Transgenic rodent gene mutation assay (*gpt* and SPI$^-$ mutation assay) (lung tissue) No standard test guideline followed. *gpt* delta transgenic mice (n=6-7, m). Exposure: Intratracheal instillation	MWNT-7 (Misui) L: 2 µm (mean; > 70 % 1-4 µm) D: 90 nm (mean; > 80 % 70-120 nm)	Administration of one, two or four doses (multiple dosing one week distance in between) of 0.2 mg/animal. Mice were sacrificed 8-12 weeks after MWCNT administration. Only the lung was analysed for genotoxicity, accompanied by pulmonary histopathology.	Positive MWNT-7 showed significant increases of *gpt* mutant frequencies in the lungs but no increased Spi$^-$ mutation frequencies. The only mutation class that was identified as significantly elevated levels compared to controls in the *gpt* gene were G:C to C:G transversions. Since these lesions are not caused by 8-oxodG, the authors assumed other oxidative products of guanine contributing to these lesions. Histopathology revealed a dose-related increasing inflammatory response, characterised by macrophage infiltration and lymphocyte infiltration, bronchiolar and visceral subpleural fibrosis, hyperplasia of bronchial and alveolar epithelia. Severe inflammation was confirmed by positive immunohistopathological staining of inflammation factors (NO synthase and nitrotyrosine).	Kato et al. (2013)
Mammalian erythrocyte micronucleus test OECD TG 474 ICR mice (n=6/group, m) Exposure: Oral gavage	1. MWNT-7 (Mitsui) L: not provided D: 70 nm (containing 3600 ppm Fe, 14 ppm Cr, 6 ppm Bi, 4 ppm Ni) 2. N-MWCNT (Nikkiso) L: not provided (cf. Ema et al. 2013) D: 44 nm (containing 176 ppm calcium, 80 ppm aluminium, 53 ppm iron, 16 ppm cadmium and 0.5 ppm lithium) Well dispersed MWCNT	Dosing: 5, 10, 20 mg/kg bw, administered twice via oral gavage at 24 h intervals Sampling time: 24 h after second gavage Positive control: Mitomycin C (5 mg/kg bw) Negative control: 0.3 % sodium carboxymethyl cellulose (20 mg/kg bw)	Negative Neither N-MWCNT nor MWNT-7 induced an increase in the frequency of micronucleated immature erythrocytes. The 20 mg/kg bw dose of MWNT-7 or N-MWCNT did not cause significant toxicity.	Ema et al. (2012)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Mammalian micronucleus test Bone marrow polychromatic erythrocytes (PCE) in 1st exp. Alveolar type II lung cells or in tail blood erythrocytes in 2nd exp. OECD TG 474, automated (PCE) C57BI/6 mice (n=6/group) Exposure: Inhalation	1. "MWCNT-S" (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = "NRCWE-6") L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.) → Straight MWCNT (= MWNT-7) 2. "MWCNT-T" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) → Tangled MWCNT	Dosing: 1st exp.: 8.2 ± 1.7 mg/m^3 MWCNT-S or 17.5 ± 2.0 mg/m^3 MWCNT-T (n= 8/group, f) 2nd exp.: 10.8 ± 2.8 mg/m^3 MWCNT-S (n=6/group, f) Exposure for 4 days (4h/day) and sacrifice after 24 (1st exp.) or 72 h (2nd exp.) of exposure Positive control: 1mg/mouse tungsten carbide-cobalt mixture (by pharyngeal aspiration) or 2 mg/kg mitomycin C (by intraperitoneal injection). Samples were collected 24 h after exposure.	Positive (local) and negative (systemic) MWCNT-S did not induce a significant systemic genotoxic effect in bone marrow or in blood tail samples but was able to induce a significant increase in micronucleated type II cells. In contrast, MWCNT-T induced a significant decrease (!) in micronucleated cells s in bone marrow Histopathology of the lung showed dose-dependent accumulation of both MWCNT materials after pharyngeal aspiration, mainly inside the bronchia and, to a lesser extent, in the alveolar lung tissue. After the inhalation exposure, both MWCNTs were distributed mainly in the alveolar lung tissue. MWCNT-S, but not MWCNT-T, showed infiltration of eosinophils as well as activated macrophages, in the peribronchial and perivascular areas of the lungs, indicating a lung inflammatory response. In all the exposures, the percentage of PCEs was similar to the values of unexposed mice, indicating no bone marrow toxicity Authors assumed that the erythrocyte micronucleus assay may not be appropriate for detecting the genotoxicity of nanomaterials, but should be replaced by techniques revealing local effects.	Catalán et al. (2016)
DNA adduct analysis (lung tissue) No standard test guideline followed. ICR mice (=5/group, m)	MWNT-7 (Misui) L: 2 µm (mean; > 70 % 1-4 µm) D: 90 nm (mean; > 80 % 70-120 nm)	Single dose of 0.2 mg/animal 3, 24, 72 or 168 h later the mice were sacrificed. Only the lung was analysed for genotoxicity, accompanied by pulmonary histopathology.	Positive	Kato et al. (2013)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Exposure: Intratracheal instillation			DNA adducts related to oxidative stress (8-oxo-7,8-dihydro-2'-deoxyguanosine, [8-oxodG]) and lipid peroxidation (heptanone etheno-deoxyribonucleosides HεdC and HεdG) were significantly increased and maintained for 72 h due to MWCNT exposure, as quantified by stable isotope dilution LC-MS/MS, suggesting that inflammatory oxidative stress is involved in MWCNT-induced genotoxicity. Histopathology revealed a dose-related increasing inflammatory response, characterised by macrophage infiltration and lymphocyte infiltration, bronchiolar and visceral subpleural fibrosis, hyperplasia of bronchial and alveolar epithelia. Severe inflammation was confirmed by positive immunohistopathological staining of inflammation factors (NO synthase and nitrotyrosine).	
Mammalian erythrocyte micronucleus test OECD TG 474 ICR mice Exposure: Oral gavage	1. "MWCNT-S" (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = "NRCWE-6") L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.) → Straight MWCNT (= MWNT-7) 2. "MWCNT-T" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) → Tangled MWCNT	Dosing: 1st exp.: 8.2 ± 1.7 mg/m^3 MWCNT-S or 17.5 ± 2.0 mg/m^3 MWCNT-T (n= 8/group, f) 2nd exp.: 10.8 ± 2.8 mg/m^3 MWCNT-S (n=6/group, f) Exposure for 4 days (4h/day) and sacrifice after 24 (1st exp.) or 72 h (2nd exp.) of exposure Positive control: 1mg/mouse tungsten carbide-cobalt mixture (by pharyngeal aspiration) or 2 mg/kg mitomycin C (by intraperitoneal injection). Samples were collected 24 h after exposure.	Positive (local) and negative (systemic) MWCNT-S did not induce a significant systemic genotoxic effect in bone marrow or in blood tail samples but was able to induce a significant increase in micronucleated type II cells. In contrast, MWCNT-T induced a significant decrease in micronucleated cells s in bone marrow Histopathology of the lung showed dose-dependent accumulation of both MWCNT materials after pharyngeal aspiration, mainly inside the bronchi and, to a lesser extent, in the alveolar lung tissue. After the inhalation exposure, both MWCNTs were distributed mainly in the alveolar lung tissue. MWCNT-S, but not MWCNT-T, showed infiltration of eosinophils as well as activated macrophages, in the peribronchial and perivascular areas of the lungs, indicating a lung inflammatory response.	Catalán et al. (2016)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			In all the exposures, the percentage of PCEs was similar to the values of unexposed mice, indicating no bone marrow toxicity Authors assumed that the erythrocyte micronucleus assay may not be appropriate for detecting the genotoxicity of nanomaterials, but should be replaced by techniques revealing local effects.	
Alkaline comet assay (BAL and lung cell suspensions) OECD TG 489 C57BI/6 mice Exposure: Inhalation and Pharyngeal aspiration	1. "MWCNT-S" (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = "NRCWE-6") L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.) → Straight MWCNT (= MWNT-7) 2. "MWCNT-T" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) → Tangled MWCNT	<u>Inhalation conditions:</u> 1st exp.: 8.2 ± 1.7 mg/m^3 MWCNT-S or 17.5 ± 2.0 mg/m^3 MWCNT-T (n= 8, f) 2nd exp.: 10.8 ± 2.8 mg/m^3 MWCNT-S (n=6/group, f) Exposure for 4 days (4h/day) and sacrifice after 24 (1st exp.) or 72 h (2nd exp.) of exposure Positive control: 1mg/mouse tungsten carbide-cobalt mixture (by pharyngeal aspiration) or 2 mg/kg mitomycin C (by intraperitoneal injection). Samples were collected 24 h after exposure. <u>Aspiration conditions:</u> 1st exp.: 1, 10, 40 µg/mouse MWCNT-S 2nd exp.: 50, 100 and 200 µg/mouse MWCNT-S 3rd exp.: 10, 40, 100 and 200 µg/mouse MWCNT-T (n = 6/group, f). Positive control:	Positive MWCNT-S dose-dependently induced DNA damage in lung but not in BAL cells after pharyngeal aspiration, which became significant at the highest dose tested (200 µg/mouse). After inhalation, the level of DNA damage was significantly increased by MWCNT-S both in lung and BAL cells. MWCNT-T induced no significant increase in DNA damage but rather a decrease, both in BAL and in lung cells. Histopathology of the lung showed dose-dependent accumulation of both MWCNT materials after pharyngeal aspiration, mainly inside the bronchia and, to a lesser extent, in the alveolar lung tissue. After the inhalation exposure, both MWCNTs were distributed mainly in the alveolar lung tissue. MWCNT-S, but not MWCNT-T, showed infiltration of eosinophils as well as activated macrophages, in the peribronchial and perivascular areas of the lungs, indicating a lung inflammatory response. Authors assumed that the erythrocyte micronucleus assay may not be appropriate for detecting the genotoxicity of nanomaterials, but should be replaced by techniques revealing local effects.	Catalán et al. (2016)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		1 mg/mouse tungsten carbide-cobalt mixture Negative control: PBS plus 0.06 % BSA Mice were sacrificed 24 days after exposure		
Comet assay (lung tissue) C57BL mice No standard test guideline followed Exposure: Intratracheal instillation	1. NM-401 ("CNT_{Large}") (CP0006-SG; IO-LE-TECNanomaterials,) L: 4.05 ± 2.40 µm D: 67 ± 26.2 nm (physicochemically similar to MWNT-7 but more flexible) Impurities 3 wt.% 2. NRCWE-026 ("CNT_{Small}") (NC-7000, Nanocyl) L: 0.85 ± 0.457 µm D: 11 ± 4.5 nm Impurities 13 wt.%	Dosing: 18, 54, 162 µg/mouse Single administration (n=6/does group, f) Sampling time: 1, 3 or 28 days postexposure	Positive: A significant, dose-dependent increase in tail length was observed for NM-401 1 day post-exposure, whereas NRCWE-026 induced positive effects at day 3 post-exposure and at the middle and high dose level. Both MWCNT types induced similarly strong acute phase and inflammatory responses in the lung as indicated by elevated BAL cell levels (neutrophil levels persisted 28 d p.e.), fibrosis-related gene expression profile changes and interstitial pneumonia. The outcome of a dichlorodihydrofluorescein diacetate (DCFH-DA) assay, which detects the presence of acellular free radicals, indicated that DNA strand breaks by NM-401 may have resulted from reactive oxygen species (ROS) generated during (ineffective) host defense rather the from the test material itself, as metal contamination was low. In contrast, NRCWE-026 induced a strong dose-dependent DCFH-DA response, which was speculated to be caused by metal impurities.	Poulsen et al. (2015)
Alkaline comet assay Japanese standard protocol ("International Validation of the In vivo	N-MWCNT (Nikkiso) L: 2.7 µm (median: min.: 0.3 µm, max.; 23 µm) D: 44 nm (containing 176 ppm Ca, 80 ppm Al, 53 ppm Fe, 16 ppm Cd and 0.5 ppm Li)	Dosing: single exposure: 0.2 and 1.0 mg/kg bw Repeated exposure; 0.04 and 0.2 mg/kg bw once a week for 5 weeks Sampling time:24 h after single exposure or 3h last instillation of repeated exposure	Negative: No changes in DNA damage were observed in lung cells. Inflammatory responses at all tested doses (deposition of test substances in macrophages, thickening of the alveolar wall, and infiltration of the alveolus by macrophages and neutrophils, multifocal hemorrhage in the alveolus).	Ema et al. (2013)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens", Japanese Center for the Validation of Alternative Methods) Crl/CD(SD) rat (males, n=5/group) Exposure: Single or repeated intratracheal instillation	Individually dispersed MWCNT	Positive control: Ethyl methanesulfonate (500 mg/kg, 3h before sacrifice) Negative control: aqueous solution of Triton X-100 at 0.5mg kg-1 by a single or repeated intratracheal instillation		
Alkaline comet assay (lung tissue) No standard test guideline followed. ICR mice (n=5, m) Exposure: Intratracheal instillation	MWNT-7 (Mitsui) L: 2 µm (mean; > 70 % 1-4 µm) D: 90 nm (mean; > 80 % 70-120 nm)	Single doses of 0.05 or 0.2 mg/animal (n=5, m). 3 h later the mice were sacrificed. Only the lung was analysed for genotoxicity, accompanied by pulmonary histopathology.	Positive MWNT-7 significantly induced DNA damage in the lung of ICR mice in a dose-dependent manner. Histopathology revealed a dose-related increasing inflammatory response, characterised by macrophage infiltration and lymphocyte infiltration, bronchiolar and visceral subpleural fibrosis, hyperplasia of bronchial and alveolar epithelia. Severe inflammation was confirmed by positive immunohistopathological staining of inflammation factors (NO synthase and nitrotyrosine).	Kato et al. (2013)
γ-H2AX assay (marker assay on peripheral blood mononuclear cells and lung cell suspensions)	1. "MWCNT-S" (MWCNT-XBRI-7, Mitsui, Lot# 05072001K28; = "NRCWE-6") L: 7 ± 4.5 µm (av.) D: 60 ± 15 or 71 ± 21 nm (av.)	<u>Inhalation conditions:</u> 1st exp.: 8.2 ± 1.7 mg/m^3 MWCNT-S or 17.5 ± 2.0 mg/m^3 MWCNT-T (n= 8, f) 2nd exp.: 10.8 ± 2.8 mg/m^3 MWCNT-S (n=6/group, f)	Negative MWCNT-S did not significantly increase the frequency of γ-H2AX positive cells at any of the tested doses (1–200 µg/mouse), either in blood cells or in lung cells. However, a borderline significant increasing linear dose–response was found when higher doses of MWCNT-S were tested (second experiment) in the lung cells, but not in the blood cells.	Catalán et al. (2016)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No standard test guideline available C57BI/6 mice Exposure: Inhalation and Pharyngeal aspiration	→ Straight MWCNT (= MWNT-7) 2. "MWCNT-L" (8-15 nm OD, Cheap Tubes, = "NRCWE-7") L: 0.37 µm (av. from literature) D: 21 ± 9 or 21 ± 17 nm (av.) → Tangled MWCNT	Exposure for 4 days (4h/day) and sacrifice after 24 (1st exp.) or 72 h (2nd exp.) of exposure Positive control: 1mg/mouse tungsten carbide-cobalt mixture (by pharyngeal aspiration) or 2 mg/kg mitomycin C (by intraperitoneal injection). Samples were collected 24 h after exposure. <u>Aspiration conditions:</u> 1st exp.: 1, 10, 40 µg/mouse MWCNT-S 2nd exp.: 50, 100 and 200 µg/mouse MWCNT-S 3rd exp.: 10, 40, 100 and 200 µg/mouse MWCNT-T (n = 6/group, f). Positive control: 1 mg/mouse tungsten carbide-cobalt mixture Negative control: PBS plus 0.06 % BSA Mice were sacrificed 24 days after exposure	Histopathology of the lung showed dose-dependent accumulation of both MWCNT materials after pharyngeal aspiration, mainly inside the bronchia and, to a lesser extent, in the alveolar lung tissue. After the inhalation exposure, both MWCNTs were distributed mainly in the alveolar lung tissue. MWCNT-S, but not MWCNT-T, showed infiltration of eosinophils as well as activated macrophages, in the peribronchial and perivascular areas of the lungs, indicating a lung inflammatory response.	

<u>Further in vivo information</u>

Below, data from in vivo genotoxicity studies is summarised that tested low-diameter MWCNT types (< 30 nm).

Muller et al. (2008) investigated MWCNT, supplied by the laboratory of Nuclear Magnetic Resonance, Facultés universitaires Notre-Dame de la Paix, Namur with a carbon content of 97.8 % (0.95 % cobalt, ~ 0.5 % Fe), an average length and outer diameter of 0.7 µm and 11.3 nm, respectively, in a cytokinesis-block micronucleus assay in MCF-7 (48h) or immortal rat liver epithelial cells (24h) at 10, 25 and 50 µg/mL. MWCNT treatment dose-dependently increased micronucleus formation in both cell types. Cytotoxicity and apoptosis was also induced at 50 µg/mL and 25 µg/mL doses, respectively. A pancentromeric FISH probe demonstrated that MWCNT induced both, clastogenic and aneugenic effects in MCF-7 cells. In concomitant in vitro assays, the same MWCNT induced micronuclei in rat lung epithelial cells as well as centromere-

positive and –negative micronuclei in the human carcinoma cell line MCF-7. In a separate study it was shown that genotoxicity in vitro and in vivo was reduced upon prior heating of this test material to 2400 °C - which led to annealing of structural defects and elimination of metal impurities – but restored upon grinding, which introduced structural defects in the carbon framework (Muller et al., 2008a).

Kim et al. (2011) injected mice intraperitoneally with up to 50 mg/kg with well dispersed thin (10-15 nm) MWCNT of 20 µm (CM-95, Hanwa Nanotech, Inc.) or 50 µm length (size selected fraction of CM-95) but failed to induce micronuclei in bone marrow cells (as tested according to OECD TG 474) with either test material.

In a second study (Kim et al. 2012) exposed rats to well dispersed long and thin MWCNT (D: 10-15 nm, L: ~ 20 µm) of CM-95 by whole body inhalation up to 0.94 mg/m^3 for 5 days and analysed isolated lung cells one and two month post exposure in the comet assay. Statistically significant increase of DNA damage in lung cell at the highest tested 1 month after the last exposure was observed. Concomitantly increasing H_2O_2 formation in BALF indicated involvement of an indirect genotoxic mechanism. Impurities of the MWCNT contained iron (< wt 2 %), cobalt (< wt 2 %) and Al_2O_3 (< wt 4 %).

In a later study, Kim et al. (2014) exposed rats by whole body inhalation with CM-100 (similar dimensions and impurities as CM-95; Hanwa Nanotech, Inc.) for 28 days. Lung cells were sampled 28 and 90 days postexposure, respectively. DNA damage in lung cells was found at all tested doses (0.17, 0.49 and 0.96 mg/m^3) in the comet assay 28 days postexposure. DNA damage was still observed 90 days postexposure at the higher doses.

Embedded in a subchronic inhalation toxicity study (OECD TG 413), Pothmann et al. (2015) investigated the local and distal genotoxicity of Graphistrength C100 (Arkema), a highly tangled and agglomerated MWCNT (L: 750 ± 623 nm, max. 2.9 µm; D: 11.8 ± 3.0 nm. Residual metals content: Al: 3.0, Fe: 2.1 % w/w). After 90 days of exposure of male rats to 0.05, 0.25 and 5.0 mg/m^3 neither an increase in the frequency of micronucleated polychromatic erythrocytes (PCE) in bone marrow, nor signs of cytotoxicity was observed in rats of either sex. Likewise, a comet assay, in the absence and presence of human 8-hydroxyguanine DNA-glycosylase, was negative in isolated lung, liver and kidney cells. However, no MWCNT deposits were detected in the liver, kidneys and bone marrow and other organs. Exposure to the highest concentration resulted in a persistent inflammatory lung response, the release of inflammatory factors in the BALF, and changes in the differential white blood cells counts. The slight changes in BALF parameters at 0.25 mg/m^3 recovered and signs of lung clearance of the MWCNT were observed. No pathological changes were observed on the pleura.

Human data

Human data is not available.

Mechanistic studies

Two in vitro studies may shed light on the mode of genotoxic action in terms of aneuploidy.

Siegrist et al. (2014) reported that MWCNT of an average length of 825 nm and an average diameter of 15 nm induced a dose-responsive increase in disrupted centrosomes, abnormal mitotic spindles and aneuploidy chromosome number in immortalised lung epithelial cells (BEAS-2B) or primary epithelial cells (SEAC) following treatment at 0.024, 0.24, 2.4, or 24 µg/cm^2 for 24 h. 3D imaging indicated that MWCNT integrated with microtubule, with DNA and within the centrosome structure. Cell cycle analysis implied induction of cell-cycle arrest in G1/S transition in treated BEAS-2B cells. SEAC cells recovered from an initial reduction in viability and colony formation dramatically increased in all but the highest exposure dose, indicating a potential to pass the genetic damage to daughter cells. The predominance of monopolar spindles with fragmented centrosomes and the cell cycle block were distinct from the disruption pattern induced by (thinner) SWCNT, which rather induce multipolar spindles and a G2 cell cycle block. It was hypothesized that the integration of the more rigid MWCNT into centrosomes may cause centrosome fracture and prevents their separation, whereas the incorporation of this stiffer material into microtubules may reduce the elasticity of the

mitotic spindle apparatus to a greater degree than the SWCNT. Both effects may seriously affect the chromosomal separation during cell division.

Yasui et al. (2015) provided an explanation for the high frequency of aneuploidism and polyploidism as well as binucleated cells observed when treated with fibre-like MWCNT. They administered MWNT-7 (Mitsui) to MDA-435 human breast cancer cells and followed their endocytosis and mitosis by live-cell imaging analysis. It was shown that during anaphase, relatively short MWCNT fibres (approximately 5 µm) migrated rapidly to either of the daughter cells, whereas some long MWCNT fibres (approximately 20 µm) remained inside the contractile ring and induced the formation of binucleated cells through impairment of cytokinesis. The authors concluded that the mechanism of polyploidisation by MWCNTs appears to very similar to that observed with crocidolite asbestos.

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

In vitro

A number of studies tested the genotoxic potential of long (> 5 µm) and high diameter (> 30 nm) MWCNT in vitro, either alone or in comparison to other (shorter and/or thinner) MWCNT or asbestos fibres (cf. Tables 9 and 9a for tube dimensions of test materials used).

Few data is available from mutagenicity tests (Asakura et al., 2010; Ema et al., 2012). MWNT-7 as well as shorter but also inflexible MWCNT (N-MWCNT, Nikkisho) proved negative in the Ames test. However, internalisation of the tested MWCNT was not proven. For the same reason, the negative results in bacterial mutagenicity assays, which tested commercial low-diameter MWCNT (CM-95 and Baytubes) are unreliable (Wirnitzer et al., 2009; Kim et al., 2011). More informative, MWNT-7 proved negative in the gene mutation test (HPRT locus) up to 100 µg/mL using hamster lung cells (Asakura et al., 2010). Though a standard test guideline was not followed, cellular uptake of the well-dispersed nanotubes was demonstrated. Concomitantly tested chrysotile asbestos fibres also did not induce mutations in the HPRT locus. Apart from the absence of mutagenicity, both materials were dose-dependently cytotoxic, possibly due to incomplete internalisation of isolated fibrous material.

Both fibre-like MWCNT types that were negative in the gene mutation tests (MWNT-7, N-MWCNT), also did not induce structural aberrations in mammalian chromosomal aberration assays (Asakura et al., 2010; Ema et al., 2012). However, the incidence of polyploid cells significantly increased (similar to chrysotile asbestos), indicating induction of numerical aberration. In contrast, short (1-2 µm) and low diameter (10-30 nm) MWCNT induced structural chromosomal aberrations in human lymphocytes (Catalán et al., 2012). MWCNT with a broad size range appear to induce both, structural and numerical aberrations, as demonstrated by the occurrence of aneuploidy metaphases (Di Giorgio et al., 2011).

Micronucleus tests (with or without cytokinesis block), which are able to detect clastogenic and aneugenic effects, yielded conflicting results. MWNT-7 induced micronuclei in human lung carcinoma cells (Kato et al., 2013) and peripheral lymphocytes (Tavares et al., 2014) but not in transformed human bronchial epithelial cells (Catalán et al., 2016) or in hamster ovary cells (Asakura et al., 2010), indicating cell type specificity as observed in the NANOGENOTX project. NM-401, MWCNT of similar diameter but shorter than MWNT-7, was the only MWCNT of five types tested which resulted in a significant increase in micronuclei of human lymphocytes (Poulsen et al., 2015). The other four negative MWCNT were much thinner and thus presumably of less marked fibrous morphology. A new publication based on results of the NANOGENOTOX project reported that the two longest MWCNT tested, NM-401 and NM-402, induced micronuclei in the human lung adenocarcinoma cell line A549 but not shorter NM-400 and NM-403, indicating that MWCNT length (irrespective of rigidity) may be a determinant of genotoxicity (Louro et al., 2016).

However, it should be noted that increases were subject to high limits of variation, which in case of another human lung cell line used (BEAES-2B) led to the conclusion of micronuclei-decreasing effect of MWCNT. Inconsistent findings with MWCNT of diverging dimensions and shapes were reported by Di Giorgio et al.

(2011) and Ponti et al. (2013). Hence, a clear morphological relationship cannot be concluded from the available testing results. It also remains unclear, if negative outcomes were a result of a combination of lack of cellular uptake, agglomeration, sample preparation and cellular toxicity rather than absence of genotoxicity. Interestingly, MWNT-7 treatment resulted in a significantly increased number of bi- and multinucleated cells (the same was found for chrysotile treatment), indicating possible interference with cell cleavage (see also under "mechanistic studies").

With regard to indicator assays, two studies are available that provided evidence for a clastogenic potential of MWNT-7. A comet assay in transformed human bronchial epithelial cells (BEAS-2B) revealed DNA damage, which was induced by MWNT-7 at considerably lower doses compared to a short nanotube type MWCNT, though MWNT-7 did not induce micronuclei in these cells (Catalán et al., 2016). These findings are in contrast to recent findings by Louro et al. (2016), who tested NM-401negative in the comet assay or the FPG-modified comet assay in BEAS-2B or in A549 cells, even at higher concentrations than Catalán et al. (2016). Similarly, NM-401 induced no increased micronuclei in BEAS-2B cells but did so in A549 cells at higher concentrations, indicating cell-specificity (see below). MWCNT type specificity was also highlighted by the study of Ursini et al. (2012), who tested pristine and OH-functionalised (thus more soluble and less aggregated) MWCNT of "bamboo-like" shape of apparently little flexibility. Both MWCNT types proved positive in the direct comet assay but did not reveal oxidative damage in a FPG modified comet assay. OH-MWCNT showed a higher genotoxic potential, which was explained by differences in the uptake mechanisms compared to the pristine MWCNT. Another study, which tested sister chromatid exchanges (SCE) in a non-guideline test reported that MWNT-7 significantly increased the SCE frequency in hamster ovary cells already at 0.1 µg/mL, compared to control treatment (Kato et al., 2013). However, it should be noted that SCE has been discouraged by OECD as a reliable test method for genotoxicity in 2014. MWCNT with high aspect ratio variability were also positive in the comet assay at low concentrations (1 µg/mL) in phagocytosing RAW mouse macrophages, indicating direct DNA damage which was correlated with increased intracellular ROS production (Di Giorgio et al., 2011). Interestingly, Wang et al. (2016) have shown that high diameter MWCNT (~ 50 nm), which proved to be mesotheliomagenic in vivo (Nagai et al. 2011), induced DNA strand breaks in mesothelial cells, when coated with haemoglobin and transferrin but not without coating. This was attributed to cellular uptake of the coated in contrast to the pristine test material, possibly involving transferrin receptor-mediated endocytosis, resulting in high intracellular iron levels promoting oxidative DNA damage. Unfortunately, the study missed important details on dosing.

MWCNT consisting of short (0.7 µm), low diameter (11.3 nm) nanotubes were shown to act both, as clastogen and as aneugen *in vitro*, evidenced by differential centromer-immunostaining in MCF-7 cells, but failed to induce mesotheliomas in a 2-year intraperitoneal bioassay (Muller et al., 2008, 2009; see section 10.9). However, the induction of aneuploid cells could have been due to high cobalt impurities of the tested MWCNT, as cobalt is known to cause chromosome loss at low doses.

In vivo

A transgenic rodent gene mutation assay following multiple intratracheal instillations of *gpt* delta mice is available for MWNT-7 (Kasai et al., 2013). Mutation analysis was confined to the lung, germ cells were not examined. A higher mutagenic frequency in the *gpt* locus of the lung was demonstrated compared to vehicle controls, which however did not show clear dose dependency. The same study analysed DNA adducts in the lungs of ICR mice after single intratracheal instillation. MWNT-7 induced oxidative and lipid peroxide-related adduct formation (8-oxodG, HεdA, HεdC, HεdG). Apart from DNA adducts, several findings supported the assumption that mutagenicity involved oxidative stress and inflammation, such as increased G:C to C:G transversions in transgenic mice as well as positive NO synthase, nitrotyrosine staining the lung of similarly treated ICR mice, supported by (immune-) histopathologic evidence of pulmonary inflammation.

Two in vivo micronucleus tests in mice provided more heterogeneous results. Following OECD TG 474, Ema et al. (2012) were unable to detect an increase in the frequency of micronucleated bone marrow erythrocytes after oral exposure to well-dispersed high-diameter (MWNT-7 [70 nm] or N-MWCNT [44 nm]). Likewise, Catalán et al. (2016) did not find a significant increase of micronucleated erythrocytes (neither in bone marrow nor in tail vein specimen) after inhalation or pharyngeal aspiration exposure (this was also true for concomitantly tested low-diameter Cheap Tubes). However, a significant increase of micronuclei was found

in alveolar type II cells isolated from the lung of mice exposed to MWNT-7 by inhalation, indicating site-of contact genotoxicity, which coincided with signs of lung inflammation.

Several in vivo comet assays demonstrated a DNA damaging potential of MWNT-7 (cf. Table 10 for tube dimensions). The aforementioned study by Catalán et al. (2016) reported a dose-dependent increase in the induction of DNA strand breaks in suspended lung cells but not in isolated BAL cells of mice exposed by pharyngeal aspiration following OECD TG 489 which, however, became significant only at the highest exposure concentration (200 µg/mouse). After inhalation, MWNT-7 induced DNA damage in both lung and BAL cells, which was particular prominent in the latter. As part of the NANOGENOTOX project, Poulsen et al. (2015) compared large, thick MWCNT ("NM401", similar to MWNT-7) with small, curled MWCNT ("NRCWE-026") in the comet assay with automated scoring after intratracheal instillation of mice. An instillation of NRCWE-026 mainly affected the level of DNA strand breaks at the middle and high dose on post-exposure day 3, whereas instillation of NM401 affected all doses at post-exposure day 1 only. As demonstrated by transcriptome analysis and histopathology, both MWCNT types induced an acute phase and a persistent inflammatory response; however, the onset was earlier for the former, which also induce more fibrosis. Differences in the DNA damaging potential of the two MWCNT types were attributed to the contribution of intrinsic and extrinsic ROS generation. Cell-free ROS generation was assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The authors speculated that in particular the lack of metal impurities of the NM-401 nanotubes may have induced a biological ROS response in the lung caused by disruption of phagosomes and lysosomes in phagocytosing alveolar macrophages (it was assumed that the tube length of ~ 4 µm of NM-401 was too short to cause frustrated phagocytosis). In contrast, NRCWE-026 induced a strong dose-dependent DCFH-DA response, indicating that ROS generation and (oxidative) DNA damage was primarily caused by metal contaminants. Kato et al. (2013) also tested MWNT-7 positive in the alkaline comet assay of isolated lung cell suspensions after single intratracheal instillation of mice in the same study, which provided evidence for oxidative DNA adduct formation under these exposure conditions, which resulted in a severe inflammatory response. MWNT-7 significantly and dose-dependently induced DNA damage in the lung of ICR mice. In contrast, using a Japanese standard protocol, Ema et al. (2013) showed that well-dispersed N-MWCNT (Nikkiso) did not cause DNA damage in isolated lung cells in the comet assay after single intratracheal installation of rats at doses eliciting an inflammatory response. It is unclear if this distinct outcome is due to differences in fibre dimensions (median length 2.7 µm, range: 0.3 – 23 µm, mean diameter: 44 nm).

In addition to positive site-of-contact genotoxicity in mice (micronuclei and DNA in comet tail) after respiratory tract exposure, Catalán et al. (2016) also applied a non-standard indicator γ-H2AX assay to assess phosphorylated (serine 139) H2A histone family member in lung cell suspensions and peripheral blood leukocytes. However, a clearly positive result was not obtained, either for MWNT-7 or for tangled Cheap Tubes, independent of the cell origin. However, a non-significant borderline dose response was observed at higher doses, which was limited to lung cells exposed to MWNT-7.

Overall conclusions

There is considerable evidence that exposure to fibre-like MWCNT induces genotoxicity in vivo. However, genotoxicity is limited to site-of-contact effects after pulmonary exposure and is only partially supported by corresponding in vitro data.

A transgenic rodent assay proved positive in the lung after intratracheal installation (germ cells were not investigated) but without clear dose dependency. Likewise, several positive micronucleus and comet assays supported a clastogenic as well as an aneugenic activity for MWNT-7 in the lung but not in distant organs, such as bone marrow. This is possibly due to a lack of systemic availability of nanotubes and thus target organ reachability issues (which, for instance, prompted Catalán et al. (2016) to doubt the usefulness of OECD TG 474 for testing nanomaterials towards bone marrow or peripheral erythrocytes).

Negative or ambivalent test results in vitro should be taken with care, when assessing particles. A negative Ames assay may result from lack of particle uptake. In fact, several experts even discouraged the Ames assay for testing nanomaterials (Doak et al., 2012; Clift et al., 2013). Furthermore, the negative *hgprt* mutation assay and ambiguous findings in indicator tests using mammalian cells may indicate cell type specificity of genotoxic response but also indicate that other factors are required to bring about mutagenicity and/or genotoxic effects

in vivo. It should also be considered that ignorance of following OECD test guideline and GLP in many research-oriented studies as well as differences in storage and handling of nanomaterials between laboratories favour heterogeneous findings.

Positive in vivo findings were frequently associated with oxidative stress and inflammation in the lung, thus secondary genotoxicity cannot be excluded. This is supported by positive findings of test material with apparent low intrinsic oxidizing potential. However, the role of an intrinsic ROS potential and the role of metal impurities on a direct DNA damaging potential of MWCNT is currently not well understood and cell-based as well as cell-free data are inconsistent.

Other than for clastogenicity, there is evidence to assume a direct genotoxic potential of MWCNT as an aneugen from micronucleus and chromosome aberration tests. This is supported by mechanistic studies which provided evidence that MWCNT may interfere with the mitotic spindle apparatus, perhaps by mimicking microtubuli. A similar aneugenic activity is also known from other fibrous materials such as asbestos (Yegles et al., 1995, Cortez et al., 2011, Donaldson et al., 2013). N-MWCNT, which was recently shown to induce mesothelioma in instilled rats (Suzui et al., 2016; see section 10.9), proved negative in vivo in the comet assay under conditions of pulmonary inflammation but induced numerical aberrations indicative of an aneugenic action. It should be noted that for aneugens which act on non-DNA targets, in general the existence of a threshold is assumed (Elhajouji et al., 1995).

Taken together, the genotoxicity data show that exposure to fibre-like MWCNT may favour clastogenic as well as aneugenic effects in the lung when inhaled. DNA damage in vivo may be directly induced and/or secondary due to oxidative stress and inflammation response, whereas it is plausible to assume direct interference of nanotubes with mitotic spindle formation resulting in aneuploidy.

This dossier is not assessing classification of germ cell mutagenicity of MWCNT. However, it is concluded that based on the available data, a genotoxic mode of action cannot be excluded, supporting the carcinogenic potential of MWCNT.

10.8.2 Comparison with the CLP criteria

Classification for germ cell mutagenicity is not subject of this dossier

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Classification for germ cell mutagenicity is not subject of this dossier

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS provided a non-exhaustive database of *in vitro* and *in vivo* genotoxicity and mutagenicity studies to support the proposed classification for carcinogenicity. However, assessment of the need for classification as a germ cell mutagen was not intended and was therefore outside the scope of the assessment by RAC.

The DS concluded that the genotoxicity data show that exposure to fibre-like MWCNT may favour clastogenic as well as aneugenic effects in the lung when inhaled. DNA damage *in vivo* may be directly induced and/or may be secondary due to oxidative stress and an inflammation response, whereas it is plausible to assume direct interference of nanotubes with the mitotic spindle formation, resulting in aneuploidy. Based on the available data, a

genotoxic mode of action cannot be excluded, supporting the carcinogenic potential of MWCNT.

Comments received during consultation

As germ cell mutagenicity was outside the scope of the CLH proposal, no specific comments on genotoxicity and/or mutagenicity were provided. It was commented that genotoxicity has been observed in rats and mice for MWCNT with a length less than 5 µm (the proposed lower limit). Furthermore, some additional references to genotoxicity and mutagenicity studies were provided.

Assessment

As this hazard class was outside the scope for this proposal, the RAC assessment was focused on the mutagenicity data as support for the classification for carcinogenicity. Therefore, the focus of the RAC assessment was on *in vivo* studies and on the lung tissue. In addition, the effect of the dimensions of the MWCNT to induce mutagenicity *in vivo* was assessed to support the definition of the MWCNT to which such a classification would be applicable.

The available *in vitro* studies indicate that MWCNT induce numerical chromosomal changes and polyploidy in chromosome aberration tests. Micronucleus tests and comet assays gave inconsistent results, depending on the tested cell line and the dimensional properties of the MWCNT.

In vivo results in lung tissue after inhalation exposure were mainly positive in several types of tests including a transgenic rodent assay, a micronucleus assay and several comet assays. The results indicated the formation of reactive oxygen species which could either be related to inflammation but also to the presence of metal impurities in the MWCNT. Based on the positive outcome of some *in vivo* micronucleus tests in lung cells and the in numerical chromosomal changes *in vitro*, clastogenic and/or aneugenic results were observed. The aneugenic effect could be explained by interference of the fibres with the mitotic spindle apparatus. The available *in vivo* studies with multiple MWCNTs indicated some differences in mutagenicity between straight and tangled MWCNT and the related fibre dimensions with stronger effects in straight MWCNTs. In a direct comparison, Catalan *et al* (2016) showed that straight MWCNT (mean 7 µm * 71 nm) induced DNA strand breaks in lung cells, as detected in the comet assay after a single pharyngeal administration in mice (high dose only), whereas a decrease was observed for a tangled type of MWCNT (mean 0.37 µm * 21 nm) in lung cells and bronchoalveolar lavage cells. After inhalation exposure, straight MWCNTs induced an increase in DNA strand breaks in lung and bronchoalveolar lavage cells and an increase in micronucleated alveolar type II cells. No such increases in strand breaks was observed for the tangled type of MWCNT. The formation of micronuclei was not determined for tangled MWCNTs. Poulsen *et al*. (2015) compared the effects of small curled MWCNT (mean length 0.8 µm) with large thick MWCNT (mean length 4 µm) at several dose levels and several timepoints (days 1, 3 and 28) after a single intratracheal exposure. Large MWCNTs induced an increase in strand breaks in lung cells only at day 1 (all dose levels) whereas small MWCNTs induced an increase on day 3 (middle and top doses). As indicated by transcriptome analysis and histopathology, both MWCNT types induced an acute phase and a persistent inflammatory response. There

were some indications that the mutagenic effect was also dependent upon the presence of metal impurities.

Overall, the available *in vivo* mutagenicity tests support lung carcinogenicity of rigid type MWCNTs via a mutagenic mechanism. It is to be noted that on basis of the information available, no strict borders for tube dimensions can be determined, as positive results were also observed for thin tangled MWCNT (Poulsen *et al*., 2015).

Supplemental information - In depth analyses by RAC

RAC identified some mistakes in the provided summaries of the genotoxicity studies. These did not affect the overall outcome of the assessment. The following mistakes were identified:

Table 9: Catalan *et al*., 2016, studied micronucleus induction in BEAS 2B cells which is an immortalised human bronchial epithelial cell line. Micronuclei were evaluated only after 48 h exposure, not after 4 and 24 h exposure (4 and 24 h exposure times were assessed only for cytotoxicity, but not for the induction of micronuclei). MWCNT-S was MWCNT-XNRI-7 (Mitsui), not MWCNT-XBRI.

Table 10: Catalan *et al*., 2016 is reported twice. The second description indicated that also oral exposure was used. This is incorrect, and the description should only refer to inhalation exposure.

Table 10, p.33: Catalan *et al*., 2016, alkaline comet assay: The Comet assay was not done in the second experiment and the MWCNT-S was MWCNT-XNRI-7 (see above). These same comments apply also ϒ-H2AX assay on p. 35 of the CLH report. The study reports only ϒ-H2AX assay results after pharyngeal aspiration (not inhalation).

Also to note that these results reported in Catalan *et al* (2016) were not generated within the NANOGENOTOX project. However, they used two materials provided by the NANOGENOTOX (i.e. Catalan *et al,* 2016 can be deleted from the list on p 25, before table 9a).

10.9 Carcinogenicity

Table 11: Summary table of animal studies on carcinogenicity (see also Annex I, 3.9.1.1.-3.9.1.12)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
Inhalation Long-term study OECD TG 451/GLP Rat	MWNT-7 (Hodogaya; Lot No. 080126 for 88 weeks, Lot No. 071223 from week 89 Average length:	0, 0.02, 0.2, 2 mg/m^3, 104 weeks (whole body exposure)	Significantly increased incidence of lung tumours (mainly bronchiolo-alveolar carcinoma, and combined carcinomas and adenomas) at MWNT-7 exposure of males at 0.2 and 2 mg/m^3 and at 2 mg/m^3 in females. No development of pleural mesothelioma.	Kasai et al. (2016)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
F344/DuCrlCrlj, m/f (age at exposure start: 6 weeks) n= 50/sex and dose group	5.2/5.7 µm, with 45.1/48.7 % of tubes > 5µm Av. diameter: 83.8/90.7 nm. Dispersed fibers collected from inhalation chamber Av. length: 5.4-5.9 µm Av. diameter: 92.9-98.2 nm MMAD (SD): 1.2-1.4 µm (2.6-3.0) Purity: > 99.6 %/>99.8 % (Fe: 4,400 ppm Cr: 48 ppm Ni: 17 ppm)	Dry-aerosol generated and well dispersed MWNT, constant in concentration and separation of individual tubes in exposure chamber over exposure period No positive control	Dose-dependent lung toxicity, such as epithelial hyperplasia, granuloma and focal fibrosis development, accompanied by altered BALF parameters. No macroscopic findings in any other organs, including pleura and peritoneum. Increased lung weights from 0.2 mg/m3 in males and at 2 mg/m3 in females. MWNT-7 lung burdens increased with dose and duration of exposure.	
Transtracheal intrapulmonary spraying Short-term study No standard test guideline followed Rat F344, m n=6/group	1. MWCNT-M (= MWNT-7, Mitsui) Average length: 5.11 µm 3.82×10^{11} fibres/g (Diameter according to WPMN report: 88 nm) 2. MWCNT-N (Nikkiso) Average length: 3.64 µm 3.46×10^{11} fibres/g (Diameter according to WPMN report: 48 nm)	Exposure to 1.25 mg MWCNT/rat: 5 x 250 µg over a period of 9 days Positive control: Crocidolite (1.35 mg)	Both MWCNT types translocated to the pleura as evidenced by pleural cavity lavage, predominantly as phagocytosed material in alveolar macrophages. Both MWCNT types and crocidolite induced hyperplastic visceral mesothelial proliferation (PCNA immunostaining), associated with inflammatory cell infiltration and inflammation and fibrotic lesions of the pleural tissues. However, MWCNT were not found in the mesothelial proliferative lesions themselves, as evidenced by polarized light microscopy and scanning electron microscopy of H&E-stained histology slides. It was assumed that inflammatory action by activated macrophages in the pleura rather than the fibres themselves induced mesothelial lesions (conditioned cell culture media of macrophages treated with MWCNT and crocidolite and the supernatants of pleural cavity lavage fluid from the dosed rats increased mesothelial cell proliferation	Xu et al. (2012)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			in vitro).	
Transtracheal intrapulmonary spraying Medium-term study No standard test guideline followed Rat F344, m n=6/group	1. MWCNT-L D: 150 nm L. 8 µm (needle-shaped) 2. MWCNT-S D: 15 nm L: 3 µm Mean length: 7.34 µm (cotton candy like aggregates)	Exposure to 1.625 mg MWCNT/rat: 13 x 250 µg over a period of 24 weeks No positive control	Needle-shaped MWCNT-L, but not cotton candy-like MWCNT-S, translocated into the pleural cavity and induced fibrotic thickening of the parietal and visceral pleura as well proliferation of both the parietal and visceral mesothelium (PCNA). Inflammatory pleural lavage parameters indicated that MWCNT-L elicited a stronger inflammatory reaction in the pleural cavity than MWCNT-S. In contrast, MWCNT-S caused higher inflammatory reactions (cell number and cytokine/chemokine levels) and 8-OHdG formation in the lung compared to MWCNT-L.	Xu et al. (2014)
Transtracheal intrapulmonary spraying Long-term (observation) study No standard test guideline followed Rat F344, m n=20/group	MWCNT-N (Nikkiso) 3 sieve fractions: 1. "Unfiltered" L: 4.2 ± 2.9 µm D: 30-80 nm (93.4 %) 2. "Flow through" L: 2.6 ± 1.6 µm D: 30-80 nm (93.4 %) 3. "Retained" L: > 2.6 µm (not measurable → dense agglomerates) D: 30-80 nm (93.4 %) Needle or fibre-like appearance Ion content: 0.004-0.005 %	Exposure to 1 mg/rat (8 administrations over a 2-week period). Post-exposure observation up to 109 weeks (animals surviving ≥ 63 weeks were included in the study). Negative controls: no treatment and vehicle control (0.5 % Pluronic F68)	MWCNT remaining in the lungs of rats administered unfiltered MWCNT-N and the flow-through and retained MWCNT fractions were 25.4, 48.0 and 26.3 %, respectively, of the amount measured at week 2 (= 486 ± 44 µg; 426 ± 116 µg; 268 ± 43 µg, respectively). Pleural malignant mesothelioma as well as lung tumours developed. The incidence of lung tumours (bronchiolo-alveolar adenoma and carcinoma) (14/38; 36.8 %) together was significantly higher than the control group (0/28; 0 %) while no significant differences in the incidence of lung tumours or total tumour burden was found among the three groups administered the different MWCNT-N sieve fractions. The incidence of malignant mesothelioma in the three	Suzui et al. (2016)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			MWCNT groups of different sieve fractions combined, 6/38 (15.8 %), was significantly higher compared to the two control groups combined, 0/28 (0 %). The groups administered the unfiltered and flow-through fractions had incidences of 3 mesothelioma cases each and the group administered the (highly agglomerated) retained fraction did not have any cases of mesothelioma. The mesotheliomas were localised in the mediastinal space (*Cavum mediastinale*) and were shown to originate from mesothelial tissue outside of the lung as shown by negative immunostaining for the lung tumour marker thyroid transcription factor-1 (TFF-1). Tumour incidence in other organs was not significant compared to controls.	
Intraperitoneal injection Long-term and short-term observation No standard test guideline followed Rat Male SPF Wistar rat n=50 (long-term) or n= 4-5 (short-term) test Mouse C57BL/6	MWNT-7 ("CNT-7"; MWCNT-XNRI-7, Mitsui) = NRCWE-006 A subset ("short CNT-7") of MWNT-7 was produced by grinding. Both types were annealed at 1500°C.	<u>Long-term experiment:</u> Single dose of 6 mg of CNT-7 ($2x10^9$ WHO fibres) or short CNT-7 ($0.36 x10^9$ WHO fibres); sacrificed after 12 months. (Dose for mice not provided) Positive control: 2 mg crocidolite ($6 x 10^9$ WHO fibres) <u>Short-term experiment:</u> 2 mg (CNT-7: $0.67 x 10^9$ WHO fibres; short CNT-7: $0.12 x 10^9$ WHO fibres; sacrificed after 1, 7, 15 and 30 d.	Both, CNT-7 and short CNT-7 induced mesothelioma, the latter to a lesser extent. First tumours after CNT-7 injection developed mesothelioma after 6 months and the majority of animals developed tumours. In contrast, only one animal developed mesothelioma 12 months after crocidolite injection.	Huaux et al. (2016)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
Sex and group size as well as study duration for long term experiment not given. for short-term experiment: n= 4-5	In addition, two MWCNT types were tested for their immunosuppressive potential in short-term tests, which proved non-carcinogenic in previous i.p. mesothelioma experiments "CNT-M (Muller et al, 2009) and CNT-T (tangled, same as "NTtngl" in Nagai et al., 2011). 1. "CNT-7": L: 7.1 µm (median) D: 75 nm Fibres > 5 µm: 75 % Metallic impurities: Fe: 0.35 w% Co, Ni, Mo: < 0.001 w% 2. "Short CNT-7": L: 2.8 µm (median) D: 75 nm Fibres > 5 µm: 14 % Metallic impurities: Fe: 0.35 w% Co, Ni, Mo: < 0.001 w% 3. "CNT-M" (aggregated): L: 0.7 µm (median) D: 11.3 nm Fibres > 5 µm: <1 %	Positive control: 2 mg crocidolite (6 x 10^9 WHO fibres) Dose for mice: 0.2 mg of CNT-7 (0.67 x 10^8 WHO fibres); sacrificed after 1, 7, 15 and 30 d.	Administration of mesotheliomagenic CNT-7 and short CNT-7 resulted - like asbestos - in an early and persistent recruitment of monocytic Myeloid Derived Suppressor Cells (M-MDSC) as detected in peritoneal lavage. In contrast, non-carcinogenic CNT-M and CNT-T did only transiently induce M-MDSC. The authors speculated that the immunosuppressive activity of these monocytes towards T-lymphocytes, which was verified by in vitro proliferation tests, is an important step in mesothelioma formation by MWCNT in addition to an early inflammatory neutrophil recruitment. The specificity of the early M-MDSC accumulation by MWCNT or asbestos was demonstrated by injection of other substances, such as silica and LPS, which only induced an inflammatory but no immunosuppressive response. Mice did not develop mesothelioma, even after multiple injections of CNT-7 (data not shown). This was attributed to the observation that the monocytic cells harvested from the peritoneum of treated mice did not possess a significant and persistent immunosuppressive activity (no accumulation of M-MDSC).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
	Metallic impurities: Fe: 0.48 w% Co: 0.49 w% Ni, Mo: < 0.001 w% 4. "CNT-T" (tangled) L: 3 µm (median) D: 15 nm Fibres > 5 µm: n/a Metallic impurities: n/a Positive control: crocidolite (L: 3 µm, D: 200 nm, Fibres > 5 µm: 5 w%)			
Intraperitoneal injection Long-term observation No standard test guideline followed Rat Male Wistar Han rCrl:W1 [Han], m n=50/group	1. "MWCNT A" $L_{WHO\ fibres}$: ~ 8.6 µm $D_{WHO\ fibres}$: ~ 85 nm No. of fibres > 20 µm: ~ 3.1 % 2. "MWCNT B" $L_{WHO\ fibres}$: ~ 9.3 µm $D_{WHO\ fibres}$: ~ 62 nm No. of fibres > 20 µm: ~ 9.4 % 3. "MWCNT C" $L_{WHO\ fibres}$: ~ 10.2 µm $D_{WHO\ fibres}$: ~ 40 nm No. of fibres > 20 µm: ~ 11.8 % 4. "MWCNT D" $L_{WHO\ fibres}$: ~ 7.9 µm $D_{WHO\ fibres}$: ~ 37 nm No. of fibres > 20 µm: ~ 2.1 %	Single dose exposure of 1 x 10^9 and 5 x 10^9 WHO fibres per animal, respectively This corresponds to: 0.2 and 1 mg (MWCNT-A) 0.6 and 3 mg (MWCNT-B) 0.08 and 0.4 mg (MWCNT C) 0.25 and 1.4 mg (MWCNT D) Samples dispersed in surfactant-like 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to prevent aggregation Observation period: two-years Positive control: 1x 10^8 WHO fibres of amosite asbestos	The test tailor-made MWCNT types exhibited a rigid-fibre like morphology with slight geometrical variations and a considerable share of WHO fibres. All types induced malignant mesotheliomas in all dose groups. Though a clear dose-dependency was not observed, mesothelioma development was more rapid and severe in case of more straight MWCNT types compared to more flexible ones and even to amosite asbestos. MWCNT D – the most curved type compared to the rather straight types A-C - showed the highest latency in mesothelioma development, indicating that curvature may be an additional factor of fibre geometry for carcinogenic potency of MWCNT. Immunohistochemical marker staining demonstrated similarity to mesotheliomas induced by asbestos that occurred in humans : 64 % of mesotheliomas were of the sarcomatoid type, 32 % of the biphasic and 4 % of the epitheloid type.	Rittinghausen et al. (2014)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			Most tumours invaded peritoneal organs, the diaphragm in particular.	
Intraperitoneal injection Long-term observation No standard test guideline followed Rat Fisher 344, f Brown Norway F1 hybrid, m n=11-60/ group	1. "NT50a" = MWNT-7 (Mitsui) Needle-like fibres of high crystallinity, highly aggregated D: 49.95 nm, L: 5.3 µm 2. NT50a(-agg*)] subfraction of NT50a 3. „NT50b"(Showa Denko) Fibres of high crystallinity, highly aggregated D: 52.4 nm, L: 4.6 µm 4. "NT145" (Showa Denko) "thick" nanotubes, aggregation low D: 143.5 nm; L: ~4.3 µm 5. "NTtngl" (Sowa Danko) Tangled nanotubes, very high aggregation. D: 15 nm; L: 3 µm	1 or 10 mg/rat NT 50a, NTtngl or NT145, 10 mg/ rat NT50b. In addition, a non-agglomerated subfraction of NT50a with fibre numbers equivalent to 1 mg NT145 was tested. [= eq. NT50a(-agg*)] Two administrations in in a 1-week interval Observation period: 1 year. No positive control	1 mg of crystalline NT50a or an equivalent amounts (eq.) of NT50a(-agg*) induced malignant mesothelioma with a higher frequency and earlier progression than 1 mg of NT145. (based on survival rates). Incidences of 10 mg NT145 or of NT50b were as high as of 1 mg. NT50a or eq. NT50a(-agg*). 10 mg of NTtngl did not induce mesotheliomas. 86 % of mesotheliomas exhibited a sarcomatoid histology (0.9 % had an epithelioid, 12.1 % a biphasic phenotype). MWCNT-induced mesotheliomas shared homozygous deletion of Cdkn2a/2b tumour suppressor genes, similar to asbestos-induced mesotheliomas. It is noted that early half of the rats administered 10 mg i.p. of either MWCNT type died within several days and the remaining animals were used as the 10 mg-injection group. The mechanism for acute death is unknown, though arterial and/or lymphatic embolism is suspected (Nagai et al., 2013).	Nagai et al. (2011)
Intraperitoneal injection Short-term observation No standard test guideline followed Rat Fisher 344, f/	1. "NT50a" = MWNT-7 (Mitsui) Needle-like fibres of high crystallinity, highly aggregated D: ~50 nm, L: 5.3 µm 2. "NT145" (Showa Denko) "thick" nanotubes, aggregation low D: 143.5 nm; L: ~4.3 µm	1 mg/rat NT 50a 1 or 5 mg NT145 or NTtngl. Single exposure Observation period: 1 month No positive control	The needle-like NT50a was a potent inducer of fibrotic inflammation, causing severe fibrotic peritonitis and mesothelial proliferation. Thicker or tangled MWCNT (NT145 and NTtngl, respectively) induced mild inflammatory fibrosis but no mesothelial proliferation. All tested MWCNT types were phagocytosed by macrophages and caused local granuloma formation.	Nagai et al. (2011)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
Brown Norway F1 hybrid, m n= 3-6/ group	3. "NTtngl" (Sowa Danko) Tangled nanotubes, very high aggregation. D: 15 nm; L: 3 µm		Concomitant in vitro cytotoxicity studies suggested that the difference in the extent of inflammation between the MWCNT types was related to the ability to induce direct mesothelial injury at the end of the one month observation time.	
Inhalation Short-term tumour promotion study No standard test guideline followed Mouse B6C3F1 hybrid, m	MWNT-7 (Mitsui-7, Hodogaya, lot # 061220-31) MMAD: 1.59 µm, CMAD: 0.42 µm Trace metal contamination 1.32 % (Fe: 1.06 %)	Two step treatment: 1. Initiation: Single i.p. dose of MCA (methylcholanthrene; 10 µg/g bw) or vehicle (corn oil). 2. Challenge (1 week after initiation): whole body inhalation of MWNT-7 (5 mg/m^3, 5 hours/ day) or filtered air (controls) for 15 days. At 17 months post-exposure, mice were sacrificed and examined.	MWNT-7 proved to be a strong promoter of pulmonary adenomas and adenocarcinomas in mice after short-term inhalation. 90.5 % of MWCNT-exposed mice developed one or the other tumour when pre-treated with MCA, compared to 14 % without pre-treatment, which was close to the air-control group (13 %). Several pre-treated mice also developed malignant serosal tumours consistent with sarcomatous mesothelioma, as demonstrated by podoplanin immunostaining. Early events encompassed focal adenomatous hyperplasia (focal alveolar epithelial hyperplasia resembling human atypical adenomatous hyperplasia), macrophage infiltrations into the lung as well as lesion-associated foreign material in the alveolar tissue, the interstitium, and also within alveolar macrophages. Incidences for the (primary) hyperplasia were 27 % for MCA + MWCNT; compared to 5 % MWCNT alone and 2 % each for MCA and air control. The significantly increased incidence of focal adenomatous alveolar hyperplasia after 15 days of inhalation exposure by MWNT-7 w/o MCA compared to air-exposed controls indicated an initiator role of MWNT-7 in carcinoma development in mice.	Sargent et al. (2014)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			The particle lung burden in MWCNT-exposed mice was equivalent to occupational settings (31.2 µg/lung).	
Intraperitoneal injection Medium-term observation No standard test guideline followed Mouse SLC mutants, m ($p53^{+/-}$ heterozygosity) n=19/group	MWNT-7 (Mitsui) D: 100 nm L: 27.5 % > 5 µm (100 % < 20 µm) Fe 0.35 % Aggregates among dispersed rod-shaped or fibrous particles in administered suspension (0.5 % methyl cellulose).	Single injection of 3 mg/mouse (1×10^9 MWCNT Observation period: 25 weeks (due to 100 % mortality) Positive control: crocidolite (3 mg/mouse)	MWCNTs induced mesothelioma, which were invasive to the abdominal wall, diaphragm, liver parenchyma and pancreas, and in some case involving the thoracic cavity. Distant metastasis was not observed (day 172 after injection). All MWCNT-treated animals died before the end of the observation period. Large tumours invaded the abdominal wall, diaphragm, liver parenchyma, and pancreas, rarely also involving the thoracic cavity. Distant metastases were not observed. Fibre-laden cells were not only found in peritoneal lesions but also in the liver and in mesenteric lymph nodes. The overall mesothelioma incidence at day 84 post i.p. treatment was even higher for MWCNT (87.5 %) compared to Crocidolite (77.8 %).	Takagi et al. (2008)
Intraperitoneal injection Mouse Long-term observation No standard test guideline followed SLC mutants, m ($p53^{+/-}$ heterozygosity) n=20/group	MWNT-7 (Mitsui) D: 100 nm L: 27.5 % > 5 µm (100 % < 20 µm) Fe 0.35 % Aggregates among dispersed rod-shaped or fibrous particles in administered suspension (0.5 % methyl cellulose).	Single intraperitoneal injection of 3, 30, 300 µg/mouse, corresponding to $1x10^6$, $1x10^7$, $1x10^8$ particles/mouse Observation period: up to 1 year No positive control	Dose-dependent mesothelioma induction with cumulative incidence of 5/20, 17/20 and 19/20, respectively (increasing particle numbers). Most mesothelioma were lethal. The 15 surviving mice at low dose treatment showed focal mesothelial atypical hyperplasia. No mesothelioma was observed in the vehicle control group. Histology of the mesotheliomas ranged from a differentiated epithelioid type to an undifferentiated sarcomatous type. Osteoid and rhabdoid differentiations, both known in human asbestos mesothelioma cases, were found in nine mice (across all dose groups). Peritoneal fibrosis, peritoneal adhesion and formation of foreign body granulomas towards agglomerated MWCNT were dose dependent and minimal in the low-dose group.	Takagi et al. (2012)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
			Severity of peritoneal adhesion and granuloma formation was dose-dependent, as estimated by logarithmic approximation of mesothelioma mortality plots. However, the time of tumour onset was apparently independent of the dose, which let the authors favour an indirect carcinogenic effect by humoral stimuli on mesothelial cells near loci of frustrated phagocytosis. As for asbestos, the authors discouraged a practical threshold due to mesothelioma induction at a dose as low as 3µg/mouse.	
Intrascrotal injection Long-term observation No standard test guideline followed Rat Fischer 344 DuCrlCrlj, m n=7/group	MWNT-7 (Mitsui) D : 82 % in range 70-110 nm L : 72.5 % in range 1-4 µm Fe 0.35 % Agglomerates and dispersed as multi-sized rod-shaped or fibrous particles in administered suspension (2 % carboxymethyl cellulose)	Single injection of 1 mg/kg bw Observations period: 52 week Positive control: crocidolite (2 mg/kg bw)	MWCNTs induced mesotheliomas in 6 of 7 treated rats that died prior to the end of the study. The overall incidence of mesothelioma was even significantly higher than those of crocidolite-treated rats. These advanced stage tumours, which developed from mesothelial hyperplasia over polyploidy or papillary mesotheliomas, invaded into adjacent tissues and organs and metastasized into the pleura. Beside these mesothelial proliferative lesions, granulomas with high cellularity, including macrophages and multinucleated giant cells were observed. Rats treated with 2 mg/kg bw crocidolite also developed granulomas but no mesotheliomas (the authors explained the absence of mesotheliomas with the low particle number concentration of crocidolite).	Sakamoto et al. (2009)

Recently, findings of a carcinogenicity study following OECD TG 453 and GLP became available, which demonstrated that MWNT-7 induced carcinomas and adenomas in the lung of F344 of rats following 104 weeks of whole body inhalation (Kasai et al., 2016). Three doses of well dispersed, dry aerosol-generated test material were tested (0.02, 0.2 and 2 mg/m^3) and both sexes developed lung tumours. However, male rats turned out to be more sensitive than females, as carcinomas in males were found already at mid-dose whereas females developed a similar number of malignant tumours at the highest dose only. In addition to the

bronchiolo-alveolar carcinomas, the low incidence of rare tumour types in this rat strain, such as adenosquamous carcinoma, poorly differentiated adenocarcinoma and squamous cell carcinoma (the latter two tended to be multiple tumours) substantiated that tumours were induced by the test material. In addition, a clear concentration-dependent incidence of (pre-neoplastic) lung lesions was detected, such as bronchiolo-alveolar, alveolar and bronchiolar (atypical) hyperplasia, focal fibrosis of the alveolar wall and granulomatous changes (Table 11a). Likewise, BAL parameters (cell counts, ALP, LDH, total protein) were dose-dependently increased in males and females. Neither MWNT-7 related mortality nor clinical signs were observed during the exposure period. In all dose groups, growth and body weight gain were not different from the control group. Absolute and relative lung weights were significantly elevated in males (0.2 mg/m^3) and females (2 mg/m^3). Other organ weights were inconspicuous. Significantly elevated incidences of granulomatous change and focal fibrosis of the alveolar wall occurred in both sexes at 0.2 and 2 mg/m^3. Alveolar macrophages with phagocytosed MWNT-7 accumulated in the alveolar spaces in male and female rats exposed to 2 mg/m^3. In contrast to the lung, there was no significant increase in tumour incidence in the pleura or in the peritoneum (nor in any other organ). The authors explained the absence of mesotheliomas by the low numbers of MWCNT detected in the pleural region after 104 weeks of inhalation compared to the high bolus doses administered in positive intraperitoneal injection studies. About 1468 individualised tubes in pleural and 2429 tubes in abdominal lavage fluid were counted in males. When compared to the mesothelioma-inducing doses injected intraperitoneally by Rittinghausen et al. (2014) those numbers were 10^6 lower than needed to induce lung tumours (based on lung burden measurements, the authors calculated that the number of MWNT-7 that induced lung carcinoma was 1.38 x 10^9 in males at 0.2 mg/m^3 and 10.4 x 10^9 in females at 2 mg/m^3). Although mesotheliomas were not detected, there were concentration-dependent increases of proliferative lesions, such as mesothelial hyperplasia of the parietal and ventral pleura, focal fibrosis of the parietal pleura and the diaphragm as well as inflammation of the mediastinum was observed in male rats at the highest dose (it is noted that inflammation of the mediastinum of control animals in both sexes was considerable). Females only developed focal fibrosis of the ventral pleura. The gender differences observed in the development of pre-neoplastic and neoplastic lesions were accounted to a higher sensitivity of males, as the MWNT-7 fibre numbers of males and females were calculated as comparable, when related to the differences in lung weight (0.91 x 10^9 and 0.94 x 10^9 in males and females at 2 mg/m^3, respectively). The absence of clinical signs, organ growth or weight effects, together with high survival rates indicated that the top exposure dose (2 mg/m^3) fulfilled the criteria of the maximal tolerated dose. The possible mode of action was discussed in the context of dose-and duration-dependent increasingly retained MWNT-7 numbers in the lung and the specific form of continuous interaction of the indigestible fibres with alveolar macrophages, which might have produced detrimental ROS and cytokines. Interestingly, besides frustrated phagocytosis, fibre masses of several µm diameter, "cocooning" lysed macrophages, were observed in the lung, whereas test material retrieved from pleural and abdominal fluids were single fibres. Fibres (single or aggregated) were also found in the nasal cavity, larynx, trachea, lymph nodes, spleen, liver, kidneys, olfactory bulb, and brain in both sexes but little compared to lungs and mediastinal lymph nodes. It was speculated that in addition to fibre length, fibre quantity and secondary genotoxicity (oxidative stress and inflammation) was important for the MWNT-7 lung pathogenesis. The measured lung burdens were assumed to be occupationally relevant when human equivalent concentrations were calculated and related to realistic workplace exposures. The NOAEL for carcinogenicity was 0.02 mg/m^3, which did not induce lung carcinoma in male rats. For chronic toxicity, a LOAEL of 0.02 mg/m^3 was derived based on significantly increased absolute and relative lung weights in females.

Table 11a: Histopathological findings of the lung, peritoneum and pleura, 2-year whole body inhalation exposure of rats to MWNT-7 (modif. from Kasai et al., 2016)

	Male				Peto test	Female				Peto test
Dose (mg/m³)	**0**	**0.02**	**0.2**	**2**		**0**	**0.02**	**0.2**	**2**	
No. of animals examined	**50**	**50**	**50**	**50**		**50**	**50**	**50**	**50**	
Neoplastic lesions										
Lung										
Bronchiolo-alveolar carcinoma	1	1	8*	10**	↑↑	0	1	0	5**	↑↑
Adenosquamous carcinoma	0	0	0	1		0	0	0	1	
Poorly differentiated adenocarcinoma	0	0	0	0		0	0	0	1	
Squamous carcinoma	0	0	0	0		0	0	0	1	
Total carcinoma	1	1	8*	11**	↑↑	0	1	0	8**	↑↑
Bronchiolo-alveolar cell adenoma	1	1	7*	5		3	1	4	3	
Total adenoma and/or carcinoma	2	2	13**	16**	↑↑	3	2	4	11**	↑↑
Peritoneum										
Malignant mesothelioma	0	3	1	1		0	0	0	0	
Non-neoplastic lesions										
Lung										
Bronchiolo-alveolar hyperplasia	2	6	13†	22††		3	3	8	12†	
Atypical hyperplasia	0	0	1	10††		0	0	0	14††	
Alveolar hyperplasia	0	2	13††	41††		1	1	6	41††	
Bronchiolar hyperplasia	0	0	4	8††		0	0	4	26††	
Accumulation: alveolar macrophages	2	7	5	48††		2	6	9	48††	
Focal fibrosis: alveolar wall	0	2	43††	48††		0	3	44††	49††	
Granulomatous change	0	5	42††	50††		0	3	45††	50††	
Pleura										
Simple esothelial hyperplasia	3	3	7	12†		3	2	6	10	
Focal fibrosis: parietal (diaphragm)	0	0	2	6†		0	0	0	3	
Focal fibrosis: ventral (lung)	0	2	4	19††		0	2	2	20	
Inflammation: mediastinum	15	18	21	26†		17	17	16	19	
Inflammation: diaphragm	0	0	1	1		0	1	1	1	

*: p≤0.05, **: p≤0.01 by Fisher Exact Test; †: p≤0.05, ††: p≤0.01 by Chi-square test; ↑: p≤0.05, ↑↑: p≤0.01 by Peto's test (Source: Kasai et al., 2016)

Earlier, Sargent et al. (2014) provided evidence that MWNT-7 acted as tumour promoter in rodents following inhalation. Mice were treated in a two-step initiation/promotion protocol first with the tumour initiator methylcholanthrene (MCA, 10 µg/g bw by single intraperitoneal injection). One week later the mice were exposed by inhalation to MWNT-7 (5 mg/m^3, 5 hours/day, 5 days/week) for 15 days. After 17 months post-exposure, they were sacrificed and examined for tumour formation. Incidences of adenomatous hyperplasia, adenomas and adenocarcinomas in the lung as well as serosal tumours were determined and provided clear evidence that MWNT-7 acted as a strong tumour promoter in mice. 90.5 % of the animals developed tumours by MCA + MWCNT treatment (adenomas and adenocarcinomas combined) with an average of 2.9 tumours per mouse. In contrast, exposure to MWCNT alone did not result in a significant increase in lung tumour incidence compared to air controls (26.5 % vs. 23.2 %, respectively). Tumour incidence after MCA initiation alone was 51.9 % (Table 11b). Likewise, the incidences separated by bronchiolo-alveolar adenocarcinomas and bronchiolo-alveolar adenomas were highly increased (62 % and 76 %, respectively, compared to 13 % in air control), whereas the incidences for MWCNT alone were not significantly different from controls (14 % and 18 %, respectively). In addition, histological evidence for cellular atypia, infiltration and metastasis was presented. Early events encompassed focal adenomatous hyperplasia (focal alveolar epithelial hyperplasia resembling human atypical adenomatous hyperplasia), macrophage infiltrations into the lung as well as lesion-associated foreign material in the alveolar tissue, the interstitium, and also within alveolar macrophages. Incidences for the (primary) hyperplasia were 27 % for MCA + MWCNT; compared to 5 % MWCNT alone and 2 % each for MCA and air control. Five mice (9 %) exposed to MCA and MWCNT and 1 (1.6 %) exposed to MCA developed (uncommon) serosal tumours morphologically consistent with sarcomatous mesotheliomas, as revealed by specific biomarkers (podoplanin, in particular) in a variety of tissues, including liver, pancreas and epididymis. The lung burden of 31.2 µg/ MWCNT was calculated to be equivalent to that that would be achieved in workers exposed to 7 µg/ m^3 in 13 years and thus being occupationally relevant. Significance of tumour incidences compared to spontaneous tumour formation was supported by the fact that the mouse strain taken for the study (B6C3F1 hybrid) has an intermediate sensitivity to spontaneous lung tumours and a profound historical record. It was concluded that many similarities in the carcinogenesis and tumour type between asbestos and MWCNT exist. MWCNT, like asbestos, may act as a promoter by inducing proliferation in cells genetically damaged due to oxidative and/or mitotic disruptive mechanisms followed by aneuploidy. However, since asbestos can act as a tumour promoter at low doses as well as a tumour initiator at longer and/or higher exposure levels, it was speculated that MWCNT may also act as a complete carcinogen following inhalation. An initiating potential of MWCNT was assumed based on the significantly increased incidence of adenomatous hyperplasia in MWCNT exposed animals (14) compared to air-exposed animals (7), which was rated primary and not regenerative. The low increase in MWCNT-indeuced lung tumours compared to air controls could possibly be due to the short exposure period (15 days).

Table 11b: Incidences of adenomatous hyperplasia and lung tumours in mice 17 months after exposure (Sargent et al., 2014).

	Air	MCA	MWCNT	MCA + MWCNT
No. of animals	56	54	49	42
No. of animals with focal adenomatous alveolar hyperplasia	7	8	14*	26*
No. of bronchiolo-alveolar adenoma	6	18*	9	32*
% of mice with one or more of bronchiolo-alveolar adenoma	11 %	33 %*	18 %	76 %*
No. of bronchiolo-alveolar adenocarcinomas	7	12*	7	26*
% of mice with one or more of bronchiolo-alveolar adenocarcinomas	13 %	22 %*	14 %	62 %*
No. of bronchiolo-alveolar adenoma and/or adenocarcinomasadenocarcinomas	13	28*	13	38*
% of mice with lung tumours	23.2 %	51.9 %*	26.5 %	90.5 %*

* indicates statistical significance at p< .0001 (Fisher's exact test) Source: Sargent et al., Particle and Fibre Toxicology 2014, 11:3, modified

In order to investigate the role of immunosuppression in MWCNT-induced mesotheliomagenesis, Huaux et al. (2016) injected Wistar rats intraperitoneally with MWNT-7 (NRCWE-006; median length: 7.1 µm) or a ground short fibre fraction thereof by a single dose of 6 mg (= 2 x 10^9 WHO fibres and 0.36 x 10^9 WHO fibres, respectively). The majority of animals developed mesothelioma after 12 months (no exact figures provided). First tumours occurred after 6 months. Animals treated with the short-fraction MWNT-7 (median length: 2.8 µm) also developed mesothelioma but at lower incidences over time (however, interim sacrifices were not reported). Injection of crocidolite (6 x 10^9 WHO fibres), which served as positive control, resulted in mesothelioma development in one rat only after 12 months (previous experiments showed that crocidolite did not induce mesothelioma in this rat strain until 14 months after injection). In contrast, the authors reported that 0.67 x 10^9 WHO fibres of MWNT-7 failed to induce mesothelioma in a non-mutant mouse strain (C57BL/6), even after multiple injections. Mesothelioma development in rats was associated with an early recruitment and accumulation of monocytic Myeloid-Derived Suppressor Cells (M-MDSC) in the peritoneal lavage. This subset of immunosuppressing leukocytes, recently identified to play an important role in carcinogenesis, was demonstrated indeed exerting an anti-proliferative activity towards isolated T-lymphocytes from MWCNT-7- or asbestos-treated rats. The authors speculated that this immunosuppressive response is as essential as the inflammatory and oxidative response elicited by MWCNT for mesothelioma development. Specificity of the immunosuppressive response was shown by its absence in mice, the weak and only transient influx of M-MDSC cells in rats injected "non-carcinogenic" MWCNT types, such as CNT-M (Muller et al, 2009) and tangled CNT-T (= "NTtngl" in Nagai et al., 2011; median length: 3 µm, median diameter: 15 nm). Likewise, injection of other non-mesotheliomagenic substances such as lipopolysaccharide (LPS, 5 µg) or silica (2 mg) also resulted in an accumulation of inflammatory neutrophils only (like MWNT-7 in mice) without evident accumulation of M-MDSC.

Using non-physiological intrapulmonary spraying, Xu et al. (2012) provided evidence for mesothelial proliferation following deposition of MWCNT in the rat lung. A total dose of 1.25 mg/animal of two different MWCNTfibre-like MWCNT types (MWNT-7 and poorly described MWCNT-M) to male F344 rats was administered over a period of 9 days. Both MWCNT types translocated to the pleura as evidenced by pleural cavity lavage, predominantly as phagocytosed material in alveolar macrophages and induced hyperplastic visceral mesothelial proliferation as well as inflammation of the pleural cavity, pleural fibrosis, similar to crocidolite. The authors assumed that mesothelial lesions were mediated primarily by activated macrophages in the pleura and not by the free fibres themselves, since fibres were absent from mesothelial lesions. This assumption was supported by in vitro findings using conditioned macrophage medium treated with multi-walled carbon nanotubes (or crocidolite) or the supernatants of pleural cavity lavage fluid from the dosed rats, as both of these fluids increased mesothelial cell proliferation in vitro (see also under "Other studies relevant for carcinogenicity" below). In a follow-up study, Xu et al. (2014) extended the exposure period in rats to 24 weeks, using two different MWCNT types, one forming longer needle-like fibres ("MWCNT-L": ~ 8 µm length, 150 nm diameter) and a second one forming candy cotton-like aggregates of about 3 µm length and 15 nm diameter ("MWCNT-S"). Only the former was found to translocate to the parietal pleura, causing fibrotic thickening of both the parietal and visceral pleura, and mesothelial (parietal and visceral) proliferation (Table 11c). In addition, inflammatory pleural lavage parameters indicated that the needle-like material elicited a stronger inflammatory reaction in the pleural cavity than the shorter MWCNT. On the other hand, the latter material was reported to cause a higher inflammatory response and oxidative damage (higher 8-OGdG levels) in the lung.

Table 11c: Fibrosis and mesothelial proliferation in the pleura (Xu et al., 2014)

	MWCNT-L	MWCNT-S	Pluronic F68 (dispersing agent)
Thickness of parietal pleura (µm)	28.75 ± 10.43†††***	7.28 ± 4.37	7.16 ± 4.95
Thickness of visceral pleura (µm)	18.92 ± 10.13†††***	6.16 ± 2.05*	4.57 ± 1.23
PCNA index (%)	~2.5-2.8†	~0.3-0.5	~ 0.3

*P < 0.05 versus PF68; ***P < 0.001 versus PF68; †††P < 0.001 MWCNT-L versus MWCNT-S by two-tailed Student's t-test. *P < 0.01 versus Pluronic F68 (PF68); †P < 0.01 MWCNT-L versus MWCNT-S (figures estimated from graph).

A new intrapulmonary spraying study revealed formation of both lung tumours and the pleural malignant mesotheliomas in the rat when observed for up to 2 years post exposure (Suzui et al., 2016). Furthermore, these highly relevant findings were observed with fibre-like MWCNT of similar dimensions to MWNT-7 but 10-times lower iron content. Groups of 20 male F-344/Crj rats were treated with a total dose of 1 mg/animal of one out of three sieve fractions of MWCNT-N (Nikkiso) or dispergent alone (0.5 % Pluronic F 68 [PF68]), over a period of two weeks and then observed for up to 109 weeks. A fifth no-treatment group was also included. Fractions were taken from passing MWCNT-N through a 25 µm pore-size sieve. Three fractions were tested, dispersed in 0.5 % PF68: An "unfiltered" fraction with mean tube length of 4.2 µm, a "flow-through" fraction of 2.6 µm mean tube length and a "retained" fraction, which, compared to the other two fractions, yielded dense agglomerates, which precluded length determination. The mean diameter of 93.4 % of MWCNT-N was measured as between 30 – 80 nm, resulting in a fibre or needle-like appearance. Kaplan-Meier plots did not show substantial differences in survival times between any of the groups and the vehicle control group. All animals surviving at least 63 weeks were included in the study. None of the single MWCNT-N preparations showed statistically significant tumour incidences. However, when combined, the incidence of malignant mesothelioma (pericardial and/or pleural) of the three fractions was significantly higher compared to control groups combined (6/38 and 0/28, respectively). It is noted that no mesothelioma was observed in the "retained" fraction. The authors speculated that this was possibly due to the dense agglomeration state of the MWCNT in this fraction. The incidence of lung tumours (bronchiolo-alveolar adenoma and carcinoma) was significantly higher in the combined fraction than the two control groups combined control group (14/38 and 0/28, respectively). The "retained" fraction also induced lung tumours (Table 11 d). Incidences of tumours in other organs were not statistically different from the controls and deemed age-related by the authors. Immunostaining investigations for Wilms tumour protein, podoplanin, calretinin and thyroid transcription factor-1 were applied to differentiate the epithelial and the mesothelial (sarcomatoid type) origin of the tumours in the respiratory tract, respectively. All the malignant mesotheliomas were localized in the mediastinal space (*Cavum mediastinale*), showing adhesion to the lung and heart or invasion of the lung parenchyma, pericardium and myocardium. None of the mesotheliomas were located in the lateral parietal pleura. MWCNT-N was mostly found in the lung alveoli but was also present in the mediastinal space (e.g. accumulating in mediastinal lymph nodes and periaortic connective tissue). MWCNT-N with needle-like or granular shapes was found in the lung alveoli either in the granulation tissue or macrophages. Accumulation of MWCNT-N in the lung and in the periaortic connective tissue was accompanied by fibrotic thickening. Lung retentions at week 109 for the unfiltered, the flow-though and the retained MWCNT-N fraction were equally high (25.4, 48.0, 26.3 %, respectively, compared to week 2). Related to the surface area of the rat lung, the administered dose was about 2.5 mg/m^2 alveolar surface area, which would be higher than the theoretical lung deposition in workers achieved during lifetime when exposed to 1 µg/m^3 daily (ca. 0.3 mg/m^2). Since much higher exposure levels were already determined in epidemiological studies, the administered rat dose was assumed a reasonable starting dose for further investigations. The role of iron in mesothelioma development was not specifically investigated.

Table 11d: Incidence of pleural malignant mesotheliomas and lung tumours (Suzui et al., 2016)

MWCNT fraction	Rats	MM; pericardial and/or pleural (%)	Lung tumours			Total tumour burden (%)
			Adenoma	Adeno-carcinoma	Combined (%)	
NT	15	0 (0.0)	0	0	0 (0.0)	0 (0.0)
V	13	0 (0.0)	0	0	0 (0.0)	0 (0.0)
NT + V	28	0 (0.0)	0	0	0 (0.0)	0 (0.0)
U	12	3† (25.0)	1	3†	4 (33.3)	7 (58.3)
FT	12	3 (25.0)	1	2	3 (25.0)	6 (50.0)
R	14	0 (0.0)	2	5	7 (50.0)	7 (50.0)
U + FT + R	38	6 (15.8)*	4	10	14 (36.8)**	20 (52.6)**

* P < 0.05; ** P > 0.001 vs. control groups (NT + V). † One rat had both a malignant mesothelioma (MM) and a lung adenocarcinoma. FT, flow-through fraction; NT no treatment, R, retained fraction; U, unfiltered fraction; V, vehicle.

Several experimental studies reported a mesotheliomagenic potential of MWCNT with fibre characteristics following intraperitoneal injection.

Intraperitoneal injection of rats with three different types of MWCNT ("NT50a": D:50 nm, L: 5.3 µm, long-crystalline fibres = MWNT-7, "NT145": D: 145 nm, L: ~4.3 µm, thick tubes or NTtngl: D: 15 nm, L: 3 µm tangled tubes) at 1-5 mg induced distinct responses after 1 month. NT50a but not NT145 or NTtngl caused severe fibrous peritonitis and induced altered dull edge of the liver (Nagai et al., 2011). All MWCNTs administered to rats were phagocytosed by macrophages, and local granuloma formation was observed. Fibrosis was observed around each i.p. organ, regardless of MWCNT deposition. However, NT50a was estimated to be the most potent inducer of fibrotic inflammation. Furthermore, the proliferation of mesothelial cells was observed only in rats treated with NT50a. Based on concomitant in vitro studies (see "Other studies relevant for carcinogenicity" below) it was hypothesized that the difference in the extent of inflammation induced by NT50a and NT145 was caused by differences in the ability to induce direct mesothelial injury by piercing mesothelial cells which in turn appeared to be dependent on crystallinity and diameter of the tubes. Evidence was provided that dispersed MWCNT with high (needle-shaped) crystallinity had a higher mesotheliomagenicity than thick (blunt) MWCNT. Male and female rats were treated by intraperitoneal injection with 1-10 mg of the three different MWCNT types described above and sacrificed after 1 year or when becoming moribund. In addition, a sub-fraction of non-aggregated NT50a fibres was tested at a number concentration equivalent to 1mg NT145 ("NT50a-agg*). Nearly half of rats administered 10 mg of either MWCNT type died within several days. Injections with NT50a(-agg*) or 1 mg of NT50a (= MWNT-7) induced malignant mesothelioma with a higher frequency and earlier progression than injections with 1 mg of NT145 and 10 mg of NTtngl. Based on survival rates, NT50a(-agg*) and 1 mg of NT50a were more carcinogenic than 1 mg of NT145. Regarding the tumour phenotype, 0.9 % was epithelioid, 12.1 % biphasic 86.0 % sarcomatoid (Table 11e). It was argued that the predominance of the sarcomatoid histology is typical for asbestos-induced tumours. Likewise, array-based comparative genomic hybridization (CGH) analysis revealed that MWCNT-induced mesotheliomas shared homozygous deletion of Cdkn2a/2b tumour suppressor genes, similar to asbestos-induced mesotheliomas responses.

Table 11e: Outcome of mesotheliomagenesis experiment by Nagai et al. (2011)

			Histology		
Name/dose, mg	**Total alive**	**Mesothelioma**	**Epithelioid**	**Biphasic**	**Sarcomatoid**
NT50a					
-agg*	15	12	0	2	10
1	13	13	0	0	13
10	43	43	1	7	34
NT50b					
10	6	6	0	1	5
NT145					
1	14	5	0	0	5
10	18	28	0	3	25
NTtngl					
10	9	0	0	0	0
Vehicle control (saline)	15	0	0	0	0

Source: Nagai et al., PNAS 108, no.49, suppl. (2011), modified

Rittinghausen et al. (2014) treated four tailor-made types of MWCNT, differing slightly in length and diameter and in the percentage of WHO fibre numbers into male Wistar rats by single intraperitoneal injection of 1 or 5 x 10^9 WHO tubes suspended in artificial lung surfactant to reduce aggregation. All MWCNT types and doses induced malignant mesotheliomas within the observation period of two years. Immunohistochemical marker staining using podoplanin, pan-cytokeratin, and vimentin demonstrated the similarity to mesotheliomas in humans induced by asbestos. Though a dose-dependency could not be clearly deduced (tumour incidences were high and varied between 40 and 100 %, mesothelioma-related mortality rates were also type-dependent but generally high), the tumour latency was assumed to be correlated to the deviation from the straight, needle-like morphology, as the most curved type resulted in the largest latency (first tumour detection after 20 months in the low dose group, whereas the earliest tumours occurred after 5 months in high and low dose groups of the more straight MWCNT types). The curvature or degree of circumflection was estimated by determining the inner angle of individual tubes (the larger the angle, the straighter the tube) and was used as a surrogate parameter for rigidity. Most tumours showed invasion of peritoneal organs, especially the diaphragm. With regard to histological typing, the sarcomatoid mesothelioma type prevailed (64 % of tumours compared to 32 % biphasic and 4 % epitheloid type). The lab-generated MWCNT were not functionalized and no metals could be detected at their surface, making confounding effects by contaminants less likely. Mesothelioma incidences induced by the different MWCNT in relation to the WHO fibre number dose are depicted in Table 11f, also illustrating the more rapid onset and higher severity of mesotheliomas in case of more straight MWCNT types compared to more flexible types and even to amosite asbestos.

Table 11f: Incidences of mesotheliomas for different MWCNT with WHO fibre geometry (Source: Rittinghausen et al. Particle and Fibre Toxicology 2014, 11:59)

Substance	Length (µm) WHO fibres*	Diameter (µm) WHO fibres*	Inner angle	Dose WHO fibres * 10^9 per rat (actual##)	No. of rats	Rats with mesothelioma	Mean survival time of mesothelioma-bearing rats (days)	Months of first detection of a morbid mesothelioma-bearing rat	Final month**
Medium control					50	1 (2 %)	739	24	24
MWCNT A low	8.57 ± 1.51	0.085 ± 1.60	174.85° ±5.82°	0.48	50	49 (98 %)	213	5	9
MWCNT A high				2.39	50	45 (90 %)	194	5	8
MWCNT B low	9.30 ± 1.63	0.062 ± 1.71	148.10°± 11.48°	0.96	50	46 (92 %)	294	6	13
MWCNT B high				4.80	50	45 (90 %)	207	5	9
MWCNT C low	10.24 ± 1.64	0.040 ± 1.57	144.55° ±11.48°	0.87	50	42 (84 %)	415	10	18
MWCNT C high				4.36	50	47 (94 %)	265	6	11
MWCNT D low	7.91 ± 1.40	0.037 ± 1.45	Not measured	1.51	50	20 (40 %)	666	20	24
MWCNT D high				7.54	50	35 (70 %)	585	11	24
Long amosite asbestos	13.95 ± 2.10	0.394 ± 1.83		0.14	50	33 (66 %)	623	14	24

*WHO fibres: length greater than 5 µm, diameter less than 3 µm, and a length-to-width ratio (aspect ratio) of at least 3:1.

##Actual WHO fiber number dose; deviations from target values are due to inhomogeneity during successive synthesis or suspension of test items.

**Last month when a mesothelioma-bearing rat was found in a group.

Using a p53 heterozygous asbestos-sensitive mouse model, Takagi et al. (2008) proved that fibrous or rod-shaped MWCNT of 10-20 µm length (27 % of MWCNT > 5 µm) and an average diameter of 100 nm) (MWNT-7, Mitsui) rapidly induced mesothelioma in addition to fibrogranulomatous lesions in the peritoneum after a single intraperitoneal dose of 3 mg, similar to Crocidolite asbestos which served as positive control. Large tumours invaded the abdominal wall, diaphragm, liver parenchyma, and pancreas, rarely also involving the thoracic cavity. Distant metastases were not observed. Fibre-laden cells were not only found in peritoneal lesions but also in the liver and in mesenteric lymph nodes. The overall mesothelioma incidence at day 84 post i.p. treatment was even higher for MWCNT (87.5 %) compared to Crocidolite (77.8 %). In particular, because of the unrealistically high exposure concentrations and highly artificial test conditions used, the study was challenged by Donaldson et al. (2008) in terms of human relevance. However, in a follow-up study, Takagi et al. (2012) demonstrated the dose-dependency of mesothelioma induction using the same MWCNT and animal carcinogenesis model (Table 11g). Three groups of p53 heterozygous mice (n = 20) were given a single intraperitoneal injection of 3, 30, 300 µg/mouse (corresponding to $1x10^6$, $1x10^7$, $1x10^8$ fibers/mouse), and observed for up to 1 year. The cumulative incidence of mesotheliomas was 5/20, 17/20 and 19/20, respectively. The severity of peritoneal adhesion and granuloma formation were dose-dependent and minimal in the lowest dose group; however, the time of tumour onset was apparently independent of the dose. Histology of the mesotheliomas ranged from a differentiated epithelioid type to an undifferentiated sarcomatous type. Osteoid and rhabdoid differentiations, both known in human cases, were found in nine mice across all dose groups. Peritoneal fibrosis, peritoneal adhesion and formation of foreign body granulomas towards agglomerated MWCNT were dose dependent and minimal in the low-dose group. Singular fibres were also distributed to

systemic organs including the liver and the brain. The finding that a dose as low as 3 µg of MWNT-7 was mesotheliomagenic implied the absence of a practical threshold similar to asbestos fibre carcinogenesis.

Table 11g: Mesothelioma induction in p53-deficient mice following intraperitoneal administration of MWNT-7 (Takagi et al., 2012)

Dose	Mesothelioma incidence	Remarks
Vehicle control	0/20 (0 %)	
3 µg/ mouse (= $1x10^6$ fibres)	5/20 (25 %)	4/20 mice had lethal mesothelioma (Fig. 2) and 1/20 had a nonlethal mesothelioma (found at the terminal kill on day 365). The other 15 mice that survived until the terminal kill showed focal mesothelial atypical hyperplasia.
30 µg/ mouse (= $1x10^7$ fibres)	17/20 (85 %)	17/20 (85 %) mice had lethal mesothelioma. Three mice without lethal mesothelioma died or became moribund due to other reasons including leukaemia.
300 µg/mouse (= $1x10^8$ fibres)	19/20 (95 %)	14/20 mice had single or multiple lethal mesotheliomas up to 2 x 2 cm in size located within the peritoneal cavity, invading adjacent organs and structures with or without peritoneal dissemination. The remaining mice died of ileus due to severe peritoneal adhesion and fibrosis, and among them, five had small incidental (nonlethal) mesotheliomas.

Using the same test material as Takagi et al. (2008) but non-genetically modified animals, Sakamoto et al. (2009) administered 1 mg/kg bw MWNT-7 to Fischer rats by intrascrotal injection (Table 11h). In rats the scrotal cavity is freely connected with the peritoneal cavity and thus intrascrotal injection is regarded as similar to intraperitoneal administration. 6 of 7 treated animals died before the end of the 52 weeks observation period due to intraperitoneally disseminated mesothelioma. These advanced stage tumours, which developed from mesothelial hyperplasia over polyploidy or papillary mesotheliomas, invaded into adjacent tissues and organs and metastasized frequently into the pleura. Beside these mesothelial proliferative lesions, granulomas with high cellularity, including macrophages and multinucleated giant cells were observed. In contrast, rats treated similarly with 2 mg/kg bw crocidolite also developed (inactive) granulomas but no mesotheliomas. The authors explained the absence of mesotheliomas with the low particle number concentration of crocidolite in their study.

Table 11h: Individual histological findings for mesothelial proliferative findings in the study by Sakamoto et al. (2009)

Treatment	Animal number	Timing of autopsy (week after treatment)	Mesothelial Hyperplasia	Mesothelioma			Overall mesothelioma incidence
				Develop-ment	Invasion	Osteoid change	
Vehicle (2 % carboxy-methylcellulose)	1-5	52	-	-	-	-	0/5 (0 %)
Crocidolite	1-10	52	-	-	-	-	0/10 (0 %)
MWNT-7							6/7 (86 %)
	1	50	+	+	+	+	
	2	37	+	+	+	-	
	3	40	+	+	+	+	
	4	39	+	+	+	+	
	5	52	+	-	-	-	
	6	40	+	+	+	-	
	7	40	+	+	+	+	

Source: Sakamoto et al. J. Toxicol.Sci. 34 (1), 65-76 (2009), modified

Human data

No human data available.

Table 12: Summary table of other studies relevant for carcinogenicity

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
In vivo studies				
Intraperitoneal injection No standard guideline followed C57BL/6 mice (n=3-8/ group)	1. "NT_{long1}" (MWNT-7?, Mitsui) Dispersed bundles and singlets. D: 84 nm, L: 13 µm (12 % > 20 µm; 24 % > 15 µm). 2. "NT_{long2}", non-commercial MWCNT (University of Manchester). Regular bundles and ropes. D: 165 nm, L: 56 µm (76 % > 20 µm; 84 % > 15 µm) 3. "NT_{tang1}"; MWCNT, tangled (NanoLab)	Single intraperitoneal injection of 50 µg/mouse Observations: 24 h & 7 d after administration "Controls": - 0.5 % BSA/saline (vehicle control) - Nanoparticulate carbon black (NPCB) - Short-fibre amosite (brown asbestos)	Long, non-agglomerating, fibre-like MWCNT Inflammation, foreign body giant cells (FBGCs) and granulomas at workplace-relevant concentrations similar to that observed with long-fibre amosite used as positive control. None of the above effects (apart from very slight granuloma formation) were observed in mice treated with low-diameter, tangled fibres MWCNT, independent of their length. Short amosite fibres or carbon black nanoparticles were also negative.	Poland et al. (2008)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	Short, densely packed agglomerates, a large proportion of which are in the respirable range < 5 µm. D: ~15 nm, L: 1-5 µm; 4. "NT_{tang2}", MWCNT, tangled (NanoLab) Bundles of intermediate-length agglomerating nanotubes, a large proportion of which are in the respirable range < 5 µm. D: ~10 nm, L: 5-20 µm	- Large-fibre amosite (brown asbestos)		
Intraperitoneal injection No standard guideline followed C57BL/6 mice (n=4/ group)	CNT: 1. "CNT_{Long1}" (= MWNT-7?; Mitsui & Co) D: 40-50 nm L: 13 µm (mean) Soluble metals (µm/g): Li- < 0.09; Be- < 0.007; Al-0.9; V-0.01; Cr-0.15; Mn-0.09; Fe-15.6; Co- < 0.01; Ni-0.2; Cu-0.06; Zn-2.5; As- < 0.07; Sr-0.84; Mo-0.01; Ag- < 0.05; Cd- < 0.02; Sb- < 0.06; Pb-0.03; U-0.01 Bundled or individual MWNTs of variable length, many in the 10-20 µm range or longer. Many very short fibres often decorate the longer fibres. 2. "CNT_{SPIN}" (MWCNT; CSIRO Australia) D: 8-10 nm L: 200-300 µm Soluble metals (µm/g): Li- < 0.02; Be- < 0.001; Al-0.3; V-0.01; Cr-0.07; Mn-0.02; Fe-50.1; Co- < 0.003; Ni-0.46; Cu-0.16; Zn-0.95; As-0.12; Sr-48.4; Mo-0.25; Ag- < 0.01; Cd-0.02; Sb-0.01; Pb-0.43; U-0.001	Single intraperitoneal injection of 50 µg/mouse BAL (cell counts, total protein, LDH, IL-6) and histopathology after 24 h and 7 d post-injection. Test materials were pre-incubated in Gambles solution for 0, 10 and 24 weeks to test their biodurabilty.	CNT_{SW}, CNT_{TANG2} and CNT_{SPIN} showed no, or minimal loss of mass or change in fibre length or morphology after incubation for up to 24 weeks in Gambles solution. However, CNT_{LONG1} lost 30 % of its original mass within the first three weeks of incubation, after which there was no further loss. Loss in mass was associated with a decrease in the proportion of long fibres. Fibre shortening was accompanied by a loss of pathogenicity (fibrotic plaques and elevated BAL parameters) when injected intraperitoneally in mice compared to fibres pre-incubated briefly. In contrast, tightly agglomerated bundles of short CNT_{SW} did not elicit an inflammogenic effect. LFA induced an acute inflammatory response regardless of pre-incubation which was similar to that of non-incubated CNT_{LONG1}, whereas non-durable LFC showed a reduced inflammogenic response, when pre-incubated for ten weeks.	Osmond-McLeod et al. (2011)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	Agglomerated sheets of very long fibres with a hair-like appearance. 3. "CNT_{TANG2}" (MWCNT; NanoLab Inc) D: 15 ± 5 nm L: 5-20 µm Soluble metals (µm/g): Li- < 0.08; Be- < 0.006; Al-41.6; V- < 0.01; Cr-0.03; Mn-0.05; Fe-606; Co-0.04; Ni-0.44; Cu-1.07; Zn-9.5; As- < 0.07; Sr-0.3; Mo-655; Ag- < 0.05; Cd-0.04; Sb- < 0.06; Pb-0.26; U- < 0.006 Bundles of intermediate length MWNTs. Often stellate in form with longer fibres protruding from the central tangled agglomerate, a large proportion of which are in respirable size range < 5 µm. 4. "CNT_{SW}" (SWCNT; Sigma-Aldrich) D: 1-2 nm L: 0.5-2 µm Soluble metals (µm/g): Li- < 0.04; Be- < 0.003; Al-6.2; V-0.34; Cr-2.01; Mn-15.7; Fe-185; Co-442; Ni-47.4; Cu-1.13; Zn-2.9; As-0.23; Sr-2.72; Mo-144; Ag-0.03; Cd-0.1; Sb- < 0.03; Pb-0.98; U-0.04 Bundles of tightly agglomerated SWNTs in which individual NTs cannot be seen be seen Non-CNT control fibres: 1. "X607" Glass fibres (Rockwool International) D: n.a. L: n.a.		It was concluded that CNT are generally durable but may be subject to bio-modification, which affects their asbestos-like inflammogenicity potential. Durable carbon nanotubes that are either short or form tightly bundled aggregates with no isolated long fibres are less inflammogenic. The results confirmed that biodurability and pathogenicity are not consistent across all types of CNT.	

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	Dispersed rod-like non-durable glass fibres 2. "LFA" Amosite asbestos (Archival samples) D: n.a. L: n.a. Dispersed rod-like amphibole asbestos (durable fibres) 3. "LFC" Chrysotile asbestos (Archival samples) D: n.a. L: n.a. Dispersed fibrous-looking Chrysotile asbestos (non-durable fibres)			
Intraperitoneal injection No standard test guideline followed Rat Wistar, m n=50/ group	Short, tangled MWCNT (synthesized) with or without structural defects (the latter largely depleted of Fe and Co and exerting a pronounced radical quenching activity) D: 11 nm L: 0.7 µm Both types formed entangling agglomerates	Single injections of 2 or 20 mg/animal of MWCNT (with defects) or 20 mg/animal MWCNT (without defects) Observation period: 24 months Positive control: 20 mg/animal crocidolite	MWCNT with or without structural defects did not induce mesothelioma. MWCNTs with structural defects elicited inflammatory responses similar to crocidolite, which was reversible. Crocidolite asbestos induced a carcinogenic response in 34.6 %, vehicle control 3.8 %.	Muller et al. (2009)
Intraperitoneal injection Long-term observation No standard test guideline followed Rat Fisher 344, f/ Brown Norway hybrid, m n = 21/group	"NTtngl" (Sowa Danko) Tangled nanotubes, very high aggregation. D: 15 nm; L: 3 µm	2x 5 mg Two administrations in a 1-week interval Post-observation period: 3 years No positive control	Number of animals observed after MWCNT administration was low (n=6) due to acute death of 9/21 animals after second injection (arterial and/or lymphatic embolism?). None of the surviving animals after 3 years developed malignant mesotheliomas (the rats developed other tumours which were attributed to senility). The other 6 animals which were examined already after 1 year also did not develop malignant mesothelioma.	Nagai et al. (2013)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			The authors attributed the failure of inducing mesotheliomas to the small diameter of the MWCNT (15 nm) and thus the tangled morphology of the tested MWCNT	
In vitro studies				
Assays: 1. Cellular uptake 2. Cell viability Human mesothelial cell lines, MeT5A (SV40-transformed mesothelial cells) and E6/E7 and hTERT-immortalized human peritoneal mesothelial cells (HPMCs) were used for internalisation and viability assays. Murine macrophages (RAW264.7) and canine kidney epithelial cells (MDCK II) were used as positive and negative controls, respectively.	1. "NT50a" = MWNT-7 (Mitsui) Needle-like fibres of high crystallinity, highly aggregated D: ~50 nm, L: 5.3 µm 2. "NT145" (Showa Denko) "thick" nanotubes, aggregation low D: 143.5 nm; L: ~4.3 µm 3. "NTtngl" (Showa Denko) Tangled nanotubes, very high aggregation. D: 15 nm; L: 3 µm 4. "NT115" (Showa Denko) D: 116 nm ; L: 4.6 µm "thick", low aggregation	- Cellular uptake: 5 µg/cm2 f.c. for 24 h. Measurements: Confocal and electron microscopy. - Cell viability: 5 µg/cm2 for 4 days Measurements: 4 assays: ATP luminescence, light absorbance mitochondrial activity, fluorescence cell caspase activity	Long, crystalline (rigid) MWCNT of low diameter were cytotoxic to mesothelial cells, which was attributed to direct "piercing" of the plasma and the nuclear membrane. It was concluded that non-functionalized MWCNT enter mesothelial cells by directly penetrating the cell membrane in a diameter- and rigidity-dependent manner, whereas asbestos mainly enters these cells through the process of endocytosis, which is independent of fibre diameter. Piercing of mesothelial cell membrane/toxicity to mesothelial cells (no exact figures provided): NT50a: Yes/High NT115: Low/Low NT145: Very low/Low NTtngl: Very low/Low	Nagai et al. (2011)
Cell proliferation assay Target cells: TCC-MESO1 mesothelioma cells (human) Primary alveolar cells (rat) as source for media conditioning.	1. MWCNT-M (= MWNT-7, Mitsui) Average length: 5.11 µm 3.82 x 10^{11} fibres/g (Diameter according to WPMN report: 88 nm) 2. MWCNT-N (Nikkiso) Average length: 3.64 µm 3.46 x 10^{11} fibres/g (Diameter according to WPMN report: 48 nm)	MWCNT-N, MWCNT-M or CRO suspensions (positive control) were added to the primary alveolar cells to a final concentration of 10 µg/mL for 24h. The conditioned macrophage culture media was then	The conditioned culture media of macrophages exposed to MWCNT-N, MWCNT-M or CRO significantly increased the proliferation of the human mesothelioma cell line TCC-MESO1. The concentrated supernatants of the pleural cavity lavage taken from the rats treated with MWCNT-N, MWCNT-M or CRO exhibited similar effects.	Xu et al. (2012)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		collected for in vitro cell proliferation assays (relative cell number using Cell Counting Kit-8, Dojindo Molecular Technologies)	The results indicate that factors in the pleural cavity lavage, possibly secreted by alveolar and pleural macrophages, are able to cause mesothelial cell proliferation. (cf. Xu et al. 2012, table 11)	
Assays: 1. Cytotoxicity 2. ROS production 3. Pro-inflammatory response Monocyte derived macrophages (human)	1. Cheap Tubes (Bayer) L: 1-10 µm D: 5-30 nm Morphology: bundled Contaminant metals (µg/g): Fe (0.05), Mg (0.01), Ni (0.12), Co (< 0.001) 2. NTL (NTlong2; Univ. of Manchester; see Poland et al., 2008) L: 56 µm (max.) D: 165 nm (max.) Morphology: long, straight, stiff Contaminant metals (µg/g): Fe (37.3), Cu (1.2), Ni (6.2), Co (3.4) 3. MWCNT (NanoLab) L: 5-20 µm D: 10 nm (max.) Morphology: Tangled Contaminant metals (µg/g): Fe (13.4), Cu (1), Ni (5), Zn (7.5) 4. MWCNT (Nanostructured and Amorphous Materials Inc.) L: 0.5-2 µm D: 5-10 nm Morphology: Short, straight Contaminant metals (µg/g): Cd (< 0.1), Co (< 0.1), Cr (< 0.1), Cu (< 0.1), Fe (24.2), Mn (50.3), Ni (21.6), Ti (0.4), V (< 0.1), Zn (5.3)	Treatment of cells with MWCNT: 0.005, 0.01, or 0.02 mg/mL for 24 h Measurements: - Cytotoxicity: LDH assay - ROS production: $DCFH_2$-DA assay - Pro-inflammatory response: TNF-alpha assay	None of the CNTs caused significant cytotoxicity. Only MWNT-7 caused a significant, dose-dependent cell-free ROS production. Bundled Cheap Tubes showed a significant TNF-α release at the highest concentration. No effects were observed for either tangled MWCNTs or short MWCNT. Authors concluded an important but not entirely responsible role of MWCNT morphology in cellular adversity towards macrophages.	Clift et al. (2013)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Assays: 1. Cytotoxicity 2. Radical formation/scavenging Bronchial epithelial BEAS 2B cells (human)	1. MWNT-7 (Mitsui) D: 74 ± 28 nm L: 5.7 ± 3.7 µm Long, needle-like < 0.5 wt% Na, Fe, Al, Mg, Ni 2. NM-401 (JRC) D: 64.2 ± 34.5 nm L: 4.0 ± 2.4 µm Long, needle-like < 0.6 wt% Na, Fe, Al, Ni, Mg; residues of Si 3. MWCNT 8-15 nm OD (Cheap Tubes) D: 17 ± 7 nm L: 0.5 ± 0.3 µm Long, tangled < 5 wt% Ni, Na, Fe, Al, Mg, Mn; residues of Ni, Fe 4. Baytubes C 150 HP (Bayer) D: 12.0 ± 7.0 nm L: 0.4 ± 0.2 µm Short, purified < 3 wt% Mn, Mg, Al, Na, Ni, Fe; residues of Si, Co 5. NM-400 (JRC) D: 13.6 ± 3.7 L: 0.8 ± 0.4 µm Short, non-purified < 10 wt% Al, Fe, Na, Ni O, Si, Fe, Mg, Na	Treatments: - Cytotoxicity: Trypan blue exclusion 19, 38, 190, 304, 380, 760, 950, 1140 and 1330 µg/mL for 4, 24, 48 h - Electron spin resonance (ESR) spectroscopy in combination with a spin trapping technique 80, 160, 320 and 640 µg/mL, (dispersed in 0.6 mg/mL BSA in buffered bronchial epithelial growth medium) for 30 min. in cellular (BEAS 2B) or cell-free system "Positive controls": crocidolite asbestos and glass wool	The two long-needle-like MWCNT (MWNT-7 and NM-401) were more cytotoxic to BEAS 2B cells than the other CNT types. The higher cytotoxicity coincides with an unidentified radical produced by these two MWCNT both under cell-free conditions and in the presence of cells. Large aggregate/agglomerate formation confounded the cytotoxicity results due to rapid sedimentation. BSA affects the OH radical scavenging effect of the various MWCNT is differently.	Nymark et al. (2014)
Assays: 1. Cell viability 2. Inflammatory cytokine and chemokine levels 3. MAP kinase inhibition Primary human alveolar type-II epithelial cells (ATII) and alveolar macrophages (AM)	1. “MWCNT-20 µm” (Nanothinx, Greece) L: 0.6-30.8 µm (median: 19.3 µm) D: 15.6-41.9 nm (median: 27.8 nm) Purity: 95 % (Al: 0.3 %, Fe: < 0.1 %) 2. "MWCNT-3µm (Nanothinx, Greece) L: 0.5–24.4 µm (median: 4.3 µm; D: 22.6–45.8 nm (median: 33.3 nm) Purity: 93 %; (Al: 0.5 % Fe: < 0.1 %)	Treatments: - Cell viability: 0.1 – 100 µg/mL, 24h LDH and MTT assay (interference was checked): - Mediators: 1-50 µg/mL, 24 h ELISA (hIL-6, hIL-8 and MCP-1) - MAP kinase inhibition: 50 µg/mL, 24h	Short MWCNTs (~0.6 µm in length) induced significantly greater responses from the epithelial cells, whilst AM were particularly susceptible to long MWCNTs (~20µm). These differences in the pattern of mediator release were associated with alternative profiles of JNK, p38 and ERK1/2 MAP kinase signal transduction within each cell type.	Sweeney et al. (2014)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	3. "MWCNT-0.6 µm" (Nanothinx, Greece) L: 0.23-9.7 µm (median: 1.1 µm) D: 19.5-47.6 nm (median: 30.6 nm) Purity: 87 % (Al: 0.6 %, Fe: < 0.1 %)	ELISA (Inhibitors to p38, ERK ½ and JNK at 10 mM each)	Authors concluded that both MWCNT length and cell type are important determinants of bioreactivity.	
Assays: 1. Cell viability 2. ROS generation 3. Inflammatory mediator release 4. Phagocytosis capacity 5. Migratory capacity Human primary alveolar macrophages (AM)	1. "MWCNT-20 µm" (Nanothinx, Greece) L: 0.6-30.8 µm (median: 19.3 µm) D: 15.6-41.9 nm (median: 27.8 nm) Purity: 95 % (Al: 0.3 %, Fe: < 0.1 %) 2. "MWCNT-0.6 µm" (Nanothinx, Greece) L: 0.23-9.7 µm (median: 1.1 µm) D: 19.5-47.6 nm (median: 30.6 nm) Purity: 87.5% (Al: 0.6 %, Fe: < 0.1 %)	Treatment: 10 µg/mL for 24 h 1 and 5 days posttreatment Measurements: - Cell viability: MTS assay - ROS generation: oxidation of fluorescent dihydroxoethidium - Inflammatory mediator release: ELISA - Phagocytosis capacity: E.coli uptake - Phagocytosis receptor expression: Flow cytometry - Migratory capacity: Transwell chemotaxis toward zymosan-activated serum	AM function was severely affected by treatment with long, but not short MWCNT: AM viability was significantly decreased at 1 and 5 days after treatment with MWCNT-20 µm, while superoxide levels and inflammatory mediator release were significantly increased. At the same time, there was reduced phagocytosis and migratory capacity alongside increased expression of the macrophage scavenger receptor MARCO; this coincided with frustrated phagocytosis. In contrast, the adverse bioreactivity of the shorter MWCNT-0.6 µm with AMs (and any resulting reduction in AM functional ability) was substantially less marked or absent altogether.	Sweeney et al. (2015)
Assay: Cellular uptake Human bronchial epithelial cells (HBECs) and human mesothelial cells (HMCs).	MWNT-7 (Hodogaya) L: 8 µm (av.) D: 150 nm (av.)	Treatment: 10 µg/mL for 2 and 24 h in 0.1 % gelatin or 2 % FBS Endocytosis inhibition: 10 µg/mL for 2h of cells pre-treated for 15 min. with one of the following substance (f.c.): - Chlorpromazine (2-50 µM) - Phenylarsine oxide (0.2-50 µM)	Both, bronchial epithelial and mesothelial cells actively internalized and accumulated MWNT-7 in lysosomes after 24 h of exposure. Inhibitor studies suggested that the major uptake pathway is clathrin-mediated endocytosis as proved in corresponding cell lines. Less clear evidence indicated a minor role for caveolae-mediated endocytosis and macropinocytosis.	Maruyama et al. (2015)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
For uptake inhibition studies: BEAS-2B (human bronchial epithelial cell line) and ACC-MESO-1 (human malignant pleural mesothelioma cell line)		- Indomethacin (5-100 µM) - Nystatin (1-20 µM) - 5-(N-Ethyl-N-isopropyl)amiloride (5-80 µM)		
Assays: 1. Cytotoxicity 2. Cell viability 3. Cytokine release 4. Phagocytosis 5. Phagocytic burst Monomac-6 (MM6) monocytes (human) J774A.1 macrophages (mouse) Isolated BAL cells (rat)	1. "CNTI" (= MWNT-7, Mitsui) L: 13 µm; D: < 100 nm Fe: 8.5 mg/g 2. "CNTA" (synth.) L: < 20 µm ("short"); D: < 100 nm Fe: 134 mg/g 3. "CNTB" (synth.) L: < 20 µm; ("short") D: < 100 nm Fe: 50 mg/g 4. "CNTC" (synth.) L: > 20 µm ("long"); D: < 100 nm Fe: 19 mg/g 5. "CNTD" (synth.) L: > 20 µm ("long"); D: < 100 nm Fe: 3 mg/g Ranking: - Length: CNTC = CNTD > CNTI = CNTA = CNTB - Crystallinity: CNTI > CNTD > CNTB > CNTA > CNTC - Iron content: CNTA > CNTB > CNTC > CNTI > CNTD	Treatments: 31, 62, and 125 µg/mL for 4, 24, and 48 h for MM6 cells 7.5, 15, 31, 62, and 125 µg/mL, for 4, 24, and 48 h for J774A.1 cells Measurements: - Cytotoxicity: LDH - Cell viability: WST-1 - Cytokines (ELISA or cytometric bead array: TGF-b, GM-CSF, IL-1a, IL-1b, TGFb1, MCP-1, TNF-a, VEGF (from MM6) and IL-1b, IL-6, IL-10, MCP-1, TNF-a from J774A) - Phagocytosis: E.coli - Phagocytic burst: superoxide anion Control particles: In addition to commercial MWNT-7 long and short-fibre amosite asbestos as well as Printex 90 carbon black nanoparticles were used.	The long MWCNT were more potent inductors of cellular responses than the short MWCNT with regard to: - impact on cell morphology during phagocytosis - superoxide anion production in phorbol 12-myristate 13-acetate- primed cells - E. coli phagocytosis - Production of pro-inflammatory mediators. Though not stringent, there was some evidence that an increased crystallinity (regarding CNTI) and an increased metal content (regarding CNTs A and C) were associated with increased MWCNT biological reactivity. In most of the assays, the MWCNT were more potent than the positive control, long fibre amosite asbestos. SEM images demonstrated the delivery of straight, rigid asbestos fibres to cells, while CNTs were delivered as small agglomerates, or, at times, as both straight and convoluted single fibres.	Boyles et al. (2015)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
	- Bioavailable iron release – neutral pH: CNTI > CNTC > CNTD > CNTA > CNTB - Bioavailable iron release – acidic pH: CNTA > CNTC > CNTI > CNTD > CNTB			
Assays: 1. Cytotoxicity 2. Wound-healing 3. Visualisation of intracellular catalytic Fe[II] 4. Lipid peroxidation 5. Apoptosis No standard test guideline followed. Transformed rat peritoneal mesothelial cells	1. "NT-50" (Showa-Denko) D: 52.4 nm, L: 4.6 µm Fibres of high crystallinity, highly aggregated High mesothelioma-inducing potential "NT-50" was identical to "NT50b" in Nagai et al. (2011). 2. "NT-tngl" (Showa Danko) D: 15 nm; L: 3 µm tangled nanotubes, very high aggregation. No mesothelioma-inducing potential "NT-tngl" was identical to "NT-tngl" in Nagai et al. (2011).	Dosing: 1. Cytotoxicity: 10 µg/cm2 for 72 h before dead-cell protease activity was measured. 2. Wound-healing: 10 µg/cm2 for 0, 8, and 24 h. Microscopic evaluation of wounded monolayer with ImageJ. 3. Visualisation of Fe[II]: 10 µg/cm^2 for 24 h before fluorescent staining with Rho-Nox-1. 4. Lipid peroxidation: 10 µg/cm^2 for 24h before immunelabelling with antibody against 4-hydroxy-2-nonenal (HNE) and BODIPY (581／591) C11. 5. Apoptosis: Not reported (the TACS Annexin V Kit protocol was followed)	Coating NT-50 with haemoglobin (Hb), transferrin (Tf) or lung lysate (Lys) significantly increased mesothelial cytotoxicity compared to pristine NT-50. Tf and Lys coating retarded cellular proliferation in the wound-healing assay. None of these effects were seen with coated NT-tngl, which the authors attributed to the fact that NTtngl does not enter mesothelial cells. Hb and Tf coating significantly increased the catalytic Fe(II) in mesothelial cells, which was interpreted as an indication that NT50 exposure induces high levels of intracellular oxidative stress. This is agreement with the finding that coating NT-50 with Tf significantly increased the uptake of NT-50. Tf receptor involvement was demonstrated by decreasing Tf receptor 1 with ferristatin II, which significantly decreased NT50 uptake and cytoplasmic catalytic Fe(II).	Wang et al. (2016)

Other studies relevant for carcinogenicity

Information from toxicokinetic, repeated dose inhalation and genotoxicity studies

The carcinogenic potential of fibre-like MWCNT is supported by a number of studies, which are discussed in more detail in the corresponding sections (sections 9, 10.8 and 10.12). In a nutshell it is concluded that there is sufficient evidence from animal studies that after repeated pulmonary exposure fibre-like MWCNT persist in the lung and induce irreversible inflammation and oxidative stress in the lung and the pleura. These processes result in pre-neoplastic lesions, including multifocal fibrosis, granulomatosis and bronchio-alveolar as well as mesothelial hyperplasia (Kasai et al., 2015, Poulsen et al., 2015, Umeda et al., 2013, Rydman et al., 2013, Porter et al., 2010, 2013, Mercer et al., 2013, Murphy et al., 2013). Furthermore, in vivo somatic mutagenicity and genotoxicity data provide evidence that MWNT-7 acts as a site of contact mutagen possibly involving, host defence-dependent ROS generation and/or direct interference with cell division processes (Kato et al., 2013, Catalán et al., 2016, Poulsen et al., 2015, Ema et al., 2013). Toxicokinetic lung exposure investigations in rodents demonstrated pulmonary escape and presence of individual MWNT-7 nanotubes at the (visceral) pleura, the primary target tissue of mesothelioma formation (Kasai et al., 2015, Xu et al., 2012, 2014, Porter et al., 2010, 2013, Mercer et al., 2013, 2013a, Aiso et al., 2011, Murphy et al., 2011, Ryman-Rasmussen et al., 2009), giving rise to pleural penetration, inflammation and hyperplasia (Kasai et al., 2015, Porter et al., 2013). The findings on repeated inhalation exposure resulted in an additional classification proposal for STOT RE, according to a weight of evidence approach (see section 10.12.)

Additional in vivo evidence (Table 12)

In a pilot study, Poland et al. (2008) provided first evidence for an asbestos-like pathogenicity of carbon nanotubes using an experimental animal model by using intraperitoneal injection, thus exposing the mesothelial lining of the abdominal cavity of mice. Female C57BL/6 mice received a single occupationally relevant equivalent dose (50 µg per animal) of long, large diameter MWCNT ("NT_{long1}" D:84 nm, L: 13 µm or "NT_{long2}") or low diameter tangled MWCNT ("NT_{tang1}" D: ~15 nm L: 1-5 µm, "NT_{tang2}" D: ~10 nm L: 5-20 µm). Each type of the long MWCNT samples included a substantial proportion of individual tubes with WHO fibre length (≥ 5 µm). Their potential to evoke an inflammatory response was compared to that of injected long or short fibre amosite asbestos ("LFA": and "SFA", respectively)[6] as well as nanoparticulate carbon black (NPCB). 24 h post-instillation long fibres, independent of their chemical composition (NT_{long1}, NT_{long2}, LFA) induced PMN recruitment and protein exudation in the peritoneal cavity, whereas short or tangled fibres or particles (NT_{tang1}, NT_{tang2}, SFA, NPCB) did not. Granuloma formation on the diaphragm and foreign body giant cells including frustrated phagocytes were observed 7 days post-instillation in mice treated with either type of long fibre MWCNT or amosite but not with tangled MWCNT, short-fibre amosite or carbon black (apart from a small non-significant granuloma formation induced by one of the tangled MWCNT types). Since the experiments were terminated 7 days postexposure, it could not be clarified whether the asbestos-like tissue lesions progressed into mesothelioma formation.

In a follow-up study, Osmond-McLeod et al. (2011) compared the durability of one SWCNT, three different MWCNT with that of mineral fibres of various biopersistence in vitro. One of the MWCNT types ("CNT_{LONG1}"; Mitsui) with an initial average diameter of 60 nm and an average length of 12.4 µm, was the same fibre-like MWCNT tested by Poland et al. (2008) and Murphy et al. (2011, 2013) though it is unclear if "CNT_{LONG1}" is actual identical to MWCNT-7. When incubated in Gambles solution at pH 4.5 (mimicking the acidic milieu of phagolysosomal fluid in macrophages) for 24 weeks, " CNT_{LONG1}" lost 30 % of its mass within the first 3 weeks without further loss thereafter. In comparison, the other MWCNT types remained fairly stable over the whole incubation period, similar to amosite asbestos. In contrast, less biopersistent fibres such as chrysotile asbestos or glass fibres decreased continuously in weight over time. Electron microscopy revealed that mass reduction of " CNT_{LONG1}" was accompanied by fibre shortening (about 50 % fewer fibres with lengths > 15 µm). Intraperitoneal injection into C57BI/6 mice of 50 µg of "CNT_{LONG1}" incubated for 10 weeks in Gambles solution demonstrated a marked reduction in the acute inflammatory response (significant changes in peritoneal leukocyte counts, total protein, LDH, and fibrotic plaques 24 hours after injection) as compared to 0 week-incubation samples. Incubated chrysotile (as well as tangled SWCNT) showed only minimal inflammation, whereas amosite asbestos proved inflammatory, independent of pre-incubation (despite a weight

[6] According to Donaldson et al. (British Journal of Industrial Medicine 1989;46:271-276), the percentage of fibres > 10 µm was 0.1-0.2 % in the short fibre sample (SFA) and 10-12 % in the long fibre sample (LFA) but comparable diameters (0.4 - 3 µm)

loss observed which, however, was not attributed to critical fibre shortage). The study provided evidence that in vitro durability of various CNT types may also impact fibre-specific assays. Thus, there are similarities to the pathogenicity of asbestos or other mineral fibres of various biopersistence. It was concluded that an adverse response in vivo was dependent on both the durability and the presence of discrete, long CNTs or fibre-shaped agglomerates of CNTs in the sample. Durable but tightly agglomerated bundles of short CNT elicited a minimal response in mice, while the pristine, discrete, long, thin fibres of "CNT_{LONG1}" induced an asbestos-like response, which was mitigated after chemical incubation which reduced the proportion of fibres longer than 15 µm. However, it is questionable, if such in vitro durability assays would sufficiently predict lung tumour and mesothelioma formation, respectively.

In contrast to the asbestos-like pathogenicity of large-diameter MWCNT (> 30 nm), which induce experimental mesothelioma in rodents (or preneoplastic lesions depending on dose and post-treatment observation), Muller et al. (2009) were unable to observe mesothelioma formation above control values in a two-year bioassay in rats, by injecting intraperitoneally 2 or even 20 mg of short, tangled MWCNT of low diameter (0.7 µm length, 11.3 nm diameter). This finding was independent of structural defects introduced into MWCNT by heating. The fractions of tubes > 5 µm was extremely low (average length >1 µm). In contrast to MWCNT, crocidolite asbestos induced mesotheliomas at a high incidence before study termination. Expressed in figures, mesothelioma incidences for the short tangled MWCNT with or without defects (2 or 20 mg/animal) were 4.0 and 6 %, respectively, for crocidolite (2 mg/animal) 34.6 %, for vehicle control 3.8 %. It is noteworthy that similar structural defective MWCNT were positively tested in in vivo and in vitro genotoxicity assays, indicating both, an aneugenic as well as a clastogenic potential. In fact, genotoxicity as well as acute pulmonary toxicity were reduced by high temperature treatment that annealed structural defects in the nanotubes but were restored upon grinding which re-introduced defects (Muller et al., 2008, 2008a, cf. section 10.8). The authors concluded that the absence of a carcinogenic response indicates that factors other than structural defects are relevant for carcinogenicity, thus strengthening the association to fibre dimensions and morphology.

Nagai et al. (2013) also stressed the importance of fibre morphology. In a follow up of the earlier intraperitoneal study that demonstrated that long, crystalline but not short or tangled MWCNT induced mesothelioma (Nagai et al., 2011, see above), the authors showed that tangled MWCNT of 15 nm diameter ("NTtngl") did not cause mesothelioma development in male rats even at a high dose (10 mg) over an observation period of 3 years after administration. The rats developed other tumours, which were attributed to senility. Since granuloma without iron deposition was induced by the tangled MWCNT that contain a relatively high amount of iron as impurity, it was concluded that mesothelioma induction in the preceding study was due to tube morphology (by injury of pierced cells, as assumed in Nagai et al., 2011) rather than metal impurities of MWCNT. It is noted that the number of animals observed after MWCNT administration was low due to high acute death.

In vitro investigations (Table 12)

In addition to these proof-of-concept studies, highlighting the role of fibre pathogenicity, a number of in vitro studies shed light on mechanistic aspects with regard to the interaction of various fibre-like MWCNT types with lung cells (epithelial cells, mesothelial cells, and alveolar macrophages), determinants of cellular toxicity and the role of physico-chemical properties.

Nagai et al. (2011) reported evidence for diameter and rigidity as additional critical factors in mesothelial damage and specific for MWCNT. Two human mesothelial cell lines (HPMC, MeT5A) or canine kidney epithelial (MDCK II) cells did not internalize either of 4 MWCNT types differing - primarily in diameter - or a fifth, tangled type ("NT50a": long-crystalline fibres = MWNT-7, "NT-115", "NT145": thick tubes or "NTtngl": tangled tubes) after 24 h incubation at a concentration of 5 µg/cm^2, as shown by flow cytometry analysis and confocal microscopy. On the other hand, crocidolite asbestos fibres were found to be internalised by mesothelial cells or by murine macrophages. The macrophages also internalised the different MWCNT types except NTtngl. Using different viability assays, an inverse relationship between MWCNT diameter and mesothelial toxicity was found with NT-50a exhibiting the highest cytotoxicity. Electron microscopy revealed that the thin (50 nm) and crystalline MWCNT - NT-50a in particular - penetrated plasma and nuclear membranes of mesothelial cells (HPMC), thus causing cytotoxicity by actively "piercing" the cells, independent of a vesicle-dependent process. The thicker (145 nm) NT-145 MWCNT were unable to injure the mesothelial cells. Crocidolite fibres interacted quite differently with the mesothelial cells. These fibres were

surrounded by vesicular structures and even thicker fibres than NT-145 were internalised. Other parameters, such as length, structural defects, free radical generation, fibre number, metal contamination, and surface area were considered secondary to diameter and rigidity. The authors speculated that the differences in mesothelial injury between MWCNT and asbestos may explain the shorter latency period of experimentally induced mesothelioma in rats in case of MWCNT compared to asbestos. Functionalisation (e.g. resulting in increased hydrophilicity) may alter direct cellular uptake as assumed in a review by Nagai and Toyokuni (2012): The presence of specific ligands on the carbon nanotube surface may induce ligand-mediated endocytosis, which allows large-sized nanotubes to enter non-phagocytic cells.

Recent results by Maruyama et al. (2015) challenged the findings by Nagai et al. (2011). They reported that after inhalation, human bronchial epithelial cells (HBECs) and human mesothelial cells (HMCs) - both potential target cells - actively take up MWNT-7 when the exposure time is sufficiently long. MWNT-7, dosed at 10 µg/mL dispersed in either fetal bovine serum (FBS) or gelatin solution, was internalized, accumulating in lysosomes after 24 h treatment. Viability after 24 h exposure was 85.5 % in FBS and 64.7 % in gelatin in case of HBECs. Viability of HMCs, dispersed in gelatin only, was about 75 %. The endocytic mechanism, which was further investigated in epithelial- and mesothelial-derived cell lines (BEAS-2B and MESO-1, respectively) using specific inhibitors, revealed that clathrin-mediated endocytosis appeared to be the major endocytic pathway. Inhibition experiments of the caveolae-mediated endocytosis and micropinocytosis were less convincing, nevertheless a minor contribution of these two pathways in MWCNT-uptake was assumed. In an earlier study, the group found that endocytosis of MWCNT is dependent on dispersants, reporting that BEAS-2B and MESO-1 cells did not endocytose MWCNT dispersed in carboxymethyl cellulose. To minimize interference of serum factors, the internalizing assays were mostly done in gelatin instead of FBS.

In a new study using MWCNT similar to the MWCNT tested by Nagai et al. (2011), Wang et al. (2016) provided evidence for another pathogenicity mechanism possibly contributing to mesothelial cell injury. "NT-50" (Showa Denko) corresponded to "NT50b" in the Nagai study (4.6 µm in length, 50 nm in diameter), which was proved to be highly mesotheliomagenic in rats. NT-50 was tested along with low-diameter, tangled "NT-tngl" (Showa Denko), corresponding to "NTtngl" in the Nagai study (3 µm in length, 15 nm in diameter), in a number of in vitro assays on transformed rat peritoneal mesothelial cells. Evidence was provided that coating of NT-50 with haemoglobin and transferrin significantly increased mesothelial uptake, possibly involving transferrin receptor-mediated endocytosis, as decreasing the receptor with the TfR 1-specific inhibitor ferristatin II reduced the uptake. At the same time, the coating resulted in increased mesothelial cytotoxicity of NT-50 and significantly increased intracellular catalytic iron ion in exposed mesothelial cells by uptake of the iron-carrying proteins. None of these effects were observed with pristine NT-50 or coated (or uncoated) NTtngl. The authors assumed that adsorption of iron-binding proteins to MWCNT may provide another mechanism for mesothelial injury in addition to the earlier hypothesised direct piercing by needle-like MWCNT. Accordingly, it was suggested that protein adsorption enables receptor-mediated endocytosis, resulting in an intracellular iron overload, which may initiate cell-damaging by internal oxidative stress, ultimately leading to mesothelioma formation (independent of presence of iron impurities of the pristine MWCNT).

Xu et al. (2012) provided evidence that the damaging effect on mesothelial cells of MWCNT migrating into the pleural cavity is more indirect rather than due to direct interaction with these cells. Supporting their in vivo findings on visceral mesothelial proliferation (see also table 11), they reported proliferation of human mesothelial cells induced by conditioned medium from primary alveolar macrophages phagocytosing MWCNT-N, MWCNT-M or crocidolite (positive control). Concentrated supernatants of the pleural cavity lavage taken from the rats treated with MWCNT-N, MWCNT-M or crocidolite similar proliferative effects. MWCNT-M (Mitsui) was identical to MWNT-7 (av. length: 5.11 µm, diameter presumably 88 nm); average length of MWCNT-N (Nikkiso) was 3.64 µm, diameter presumably 48 nm.

Clift et al. (2013) compared the interaction of a number of commercial MWCNT, differing in physical properties, on their interaction with human monocyte-derived primary macrophages in vitro. None of the MWCNT caused significant cytotoxicity up to 0.02 mg/mL after 24 h. Only the long straight and stiff MWCNT (NT_{long2}: cf. Poland et al., 2008, L: max. 56 µm, D: max. 165 nm) caused a significant, dose-dependent (0.005–0.02 mg/mL) reactive oxygen species production. In contrast, bundled MWCNT (Cheap Tubes; L: 1-10 µm, D: 5-30 nm) showed a significantly enhanced release of tumor necrosis factor alpha after 24 h exposure at 0.02 mg/mL compared to control. No effects were observed for either tangled (NanoLab; L: 5-20 µm, D: max. 10

µm) or short straight MWCNT (Nanostructured and Amorphous Materials; L: 0.5-2 µm, L: 5-10 nm). The authors concluded that a specific morphology of the MWCNT but also other factors contributed to the adverse biological responses observed.

Nymark et al. (2014) studied the cytotoxic potential of five different MWCNT towards transformed human bronchial epithelial BEAS 2B cells with an epithelial phenotype and correlated the cytotoxicity to the cell-free and cell-bound radical formation and scavenging potential of the different MWCNT types in the absence or presence of BSA, a widely used dispergent for nanomaterials. The test material was obtained from commercial sources or from the JRC repository and was thus well characterized. A major finding was the higher cytotoxicity of the long needle-like test materials (MWNT-7; D: 74 nm, L: 5.7 µm and NM-401, D: 64 nm, L: 4 µm) compared to tangled (MWCNT 8-15 nm OD) and short (Baytubes C 150 HP) forms. The two needle- or fibre-like materials produced a yet to be identified radical in the presence of BSA, suggesting an additional toxicological mechanism for fibre-like MWCNT (independent from the slight radical scavenging activity inherent to all MWCNT). However, agglomerate size of the MWCNT may have confounded their cytotoxicity.

Sweeney et al. (2014) investigated the biological interaction of MWCNT of different lengths but similar diameter with primary cultures of transformed human lung cells in vitro. There was a clear length dependence and cell specificity: Short MWCNT (0.6 µm) induced significantly greater responses with regard to cytotoxicity (at high doses only) and inflammatory mediator release in epithelial cells, whilst alveolar macrophages were more susceptible to long MWCNT (20 µm) at already low, physiologically relevant doses, which dramatically increased at higher doses, possibly due to "frustrated phagocytosis". Variability in biological response was also reflected by different signal transduction profiles within each cell type.

In a follow up in vitro study, Sweeney et al. (2015) demonstrated marked differences in the behaviour of human primary alveolar macrophages after treatment with either short (~ 0.6 µm) or long (~ 20 µm) MWCNT of similar diameter (~ 28-30 nm) at 10 µg/m for 24 h and 1 or 5 days posttreatment. Whereas long MWCNT underwent incomplete (“frustrated”) phagocytosis, short MWCNT did not. Long MWCNT elicited more pronounced responses with regard to ROS generation and inflammatory mediator release (IL-6, IL-8). In contrast, phagocytic and migratory capacity was markedly reduced. The expression increase of the scavenger receptor MARCO was higher and longer after treatment with long MWCNT. However, other macrophage receptors (TLR4, MSR-1, and CD36) were elevated only after treatment short MWCNT. It has to be considered that long but not short MWCNT strongly affected cell integrity (membrane blebbing and severe membrane distortion) and viability of the macrophages, though the decrease in E. coli phagocytic as well as the migratory capacity was said not to be attributable to reduced viability (~ 70 % day 1, ~ 50 % day 5) only.

Boyles et al. (2015) tested a number of lab-synthesized fibre-like MWCNT on human monocytes (MM6) and mouse macrophages (J774A.1) and compared their induction of frustrated phagocytosis, cytotoxicity and inflammation-related response to that of MWNT-7 and asbestos fibres. In general, MWCNT > 20 µm in length but not MWCNT (< 20 µm) were cytotoxic and induced pro-inflammatory and pro-fibrotic immune responses. Also, frustrated phagocytosis, accompanied by respiratory burst and reduced phagocytic activity was most evident for long MWCNT. For CNTs > 20 µm, metal content and crystallinity had less or no influence on these endpoints, suggesting a role of fibre-length in fibre pathogenicity towards macrophages. Responses by MWCNT were even more detrimental than those induced by concomitantly tested asbestos fraction (SFA, LFA). Diameters were not precisely determined. Besides graphene quality, differences in crystallinity provided hints on the rigidity of the tubes. SEM images showed that MWCNT were delivered as small agglomerates, or as both straight and convoluted single fibres. Hence, experimental evidence supported pro-inflammatory, pro-fibrotic, pro-angiogenic activity induced by MWCNT, which may contribute to macrophage cytotoxicity. Thus, a corresponding detrimental environment triggered by frustrated phagocytosis of MWCNT in a length-dependent manner may also be influenced by iron content and MWCNT crystal structure.

To summarize, the above in vitro experiments provide several mechanistic clues for the understanding of the carcinogenic potential of MWCNT towards target cells. However, currently there is no unifying picture how mesothelial cell injury (or epithelial cell damage in case of lung tumours) is brought about. Evidence has been provided for clathrin-mediated or (transferrin-) receptor-mediated endocytosis but also for direct piercing of mesothelial cells. Fibre geometry (length and diameter) and crystallinity were identified as relevant factors affecting cell injury with a tendency towards longer and more crystalline (or rigid) tubes being more cytotoxic to both mesothelial and epithelial cells. Cellular damage has been linked to intracellular ROS generation, which

in turn may be a consequence of fibre geometry. Metal impurities of MWCNT or adhesion of metal-binding proteins are also discussed as promoters of intracellular oxidative stress, possibly resulting in DNA damage. Frustrated phagocytosis was most evident in case of crystalline MWCNT > 20 µm as were subsequent effects, such as cytotoxicity, increased intracellular ROS generation, inflammatory mediator release and reduced phagocytic and migratory activity of alveolar macrophage cell cultures.

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

Table 13 summarises the available key information for the classification of MWCNT with regard to carcinogenicity. There is unequivocal evidence from a two-year study according to OECD TG 451 and performed under GLP that inhaled MWNT-7 is carcinogenic to rats. The study reported neoplastic lesions in the lung (carcinoma and adenoma) and provided data on typical inflammatory and pre-neoplastic changes in the lung, such as epithelial hyperplasia, focal fibrosis, granulomas, fractured as well as frustrated alveolar macrophages and elevated BALF parameters. Both sexes developed lung tumours though males were found to be more sensitive. Interestingly, dense aggregates of MWCNT "cocconing" around disintegrated alveolar macrophages were observed in lung tissue, indicating possible additional adverse effects towards these cells besides frustrated phagocytosis. Concomitant lung burden measurements indicated that exposure doses were occupationally relevant when re-calculated into human equivalent concentrations. The quantity and persisting retention of inhaled MWCNT in the lung were deemed crucial for carcinoma development. The same study did not observe mesothelioma in the pleural or abdominal region, contrasting findings from intraperitoneal injection experiments with MWNT-7. The lack of these mesothelial tumours could have been a matter of insufficient fibre numbers reaching the pleural and abdominal cavity when inhaled (see below), which is particularly relevant when the predominant mode of action is assumed to depend on secondary genotoxicity. It may also reflect the slow growth of mesothelial tumours known from asbestos fibre pathogenicity, as pre-neoplastic lesions such as hyperplasia and fibrosis of parietal pleura were present.

In male mice, MWNT-7 was demonstrated to have also a high lung-carcinoma-promoting potential after short-term inhalation of methylcholanthrene-initiated animals at an occupationally relevant lung burden equivalent.

To date MWNT-7 is the only type of MWCNT, which proved carcinogenic in a long-term inhalation study and mesotheliomatogenic following non-physiological administration. However, other types of MWCNT were also shown to induce mesothelioma in a variety of animal models. Interestingly, the mesotheliomatogenic potency of a number of tested MWCNT proved even higher than that of asbestos. As a common feature, all positive MWCNT types so far tested had a diameter larger than 30 nm. Table 13a summarises the available data related to carcinogenicity in more detail, highlighting the type, dimensions and impurities and doses used in each individual study.

When administering a bolus dose of 1 mg per animal of MWCNT-N (30-80 nm in diameter) into the lung by transtracheal intrapulmonary spraying both, bronchiolo-alveolar carcinoma and pleural mesothelioma developed in male rats within a post-exposure period of up to two years, the latter preceded by hyperplastic proliferation of mesothelium of the parietal and visceral pleura, as proved in separate transtracheal studies.

Several independent studies in rat or mouse (including p53-mutant mouse strains) proved a mesothelioma-inducing potential of MWCNT by intraperitoneal or intrascrotal bolus injections and subsequent long-term observation, thus providing evidence for a fibre-like pathogenicity similar to asbestos (frequently used as "positive controls"). The mesotheliomagenic potential was not restricted to MWNT-7 but was also reported for other high-diameter MWCNT types (MWCNT > 30 nm in diameter).

On the other hand, a long-term i. p. bioassay is available that demonstrated no tumour (mesothelioma) formation for MWCNT of a diameter at or below 15 nm in diameter with a tangled morphology. This CLH proposal uses the diameter as proxy for rigidity and thus fibre-like character of a carbon nanotube. Based on the available evidence, the boundary diameter for classification of MWCNT is set to ≥ 30 nm. It is hypothesized that thinner MWCNT would lose their fibre-like rigid morphology. A tube length threshold, as discussed for fibre pathogenicity in general and demonstrated for nanowires, cannot be clearly derived from the available data. The smallest average tube length with mesothelioma-inducing potential was about 2.6 µm. However, alveolar deposition and interaction with alveolar and especially pleural macrophages justify a minimum length of 5 µm for the inhalation route, in accordance with the fibre pathogenicity paradigm.

Uncertainty with regard to tube dimensions cannot be completely eliminated. For example, it cannot be decided if the diameter range between 15-30 nm is indeed a "regulatory gap". As mentioned before, tube diameter is used as a surrogate parameter for a fibre-like morphology as long as there is no better measurand available for rigidity. Furthermore, length and width data in the literature partially varies largely even for the allegedly identical MWCNT types. It should also be considered that mean or median values or neither of them are arbitrarily provided in the studies. Eventually, it is to be taken into account that those dimensional values always represent a continuum of lengths and diameters in a population of tubes, which adds uncertainty to boundary settings. It is assumed that the EU nano definition for size and size distribution (50 % in the range of 1-100 nm) with an additional cut-off value of 30 nm is sufficient to capture critical fibre-like objects (see also below on considerations on the applicability of the fibre pathogenicity paradigm).

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There is a considerable dose discrepancy between the MWCNT "fibre" number found in the pleural region at the end of long-term inhalation exposure and MWCNT "fibre" numbers administered intraperitoneally which induced experimental mesothelioma. The available bolus injection studies deposited much larger MWCNT fibre numbers in the abdominal cavity, which are in the order of magnitude of 10^9 in rats (Rittinghausen et al., 2014) or 10^6 in p53-deficient mice (Takagi et al., 2012), whereas only 10^3 individual MWNT-7 fibres were found in the pleura and the abdominal cavity after inhalation of 2 mg/m^3 for two years. A recent trans-tracheal intrapulmonary spraying study, which tested well-dispersed MWCNT-N (Nikkiso), reported the development of pleural malignant mesothelioma in addition to lung tumours (Suzui et al., 2016). The amount of MWCNT-N in the lung was about 1000 µg 2 weeks after administration, which dropped to below 500 µg at the termination of the study after 109 weeks. This amount is not substantially different from lung burden measurements at the end of the 104 week continuous inhalation study (males: ~2 00 µg/lung at 0.02 mg/m^3 exposure concentration and ~ 1800 µg at 2 mg/m^3). Nevertheless, it cannot be ruled out that the initial amount of MWCNT-N that translocated to the pleura was larger by this type of bolus administration compared to continuous low dose inhalation exposure. Despite clear discrepancies in doses between inhalation and intraperitoneal administration it should be considered that the classification proposal is based exclusively on the identification of intrinsic hazardous properties. In this context, the available database shows that the intraperitoneal test is able to discriminate between mesotheliogenic and non-mesotheliogeneic MWCNT (Muller et al., 2009). Furthermore, it is known from mineral fibre studies that the mesothelioma risk of humans is higher than that of rats. For instance, the concentration of amphibole fibres in the lungs of asbestos workers with mesothelioma was 1000 times lower than crocidolite fibre concentrations in the lung of rats in negative inhalation studies (Pott et al., 1994).

Table 13: Compilation of factors to be taken into consideration in the hazard assessment

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
Rat Strain: Fisher 344	Lung carcinoma and adenoma Highly significant compared to air controls. No pleural or peritoneal mesotheliomas Evidence based on a guideline-compliant long-term inhalation study (Kasai et al., 2016)	No No other organs developed tumours	Yes Concentration- and duration-dependent lung toxicity: Alveolar epithelial hyperplasia → granulomatous changes and focal fibrosis → adenoma → carcinoma	Onset of tumour development not examined	Bothe sexes developed neoplastic lesions of the lung. However, males were more sensitive (tumours developed at already 10-times lower exposure concentration higher tumour incidences and higher incidence than females)	No. There were no abnormal clinical signs or significant effects on body weight gain or survival rates up to highest exposure dose	Inhalation (whole body exposure)	MoA unclear but significant lung carcinoma already at low aerosol concentrations (0.02 mg/m^3) discourages pulmonary overload as relevant MoA. Absence of mesothelioma formation has been speculated to be due to the low numbers of fibre-like MWCNT observed in the pleura following inhalation exposure compared to the number of fibres that induces carcinomas in the lung (~ 10^6 times more). However, with regard to hazard identification, the absence of mesothelioma also indicates a lack of sensitivity of this study design to detect this slowly developing tumour type by a fibre-related pathology. In this context it is noteworthy that a concentration-dependent increase in mesothelial hyperplasia in the parietal pleura was observed in both sexes.
Mouse Strain: B6C3F1	Lung carcinoma Highly significant increase in lung tumour volume compared to air	(Yes) Metastases and serosal tumours (combined	(Yes) Neoplastic progression of initiated lung cells (combined	Not applicable	Only males tested	Not addressed	Inhalation Challenge following i.p. initiation with methylcholanthrene	Experimental short-term exposure study demonstrating strong tumour promoting activity, both for lung carcinoma and

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	controls 17 months after exposure after combined treatment but already significant after MWNT-7 inhalation alone In addition increased malignant sarcomatous mesothelioma formation in the lung and other organs after combined treatment compare to MCA alone. Evidence based on individual study (not guideline-compliant) (Sargent et al. (2014)	treatment)	treatment)				(MCA)	mesothelioma after inhalation. MWCNT lung burden approximated feasible human occupational exposure equivalents.
<u>Rat</u> Strain: Fisher 344	Bronchiolo-alveolar carcinoma and adenomas as well as pleural malignant mesothelioma after about 2 years post-exposure observation. Proliferative and inflammatory lesions in the pleura Evidence based on three non-guideline-compliant studies of similar design, differing primarily in	Not investigated	Yes Hyperplastic proliferation of mesothelium of the parietal and visceral pleura	Not applicable	Only males tested	Not specifically addressed but discussed as less likely	Transtracheal intrapulmonary spraying	Non-physiological pulmonary short or medium-term exposure method, demonstrating translocation of fibre-like MWCNT to the pleural cavity and asbestos-related pathogenicity. Extended post-exposure observation resulted in lung tumours and pleural malignant mesothelioma of fibre-like MWCNT similar but not identical to MWNT-7 Positive control: crocidolite asbestos, negative control: non fibre-like MWCNT

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	exposure duration (Xu et al., 2012 and 2014), Suzui et al., 2016)							Supporting evidence for relevance of fibrous size and shape of MWCNT in mesothelioma development after inhalation.
<u>Rat</u> Strains: Wistar Fisher 344 Brown Norway <u>Mouse</u> Strains: B6C3F1 SLC mutant ($p53^{+/-}$)	Malignant mesothelioma of the peritoneum (sarcomatoid, epitheloid and biphasic type) Highly significant tumours compared to controls; very rare spontaneous tumour type in rodents. (Rittinghausen et al., 2014) Additional evidence based on a number independent studies using different administration methods, MWCNT test materials and dosing schemes (not guideline-compliant) (Takagi et al., 2008 and 2012; Sakamoto et al., 2009; Murphy et al., 2011; Nagai et al., 2011: Huaux et al., 2016)	Yes Invasion of peritoneal organs and metastasising, depending on study duration	Yes Pleural fibrosis and granulomatosis → mesothelial proliferation → hyperplasia → malignant mesothelioma	Yes Time of tumour onset dependent on fibre geometry but not necessarily dependent on dose	Both sexes (however, most studies performed with male rodents)	Not specifically tested Partially high concentrations administered. A role of metal contaminants could not be excluded but were critically discussed in a number of studies as of little impact.	Intraperitoneal injection Intrascrotal injection Intrapleural injection	Strong evidence for asbestos-related pathogenicity of MWNT-7 and other MWCNT with similar characteristics in size and rigidity. Frequently, asbestos fibres used as positive, while non-MWCNTfibre-like MWCNT as negative controls, supported by observations of frustrated phagocytosis by macrophages. Experimental evidence for a common initial pathogenic event shared by mesotheliomagenic MWCNT and asbestos fibres but absent from tangled MWCNT. Positive studies from mutant mice with tumour suppressor gene heterozygosity, sensitive for asbestos, are to be regarded as proof-of – principle evidence for similar pathogenicity of MWCNTfibre-like MWCNT. Experimental mesothelioma studies use non-physiological route of exposure and only partially realistic dosing

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
								schemes. However, those bioassays have been demonstrated as relevant and highly predictive in terms of (slow growing) mesothelioma formation for humans exposed to fibres such as asbestos and mineral wool (used in hazard classification according to EU CLP regulation. The 2–year rat inhalation study on MWNT-7 (see above) and similar carcinogenicity studies on fibres demonstrate that the inhalation test design lacks sensitivity to detect pleural mesothelioma, thus underestimating human cancer risk.

Table 13a: Overview of test material characteristics, exposure conditions and study outcome with respect to the carcinogenic potential of various types of MWCNT

Test material	Length	Diameter	Shape	Fibre No.	Metal impurities	Dose	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWNT-7 **(Mitsui)**	5.2 or 5.7 µm (45.1 or 48.7 % of tubes > 5µm	83.8 or 90.7 nm	Fibres	$9.03x10^6$ fibres/µg No. of MWNT-7 that induced lung carcinoma: 1.38×10^9 in males (at 0.2 mg/m^3) and 10.4×10^9 in females (at 2 mg/m^3) No. of fibres in pleura at 0.02, 0.2, 2 mg/m^3): 23, 134, 1468 (m) and 02, 240, 847 (f), respectively	Fe: 4,400 ppm Cr: 48 ppm Ni: 17 ppm	0, 0.02, 0.2, 2 mg/m3	rat	Inhalation (whole body) 104 weeks	Yes Lung carcinoma and adenoma (mainly bronchiolo-alveolar) No pleural mesothelioma	Kasai et al. (2016)
MWNT-7 **(Mitsui)**	5.11 µm (mean)		Fibres (low agglomeration)	3.82×10^{11} fibres/g		1.25 mg/animal	rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu et al. (2012)
MWCNT-N **(Nikkiso)**	3.64 µm		Fibres (low agglomeration)	3.46×10^{11} fibres/g		1.25 mg/animal	rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu et al. (2012)
MWCNT-L **(synthesized in lab)**	8 µm	150 nm	Needles (low agglomeration)			1.625 mg/animal	rat	Transtracheal intrapulmonary spraying 24 weeks	Yes Patchy parietal mesothelial proliferation	Xu et al. (2014)

Test material	Length	Diameter	Shape	Fibre No.	Metal impurities	Dose	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT-S **(synthesized in lab)**	3 µm	15 nm	Tangled (agglomerated)			1.625 mg/animal	rat	Transtracheal intrapulmonary spraying 24 weeks	No	Xu et al. (2014)
MWCNT-N **(Nikkiso)**	4.2 µm 2.6 µm	30-80 nm	Fibres		Fe: 0.046 %	1 mg/animal	rat	Transtracheal intrapulmonary spraying 2 weeks + 109 weeks p.e.	Yes Bronchiolo-alveolar lung adenomas and carcinomas Pleural malignant mesotheliomas	Suzui et al., 2016
NT_{long2} **(Univ. Manchester)**	> 15 µm (85 % of fibres)	20-100 nm (165 nm, measured)	Long straight fibre (low agglomeration)		low	5 µg/animal	mouse	Intrapleural injection	Yes Mesothelial proliferation in parietal pleura of chest wall and diaphragm 24 weeks p.e.	Murphy et al. (2011)
NT_{tang2} **(NanoLab)**	1-5 µm	15 nm	Short, tangled (low agglomeration)		low	5 µg/animal	mouse	Intrapleural injection	No (Observation period 24 weeks)	Murphy et al. (2011)
MWNT-7 **(Hodogaya)**	Several µm	Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm.	Single fibre-like nanotubes to tangled agglomerates		Trace metal contamination 1.32 % (Fe: 1.06 %)	5 mg/m^3 for 15 days (31 µg lung burden)	mouse	inhalation	Yes (Promoting activity) Pulmonary adenomas and adenocarcinomas as well as systemic sarcomatous mesotheliomas 17 months p.e.	Sargent et al. (2014) [Porter et al., 2013]
MWCNT **(synthesized in lab, with and w/o defects)**	0.7 µm	11 nm	Tangled (agglomerated)		Al: 1.97 / 0.37 % after depletion Fe: 0.49 / < 0.01 % Co: 0.48 / < 0.01 %	2 and 20 mg / animal	rat	Intraperitoneal injection	No (observation period 24 months)	Muller et al. (2009)
NT50a **NT-50a(-agg)**	5.29 µm	49.95 nm	Needles of high crystallinity	~15 x 10^3 fibres/µL	Soluble metals in fibre solution (0.5 mg/mL):	1 mg /animal (mass concentration for	rat	Intraperitoneal injection	Yes	Nagai et al. (2011)

Test material	Length	Diameter	Shape	Fibre No.	Metal impurities	Dose	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
= MWNT-7, Mitsui)			(bulk material was agglomerated, the subfraction NT-50a(-aggr.) was not)	NT50(-agg*), a double-concentrated subfraction of NT50a of low agglomeration	Fe: < 100 ng/g Cu: < 100 ng/g Low ROS generation	subfraction NT50a(-agg* was not provided but deemed much lower (derived from centrifugation supernatant of NT50a)			Malignant mesothelioma after 1 year	
NT145 **(Showa Denko)**	4.34 µm	143.5 nm	Rods (low agglomeration)	~15 x 10^3 dispersed fibres/µL in 0.5 mg/mL fiber suspensions	Soluble metals in fibre solution (0.5 mg/mL): Fe: not detected Cu: not detected Low ROS generation	1 mg and 10 mg/animal	rat	Intraperitoneal injection	(Yes) However mesothelioma incidence after 1 year of 1mg dose much lower and latency of tumour-induced mortality higher compared to 1 mg NT50a and equivalent fibre dose of NT50a(-agg*). Furthermore, in contrast to NT50a, fibrosis index of NT 145 was not significantly increased against controls, and did not pierce mesothelial cells.	Nagai et al. (2011)
NT50b **(Showa Denko)**	4.6 µm	52.4 nm	Fibre of high crystallinity (agglomerated)	~10 x 10^3 dispersed fibres/µL in 0.5 mg/mL fiber suspensions	Soluble metals in fibre solution (0.5 mg/mL): Fe: < 100 ng/g Cu: not detected Low ROS generation	10 mg/animal	rat	Intraperitoneal injection	Yes Malignant mesothelioma after 1 year (1 mg not tested)	Nagai et al. (2011)
NTtng **(Showa Denko)l**	3 µm	15 nm	Tangled (agglomeration very high)		Soluble metals in fibre solution (0.5 mg/mL): Fe: > 100 ng/g Cu: not detected Low ROS generation	10 mg/animal	rat	Intraperitoneal injection	No (observation period up to 3 years)	Nagai et al. (2011) Nagai et al. (2013)

Test material	Length	Diameter	Shape	Fibre No.	Metal impurities	Dose	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT A **(synthesized)**	8.57 µm (WHO fibres) 2.72 µm (all fibres)	85 nm	Straight fibre (inner angle: 175°) (low agglomeration)	Fibres > 20 µm: 3.81 % of WHO fibres	No surface-bound or dissolved iron or cobalt catalyst detected (incorporation into nanotube core assumed)	0.48 x 10^9 (0.2 mg) 2.39 x 10^9 (1 mg) WHO fibres per rat	rat	Intraperitoneal injection	Yes Mesothelioma in 98/90 % of rats (low/high dose) Mean survival time: 213/194 days First detection of morbidity: 5/5 months p.e.	Rittinghausen et al. (2014)
MWCNT B **(synthesized)**	9.3 µm (WHO fibres) 2.13 µm (all fibres)	62 nm	Straight fibre (inner angle: 148°) (low agglomeration)	Fibres > 20 µm: 9.35 % of WHO fibres	No surface-bound or dissolved iron or cobalt catalyst detected (incorporation into nanotube core assumed)	0.96 x 10^9 (0.6 mg) = low dose 4.8 x 10^9 (3 mg) = high dose WHO fibres per rat	rat	Intraperitoneal injection	Yes Mesothelioma in 92/90 % of rats Mean survival time: 294/207 days First detection of morbidity: 6/5 months p.e.	Rittinghausen et al. (2014)
MWCNT C **(synthesized)**	10.24 µm (WHO fibres) 4.18 µm (all fibres)	40 nm	Straight fibre (inner angle: 146°) (low agglomeration)	Fibres > 20 µm: 11.77 % of WHO fibres	No surface-bound or dissolved iron or cobalt catalyst detected (incorporation into nanotube core assumed)	0.87 x 10^9 (0.08 mg) 4.36 x 10^9 (0.4 mg) WHO fibres per rat	rat	Intraperitoneal injection	Yes Mesothelioma in 84/94 % of rats Mean survival time: 415/265 days First detection of morbidity: 10/6 months p.e.	Rittinghausen et al. (2014)
MWCNT D **(synthesized)**	7.91 µm (WHO fibres) 2.53 µm (all fibres)	37 nm	Bent fibre (inner angle: not measured) (low agglomeration	Fibres > 20 µm: 2.13 % of WHO fibres	No surface-bound or dissolved iron or cobalt catalyst detected (incorporation into nanotube core assumed)	1.51 x 10^9 (0.25 mg) 7.54 x 10^9 (1.4 mg) WHO fibres per rat	rat	Intraperitoneal injection	Yes Mesothelioma in 40/70 % of rats Mean survival time: 666/585 days First detection of morbidity: 20/11 months p.e.	Rittinghausen et al. (2014)
MWNT-7 (Mitsui)	7.1 µm	75 nm	Fibres	78 % > 5 µm	Fe: 0.35 % Co: < 0.001 %	6 mg (2x10^9 WHO fibres)/animal	rat	Intraperitoneal injection	Yes First incidences of mesotheliomas after 6	Huaux et al. (2016)

Test material	Length	Diameter	Shape	Fibre No.	Metal impurities	Dose	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
			(modified by annealing)		Ni: < 0.001 % Mo: < 0.001 %				months, ~ 100 % of animals positive after 12 months (crocidolite asbestos negative)	
Short MWNT-7	2.8 µm	75 nm	Grounded fibres (modified by annealing)	14 % > 5 µm	Fe: 0.35 % Co: < 0.001 % Ni: < 0.001 % Mo: < 0.001 %	6 mg ($2x10^9$ WHO fibres)	rat	Intraperitoneal injection	Yes Lower incidences and longer latency compared to unground MWNT-7 (crocidolite asbestos negative)	Huaux et al. (2016)
MWNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	Rod/Fibre (agglomerates among individual nanotubes)		Fe: 0.35 %	3 mg/animal (1 x 10^9 fibres)/animal	mouse (transgenic)	Intraperitoneal injection	Yes Invasive mesothelioma after 25 weeks (100 % mortality)	Takagi et al. (2008)
MWNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	Rod/Fibre (agglomerates among individual nanotubes)		Fe: 0.35 %	3, 30, 300 µg/animal ($1x10^6$, $1x10^7$, $1x10^8$ fibres)	mouse (transgenic)	Intraperitoneal injection	Yes Dose-dependent mesothelioma induction 1 year p.e., with cumulative incidence of mesotheliomas of 19/20, 17/20 and 5/20, respectively.	Takagi et al. (2012)
MWNT-7 **(Mitsui)**	1-4 µm (72.5 %)	82 % in range 70-110 nm	Rod/Fibre (agglomerates among individual nanotubes)		Fe: 0.35 %	1 mg/kg bw	rat	Intrascrotal injection	Yes Mesothelioma in 6/7 animals after 52 weeks	Sakamoto et al. (2009)

A recently published IARC monograph (IARC, 2017) evaluated carbon nanotubes with regard to carcinogenicity. The IARC grouped MWNT-7 in Group 2B (possibly carcinogenic to humans) and other MWCNT (as well as SWCNT) excluding MWNT-7 in Group 3 (not classifiable). The evaluation identified sufficient evidence from animal data (mesothelioma induction after injection, adenocarcinoma promotion after inhalation) with regard to MWNT-7 and limited evidence for MWCNT with similar dimensions as MWNT-7 also causing mesothelioma. Other findings (pleural translocation, persistent pulmonary inflammation, granuloma formation, fibrosis, and bronchiolar or bronchiolo-alveolar hyperplasia in rodents, genetic lesions, and perturbation of the cellular mitotic apparatus) were acknowledged as relevant to humans, though the mechanistic evidence for carcinogenicity was not considered strong by the majority of the expert panel. Furthermore, the lack of coherent evidence across the various distinct CNT precluded generalisation to other types of CNT (Grosse et al. 2014). However, the IARC categorisation not yet considered highly relevant findings, in particular those by Kasai et al. (2016), Suzui et al. 2016 and Rittinghausen et al. (2014), which not only strengthen the classification for MWNT-7 as a human carcinogen but also provide sufficient evidence for extending the classification to other MWCNT with WHO fibre dimensions.

Considerations on the applicability of the fibre pathogenicity paradigm

The overall relevance of the data with regard to carcinogenicity of rigid MWCNT in humans is given by the similar pathogenicity of inhaled biopersistent asbestos[7], which induces both lung carcinoma and mesothelioma. Similar classifications have already been applied previously for other types of fibres. Asbestos-induced malignant mesothelioma is an otherwise extremely rare tumour type with large latency periods (Peto, 1985). Though human evidence is missing, MWCNT with fibre dimensions also induced lung carcinoma in rats but no mesothelioma following inhalation. However, mesothelioma was induced by intraperitoneal injection or direct intrapulmonary spraying exposure. Though these exposure methods are both non-physiological, resulting in considerable local bolus doses, they are appropriate tools for the identification of fibrous carcinogens. The intraperitoneal model has been rated superior to inhalation for identifying human fibre carcinogens (Wardenbach et al., 2005). In addition, there is evidence for pleural translocation of inhaled MWCNT in rodents and pleural plaques or thickening, granulomatosis and fibrosis, resembling pre-carcinogenic conditions asbestos-exposed workers develop. Like asbestos, rigid WHO fibre-like MWCNT are retained in the lung after exposure, elicit a persistent inflammatory response in the lung, may undergo pleural drift, provoke frustrated phagocytosis, and induce diffuse and focal fibrosis in lung and pleura. Both asbestos and MWCNT produce numerical and structural chromosomal alterations and interfere with mitotic spindle formation.

The "fibre pathogenicity paradigm" has been suggested as a unifying concept to explain the specific toxicology of inhaled respirable fibres (Donaldson et al., 2010, 2013). This concept identified a triad of inter-related parameters as necessary triggers of adversity in the respiratory tract which are responsible for the development of "asbestiform" disease, largely independent of chemical composition: length, diameter and biopersistence. With regard to length and width, the concept adheres to the WHO fibre dimensions (length: ≥ 5 µm, diameter: < 3 µm, aspect ratio $\geq 3{:}1$). Any modifications in one of these parameters is supposed to have a dramatic impact on the pathogenic and ultimately the carcinogenic potential of a fibre. According to the EC definition of nanomaterials the aspect ratio can be considered as a redundant parameter, since a MWCNT with diameter of 100 nm and length of 5 µm would still result in a aspect ratio of 50:1.

Biopersistence:

The carbon scaffold of pristine MWCNT is extremely stable, disposing mechanical strength even higher than steel. Accordingly, non-degraded MWCNT have been observed to be retained in the mouse lung > 300 days after a 12 day inhalation period (Mercer et al., 2013a) and MWCNT proved to be still

[7] All types of asbestos proved to be carcinogenic to humans as confirmed by a recent update by the IARC assessment, and seven CAS numbers of asbestos are classified as Carc. 1A/H 350 (as well as STOT RE 1/H372**) according to European CLP regulation (version: 2008R1272 — EN — 19.04.2011 — 002.001 — 1). The same carcinogenicity category applies to erionite fibres (CAS No. 12510-42-8).

mesotheliomagenic after 24 weeks of incubation in vitro in Gambles solution at pH 4.5, thus mimicking the acidic conditions of phagolysosomal fluid in alveolar macrophages (Osmond-McLeod et al., 2011). Inhaled SWCNT have been shown to be prone to degradation by myeloperoxidase from neutrophils and macrophages in vitro (Kagan et al., 2010). If rigid WHO fibre-like MWCNT in vivo are similarly attacked by cytoplasmatic enzymes, is unknown but unlikely, considering their incomplete phagocytosis and the observed long retention half-times in the lung.

Tube length:

The smallest mean tube length of MWCNT materials inducing both malignant mesothelioma and lung tumours was 2.6 ± 1.6 µm as administered as the "unfiltered fraction" and 2.0 ± 0.4 µm as measured in tumour tissue areas (Suzui et al., 2016) following intrapulmonary spraying. However, a large size range between 1-10 µm in the unfiltered fraction was reported, making it impossible to determine a critical size threshold form this data. Nagai et al. (2011) identified a tube length of 4-5 µm and a needle-like shape as important tumour-inducing factors in vivo. This was supported by a number of in vitro studies which reported that long (> 5 µm) but not short tubes are more cytotoxic for mesothelial and epithelial cells (Nagai and Toyokuni, 2012; Nymark et al., 2014) and more cytotoxic and inflammogenic for (alveolar) macrophages (Sweeney et al.; 2014, 2015; Boyles et al., 2015). Schinwald et al. (2012) and Poland et al. (2012) proposed a generic threshold or minimum length of 4 to 5 µm for fibre-induced acute pleural inflammation, based on intrapleural injections and intraperitoneal injection or pharyngeal aspiration, respectively, into mice of fibrous high aspect ratio nanomaterials of definite lengths, such as silver or nickel nanowires. The size limit is explained with the trapping of larger fibres at the stomata in the parietal pleura (the size of which ranges from 4-10 µm in rodents), where they accumulate and less by the incapability of alveolar macrophages to cope with these longer objects known as frustrated phagocytosis.

Tube diameter:

The available data suggest that there is a correlation between tube diameter and carcinogenic potential of MWCNT, as a higher diameter increases the rigidity of the tube and thus its resistance towards macrophage clearance. This is corroborated by lack of mesothelioma data for thin, low diameter MWCNT and SWCNT, which preferentially have a tangled, non-fibrous morphology. From the available data a minimum tube diameter > 15 nm is deemed critical for fibre pathogenicity. Piercing of mesothelial cells, which has been identified as an additional mechanism in mesotheliomagenesis (Nagai et al., 2011) would also depend on tube rigidity and thus its diameter.

Other relevant factors:

Material properties such as surface reactivity and metal impurities are regarded as modifying factors of toxicity, in particular in terms of ROS generation and induction of oxidative stress. MWCNT-derived iron ions have been assumed to be detrimental to mesothelial cells when MWCNT are in close contact with them or when internalized, as metals favour oxidative damage. However, systematic investigations on the impact of metal toxicity in MWCNT carcinogenicity are missing. The available data do not provide a clear picture because of the large variation in purity of MWCNT used as test materials across studies. Wang et al. (2016) demonstrated that contaminations are possibly not the only source of metals. Coating of MWCNT by host iron-binding proteins such as haemoglobin or transferrin, may not only facilitate MWCNT uptake but also promotes oxidative stress to mesothelial cells due to high intracellular iron concentrations (Dymacek et al. 2018, Snyder Talkington, 2013, Arnoldussen 2018)

It is concluded that biopersistence, length and diameter of MWCNT are critical drivers of pathogenicity, similar to hazardous fibres such as asbestos. Thus, overall the fibre paradigm is applicable to MWCNT. Other material-specific factors, such as surface reactivity, surface functionalization and metal ion release or binding

could also be relevant as putative modifiers of the fibre-induced carcinogenic potential but are deemed secondary to fibre dimensions.

10.9.2 Comparison with the CLP criteria

It is concluded that there is sufficient evidence from existing data to classify MWCNT with fibre lengths ≥ 5 µm and a minimum diameter of ≥ 30 nm as carcinogenic to humans. This evidence is based on the following findings from animal studies:

1. Dose-dependent significantly increased incidence in lung tumours (carcinoma and adenoma) in male and female rats following long-term inhalation (whole body) exposure at realistic, non-overload concentrations.
2. Promotion of lung tumour development (carcinoma and adenoma) after short-term inhalation exposure of MWCNT to methylcholanthrene-initiated mice.
3. Mesothelioma formation after intratracheal instillation of rats.
4. Induction of malignant mesothelioma in wild rats and p53-mutant mice following intraperitoneal (and intrascrotal) injection.
5. Preneoplastic changes in lung (irreversible inflammation, fibrosis, epithelial hyperplasia) in rats and mice following short-term inhalation, intratracheal instillation or transtracheal intrapulmonary spraying.
6. Length-dependent induction of fibrosis in pleura after direct intrapleural exposure of rats.
7. Localisation of MWCNT in the lung for > 1 year and in the pleural region, indicating high biopersistence and pleura drift after subacute inhalation exposure.
8. Specificity of intraperitoneal mesothelioma induction limited to fibre-like MWCNT ≥ 30 nm in diameter.
9. Principal similarities to asbestos pathogenicity (frustrated phagocytosis, stoma retention, mitotic spindle formation interference), tumour development (lung and pleura fibrosis) and to mesothelioma type (sarcomatoid, epithelial and biphasic), as demonstrated by comparative studies using asbestos as positive control fibres.

Classification criteria

A) Strength of evidence:

The lack of human exposure and hazard information does not allow conclusions on causality between human exposure and the development of cancer. However, the weight of evidence of the available data from animal studies allows the conclusion that there is sufficient evidence that fibre-like MWCNT may be carcinogenic to humans when inhaled. Key to this conclusion is an available 2 year inhalation study in rats proving an increased incidence of lung tumours but not of mesothelioma by MWNT-7 exposure in both sexes. Importantly, this study avoided exposure to doses known to elicit a particle-related overdose response. Rather, clearance function by alveolar macrophages was likely affected by incomplete phagocytosis. Length-dependent malignant mesothelioma induction by MWNT-7 and other non-curled MWCNT with fibre dimensions in rats and mice using intraperitoneal injection demonstrated the principal applicability of the fibre pathogenicity paradigm to these substances (Donaldson et al., 2010, 2013). The discrepancy between long-term inhalation and intraperitoneal injection with regard to mesothelioma formation is not surprising and a matter of dose and observation duration. Mesothelioma is a rare tumour with long latency. It could have been that in the inhalation study by Kasai et al. (2016) the number of MWCNT translocating to the pleura was too low and the post-exposure period too short to observe mesotheliomas. This assumption is in accordance with the observation of

proliferative mesothelial changes in the pleura of a number of test animals. On the other hand, high local bolus exposure of rigid MWCNT with WHO fibre dimensions by intraperitoneal injection frequently resulted in mesothelioma formation within several months (e.g. Rittinghausen et al., 2014). However, it should be noted that classification as a carcinogen relies on hazard identification and dose considerations are secondary, in particular as there is some indication that MWCNT also are genotoxic. Taken together, there is more than one reliable animal study directly demonstrating a carcinogenic potential of MWCNT with fibre dimensions in more than one species and a large number of supporting experimental findings exist in vivo and in vitro. Extrapolation to all MWCNT with defined fibre dimensions is justified by i) positive findings in mesothelioma studies using MWCNT other than MWNT-7, ii) the absence of mesothelioma when injecting thin and curled or tangled MWCNT, and iii) the similarities to asbestos pathogenicity. Analysis of all available data showed that fibre pathogenicity is limited to non-tangled MWCNT ≥ 30 nm in diameter and ≥5 µm in length, thus complying with the current WHO fibre dimensions.

B) *Additional considerations (according to Annex I: 3.6.2.6.,* Guidance on the Application of the CLP Criteria*):*

a. *Tumour type and background incidence*
There is sufficient evidence from animal studies that two tumour types are induced which are typical for inhaled fibrous materials, bronchioalveolar epithelial adenocarcinomas and slow-growing mesotheliomas. Lung carcinoma is a typical consequence of chronically inhaled biopersistent particles. For asbestos, extrathoracical tumours in humans are also rare and controversial (IARC, 2012. In terms of mesothelioma, the spontaneous incidence is particularly rare in rodents and sensitivity for chemical-induced mesothelioma development in rodents is deemed lower compared to humans (Pott et al., 1994, Wardenbach et al., 2005). Serosa tumours induced by MWCNT included sarcomatoid, epithelial and biphasic type, which are also typically induced by asbestos fibres. All relevant available studies demonstrating an increased tumour incidence reported a significant difference between substance-induced and spontaneous tumour incidence.

b. *Multi-site responses*
Malignant tumours induced by MWCNT inhalation are restricted to the lung and more specifically to the site of deposition, correlating with sites of focal fibrosis. Pleural mesothelioma formation was shown but so far only after non-physiological exposure conditions (intraperitoneal, intrapleural, intrascrotal administration) although pleura drift of MWCNT occurs in case of chronic inhalation exposure. Extrathoracic tumours following inhalation have not yet been reported.

c. *Progression of lesions to malignancy*
The data on MWCNT collectively provides evidence for a progression of lesions characteristic for inhaled biopersistent fibrous materials, ranging from persistent inflammation, epithelial hyperplasia, giant cell formation, granulomatosis, fibrosis to benign adenoma and malignant carcinoma development in the lung. Pre-neoplastic lesions such as fibrosis and mesothelial proliferative changes are also observed at the parietal pleura, the chest wall and the diaphragm after instillation, preceding malignant mesothelioma formation. Pre-neoplasia and neoplasia are dose-dependent.

d. *Reduced tumour latency*
Usually, particle-induced lung tumours develop late and mesothelioma development has a particular long latency period in humans. Using intraperitoneal injection, Rittinghausen et al. (2014), showed that the potency as well as the latency of mesothelioma induction of MWCNT with WHO fibre dimensions correlates with their rigidity, since the less curved the nanotube is the shorter was the time period to mesothelioma onset. It could be shown that when equally high numbers of MWCNT and amosite asbestos fibres were injected, MWCNT generally proved to be even more potent and had shorter latencies.

e. Responses are in single or both sexes

Lung tumours were observed in both sexes following long-term inhalation. However, male rats proved more sensitive than female rats. This can be explained by the higher respiratory frequency of males. Both sexes are also prone to mesothelioma development after intraperitoneal injection.

f. Responses are in a single species or several species

Lung carcinoma formation by inhalation exposure was shown in rats, tumour-promoting activity was observed in mice. Experimental mesotheliomagenesis has been demonstrated in different strains of rats and in mice. However, wild-type mice are not ideal animal models for mesothelioma induction because of long tumour latencies (Vaslet et al. 2002, Huaux et al., 2016). Therefore, several injection studies preferred mutant mouse strains, heterogeneous for p53 or other tumour-suppressor genes.

g. Structural similarity to a substance(s) for which there is good evidence of carcinogenicity

A number of studies included asbestos fibres as positive controls and demonstrated the principal similarity between asbestos and fibre-like MWCNT pathogenicity. The applicability of the fibre pathogenicity paradigm (Donaldson et al., 2010, 2013), stressing the relevance of material properties such as length, diameter and biopersistence, is discussed separately.

h. Routes of exposure

Fibre-like MWCNT proved to be carcinogenic when inhaled. No information is available for carcinogenesis after oral or dermal administration. Absence of tumour development by other physiological exposure routes would be in agreement with asbestos-induced cancer. Though intraperitoneal injection is a non-physiological exposure route, it is meanwhile an accepted model to demonstrate mesothelioma induction by fibrous materials (IARC also considers intraperitoneal studies for carcinogenicity categorisation). Other non-physiological pulmonary exposure techniques, such as intratracheal instillation, intrapulmonary spraying or intrapleural injection, have been applied to demonstrate fibre deposition and distribution as well as toxicity and tumour development in the lung. These studies provide useful supportive information for classification.

i. Comparison of absorption, distribution, metabolism and excretion between test animals and humans

With regard to inhalation there are principal differences between rodents and humans as the former are obligatory nose-breathers and have a higher respiratory minute volume which may explain differences in deposition of MWCNT in the respiratory tract, alveolar epithelial surface, and in lung burden. There are also differences in the alveolar macrophage pool between rodents and humans, which has been attributed a particularly critical determinant of particle clearance half-times (Pauluhn, 2011). Rats clear particulate matter from the alveolar region faster than humans, justifying a higher dose to achieve an equivalent lung burden to humans. These species differences have been taken into account by lung burden measurement to account for internal doses and calculation of human equivalent concentrations, which are subsequently related to realistic workplace exposure concentrations. However, apart from quantitative differences that may affect the carcinogenic potency, there is no reason to assume that fundamentally different kinetics of inhaled MWCNT exist between rats and humans, taking into account the high resistance of MWCNT towards degradation and their fibre-like shape, which make them highly biopersistent resulting in high lung retention and negligible metabolism. A number of rodent inhalation studies demonstrated that persistent inflammation and pre-neoplastic lesions occur at lung burdens, the human equivalent of which would be relevant for human occupational exposure situations exposed to MWCNT (Porter at al., 2010, Mercer et al., 2013, 2013a, Sargent et al., 2014, Kasai et al., 2015).

j. The possibility of a confounding effect of excessive toxicity at test doses

The aspect of excessive toxicity is to be considered. Pulmonary adenoma and carcinoma incidence increased significantly and dose-dependently following chronic inhalation of

MWNT-7, starting at 0.02 mg/m^3, differing significantly from controls after 0.2 mg/m^3 (Kasai et al., 2016). Lung burdens in this carcinogenicity study ranged from 8 to 1800 µg at the end of the 104 week exposure period, thus arguing against particle overload as the primary cause of lung tumour formation, which usually starts at particle burdens > 1mg/lung (Morrow, 1988). The lung tumour-promoting potential of MWNT-7 was evident after 15 days of inhalation in initiated mice at a lung burden of 31 µg, which was deemed occupationally relevant for (susceptible) humans. In fact, early mesothelioma experiments used high and sometimes repeated bolus doses which are unrealistically high (e.g. Poland et al., 2008, Sakamoto et al., 2009, Takagi et al., 2008). However, Takagi et al. (2012) showed that MWNT-7 induced mesothelioma at a dose as low as 3 µg/animal, a 1000-fold lower concentration than tested earlier (Takagi et al. 2008), despite the fact that they used more vulnerable p53-heterogeneous mice. Murphy et al. 2011 were able to induce mesothelial proliferation in mice by intrapleural injection of long straight MWCNT fibres (diameter: 0-200 nm, 165 nm measured) but not by short or tangled fibres (diameter: 15 nm) at a comparably low dose (5 µg). On the other hand, tangled MWCNT did not induce mesotheliomas after intraperitoneal injection of up to 20 mg/rat. Aerosol generation and exposure conditions are known to modify the morphology and the toxicology of particles (Pauluhn and Rosenbruch, 2015). It is essential to consider how the test material is generated and whether in the study efforts are made to reduce the agglomeration of tubes. Dry aerosol generation and methods to individualise fibres has been given particular weight in hazard identification of MWCNT.

k. *Mode of action and its relevance for humans*

There is sufficient evidence from the available data that the inhalation toxicology of rigid MWCNT is similar to asbestos and other mineral fibres, with biopersistence and WHO fibre dimensions as crucial determinants of carcinogenicity. Accordingly, the relevance for humans is implicitly given by this analogy and the extensive historical record of lung cancer and mesothelioma incidences due to asbestos exposure (Lippmann, 2014, Roe et al., 2015). Key to this analogy is common mechanistic effects observed between asbestos and carbon nanotubes, including frustrated phagocytosis, pleural stomata retention, ROS generation and mitotic spindle disruption, which may act independently or in concert contributing to pathogenicity. In terms of genotoxicity, the available data provide evidence for both a clastogenic and an aneugenic activity of WHO fibre-like MWCNT as well as some evidence for inflammation-mediated secondary genotoxicity. However, more reliable and standardised tests are required to clarify if MWCNT is a genotoxic carcinogen. In particular, an aneugenic action of MWCNT by interfering with microtubules in spindle formation during mitosis, which has also been reported for asbestos and other CNT, is discussed (which would be in accordance with a threshold effect). The molecular mode of action is not well understood. In taking efforts to identify predictors and adverse outcome pathways of MWCNT toxicity, a number of recent studies stressed the role of inflammasome activation in MWCNT bioactivity (Sager et al., 2014, 2016, Hamilton et al. 2013, 2013a, Vietti et al., 2016, Dong et al., 2015), though it would be premature to draw general conclusions on the mode of action and role of surface modification in terms of fibre pathogenicity. In addition, these events generally require cellular uptake or intracellular signalling which may be distinct from events associated with incomplete phagocytosis in case of fibre-like MWCNT.

10.9.3 Conclusion on classification and labelling for carcinogenicity

According to the Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, classification into carcinogenicity category 1 (Carc.1) applies, when a substance is a known or presumed human carcinogen. Carc.1A applies if a substance is known to have carcinogenic potential for humans, and classification is largely based on human evidence. Carc.1B applies, if the substance is presumed to have carcinogenic potential for humans and classification is largely based on animal evidence.

Carc.2 classification comprises suspected carcinogens, when the evidence is not sufficiently convincing to place the substance as Carc.1A or 1B but when limited evidence is available.

Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated. The strength of evidence together with additional considerations (factors a - k; see above) is the basis for classification as Carc.1 A or 1B.

The present proposal precludes classification as Carc.1A, because human information is lacking.

However, there is sufficient evidence for classification as Carc.1B. A causal relationship has been established between the agent and an increased incidence of malignant neoplasms in two and more species of animals and two or more independent studies in one species carried out at different times or in different laboratories or under different protocols.

Key to classification is a two-year inhalation study commissioned by the Japanese Ministry, which demonstrated the development of lung carcinoma after inhalation of MWCNT in male and female rats at non-overload exposure conditions (Kasai et al., 2016) as well as several mesothelioma-inducing studies in rats and (knock-out) mice. Supporting evidence comes from a lung tumour-promoting study and a number of independent findings on pre-neoplastic lesions, lung retention and pleura drift after pulmonary exposure. Comparative investigations indicated similarities to asbestos fibre pathogenicity.

Key parameters relevant to carcinogenicity classification of MWCT/MWCNT are WHO tube length, a minimum diameter that is considered critical for fibre-like properties of the nanotubes, as well as biopersistence. A practical length threshold of 5 µm[8] is in agreement with the fibre pathogenicity paradigm and the fact that smaller tubes are effectively cleared from the lung when inhaled at non-overload conditions. A threshold in diameter of 30 nm is derived empirically from the available carcinogenicity data.[9] The diameter is a useful proxy for defining a fibre phenotype of a carbon nanotube, as long as a standardised method for determining rigidity is not available. Presently no data is available which would support classification of MWCNT with diameters between 15 and <30 nm. For the 15-30 nm diameter range, evidence on carcinogenicity is currently not available. However, it is assumed that MWCNT with diameters up to 30 nm tend to coil thereby losing their straight fibre character. In terms of biopersistence, pristine carbon nanotubes are extremely stable materials and highly resistant towards degradation under physiological conditions.

It is highly improbable that exposure by the dermal or even oral route would lead to a carcinogenic response taking into account that long-term deposition of MWCT in the tissues, as can occur in lung, is a prerequisite for carcinogenicity. According to present knowledge, there is no evidence that other carcinogenic fibres meeting the WHO definition have carcinogenic properties after oral or dermal exposure.

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[8] Earlier studies derived empirically a critical fibre length of 20 µm from asbestos and MMVF exposure studies as this is a maximum length for the "frustrated phagocytosis" phenomenon by alveolar macrophages (longer fibres are not deposited in the alveolar region, whereas shorter fibres are completely internalised and removed). However, for MWCNT this length threshold cannot be reproduced and other (primary or secondary) biopersistence mechanisms need to be considered, such as pleural stoma retention.

[9] Using the experimental findings on the carcinogenic potential of the thinnest carcinogenic MWCNT with a mean diameter of 37 nm substracting three standard deviations leading to 33 nm would cover nearly 100 % of all tested fibre diameters assuming a normal distribution. 30 nm was used as rounded cut-off.

It is proposed that Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T MWCT/MWCNT are be classified as carcinogen 1B for the inhalation route (H350i).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposal on the classification as Carc. 1B is based on the evidence from animal studies on several different types of MWC(N)T with fibre dimensions (≥ 5 µm in length and a diameter range ≥ 30 nm to < 3 µm) including:

- Significantly increased incidence in lung tumours in rats following long-term inhalation of MWCNT-7 (Kasai *et al*., 2016).
- Experimental mesothelioma formation in rats and (mutant) mice following intraperitoneal injection (Nagai *et al*.,2011; Huaux *et al*, 2016; Rittinghausen *et al*., 2014; Tagaki *et al*., 2008 and 2011).
- Preneoplastic changes (fibrosis) of lung and pleura tissue and tumour types strikingly similar to those after asbestos exposure.
- Pleural drift and retention following inhalation exposure.
- Promotion of lung tumour development after short term inhalation exposure of initiated mice (Sargent *et al*., 2014).
- Mesothelioma formation after transtracheal intrapulmonary spraying (Suzui *et al*., 2016)
- Pathogenicity progress after inhalation and intratracheal instillation similar to asbestos.

The critical studies used by the DS are listed in the table below (modified from table 13a in the CLH report, but with the data on thin, tangled MWCNTs excluded).

Test material	Length	Diameter	Species	Exposure	Carcinogenicity/pre-neoplasia	Reference
MWCNT-7 **(Mitsui)**	5.2 or 5.7 µm (45.1 or 48.7 % of tubes > 5µm	83.8 or 90.7 nm	rat	Inhalation (whole body) 104 weeks	Yes Lung carcinoma and adenoma (mainly bronchiolo-alveolar) No pleural mesothelioma	Kasai *et al*. (2016)
MWCNT-7 **(Mitsui)**	5.11 µm (mean)		rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu *et al*. (2012)
MWCNT-N **(Nikkiso)**	3.64 µm		rat	Transtracheal intrapulmonary spraying 9 days	Yes Hyperplastic visceral mesothelial proliferation	Xu *et al*. (2012)
MWCNT-L	8 µm	150 nm	rat	Transtracheal intrapulmonary spraying	Yes	Xu *et al*. (2014)

(synthesized in lab)				24 weeks	Patchy parietal mesothelial proliferation	
MWCNT-N **(Nikkiso)**	4.2 µm 2.6 µm	30-80 nm	rat	Transtracheal intrapulmonary spraying 2 weeks + 109 weeks p.e.	Yes Bronchiolo-alveolar lung adenomas and carcinomas Pleural malignant mesotheliomas	Suzui *et al*., 2016
NT_{long2} **(Univ. Manchester)**	> 15 µm (85 % of fibres)	20-100 nm (165 nm, measured)	mouse	Intrapleural injection	Yes Mesothelial proliferation in parietal pleura of chest wall and diaphragm 24 weeks p.e.	Murphy *et al*. (2011)
MWCNT-7 **(Hodogaya)**	Several µm	Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm.	mouse	inhalation	Yes (Promoting activity) Pulmonary adenomas and adenocarcinomas as well as systemic sarcomatous mesotheliomas 17 months p.e.	Sargent *et al*. (2014) [Porter *et al*., 2013]
NT50a **NT-50a(-agg)** **= MWCNT-7, Mitsui)**	5.29 µm	49.95 nm	rat	Intraperitoneal injection	Yes Malignant mesothelioma after 1 year	Nagai *et al*. (2011)
NT145 **(Showa Denko)**	4.34 µm	143.5 nm	rat	Intraperitoneal injection	(Yes) However mesothelioma incidence after 1 year of 1mg dose much lower and latency of tumour-induced mortality higher compared to 1 mg NT50a and equivalent fibre dose of NT50a(-agg*). Furthermore, in contrast to NT50a, fibrosis index of NT 145 was not significantly increased against controls, and did not pierce mesothelial cells.	Nagai *et al*. (2011)
NT50b **(Showa Denko)**	4.6 µm	52.4 nm	rat	Intraperitoneal injection	Yes Malignant mesothelioma after 1 year (1 mg not tested)	Nagai *et al*. (2011)

MWCNT A **(synthesized)**	8.57 µm (WHO fibres) 2.72 µm (all fibres)	85 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 98/90 % of rats (low/high dose) Mean survival time: 213/194 days First detection of morbidity: 5/5 months p.e.	Rittinghausen *et al*. (2014)
MWCNT B **(synthesized)**	9.3 µm (WHO fibres) 2.13 µm (all fibres)	62 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 92/90 % of rats Mean survival time: 294/207 days First detection of morbidity: 6/5 months p.e.	Rittinghausen *et al*. (2014)
MWCNT C **(synthesized)**	10.24 µm (WHO fibres) 4.18 µm (all fibres)	40 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 84/94 % of rats Mean survival time: 415/265 days First detection of morbidity: 10/6 months p.e.	Rittinghausen *et al*. (2014)
MWCNT D **(synthesized)**	7.91 µm (WHO fibres) 2.53 µm (all fibres)	37 nm	rat	Intraperitoneal injection	Yes Mesothelioma in 40/70 % of rats Mean survival time: 666/585 days First detection of morbidity: 20/11 months p.e.	Rittinghausen *et al*. (2014)
MWCNT-7 (Mitsui)	7.1 µm	75 nm	rat	Intraperitoneal injection	Yes First incidences of mesotheliomas after 6 months, ~ 100 % of animals positive after 12 months (crocidolite asbestos negative)	Huaux *et al*. (2016)
Short MWCNT-7	2.8 µm	75 nm	rat	Intraperitoneal injection	Yes Lower incidences and longer latency compared to unground MWCNT-7 (crocidolite asbestos negative)	Huaux *et al*. (2016)

MWCNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	mouse (transgenic)	Intraperitoneal injection	Yes Invasive mesothelioma after 25 weeks (100 % mortality)	Takagi *et al*. (2008)
MWCNT-7 **(Mitsui)**	< 20 µm (27.5 % > 5 µm	100 nm	mouse (transgenic)	Intraperitoneal injection	Yes Dose-dependent mesothelioma induction 1 year p.e., with cumulative incidence of mesotheliomas of 19/20, 17/20 and 5/20, respectively.	Takagi *et al*. (2012)
MWCNT-7 **(Mitsui)**	1-4 µm (72.5 %)	82 % in range 70-110 nm	rat	Intrascrotal injection	Yes Mesothelioma in 6/7 animals after 52 weeks	Sakamoto *et al*. (2009)

The proposal for classification for carcinogenicity is restricted to the inhalation route as according to present knowledge the inhalation route is the only relevant one. It is highly improbable that exposure by the dermal or even oral route would lead to a carcinogenic response, taking into account that long-term deposition of MWC(N)T in the tissues, as can occur in lung, is a prerequisite for carcinogenicity. According to present knowledge, there is no evidence that other carcinogenic fibres meeting the WHO definition have carcinogenic properties after oral or dermal exposure.

Comments received during consultation

According to several industry comments, the CLH proposal should be targeted only to MWCNT-7 type tubes since majority of data comes from this type of MWCNTs and they consider that extension to other type of MWC(N)Ts is not appropriate mainly due to lack of data. One industry organisation also considered Cat 2 more appropriate in line with the IARC 2B classification. Two commenting Member states agreed with the proposed cat 1B classification but were considering the possible extension of the scope to MWCNTs with a diameter <30 nm. To support this, the recent study by Saleh *et al* (2020) was cited.

Additional key elements

See the section 'RAC general comment' in the beginning of the opinion relating to the fibre paradigm.

The study by Saleh *et al*. (2020) was submitted during the consultation of the CLH report. This study was a 2-year comparative carcinogenicity study with either straight-type MWCNT (approximately 150 nm in diameter) or tangled-type MWCNT (7.4 nm in diameter) administered via Intra-Tracheal Intra-Pulmonary Spraying (TIPS) to rats (once a week over a 7 week period, followed by a 2-year observation period). Crocidolite asbestos was used as the reference material. The rats which were administered straight type MWCNT or asbestos did not have a significant increase in bronchiolo-alveolar hyperplasia or tumours in the lung. However, tangled MWCNT did have significantly elevated incidences of bronchioloalveolar

hyperplasia and tumours in the lung. Malignant pleural mesothelioma was not induced in any of the groups.

Assessment and comparison with the classification criteria

Key studies for the assessment of the carcinogenicity of MWCNTs are:

1. Kasai *et al*. (2016), which is a guideline based, full 2-year inhalation carcinogenicity study in rats. It showed a significantly increased incidence in lung tumours (mainly bronchiolo-alveolar carcinoma, and combined carcinomas and adenomas) after exposure to MWCNT-7 (Mitsui). In males, increased incidences of tumours were seen at 0.2 and 2 mg/m^3 and in females at 2 mg/m^3. A summary of the malignant tumour findings is provided in the table below. The absence of induction of pleural mesothelioma is likely to indicate the lack of sensitivity of the study design to properly detect this slowly developing tumour type by a fibre-related pathology. Low numbers of MWCNT were detected in the pleural region after 104 weeks of inhalation compared to the numbers after high bolus doses administered in positive intraperitoneal injection studies. However, a concentration-dependent increase in mesothelial hyperplasia of the parietal and ventral pleura, focal fibrosis of the parietal pleura and the diaphragm as well as inflammation of the mediastinum were observed in male rats. Females showed a statistically significantly increased incidence of focal fibrosis of the ventral pleura. The authors did not comment the peritoneal mesotheliomas which were observed in males, especially at the low-dose.

***Table**: Tumour findings in the study by Kasai et al. (2016).*

	Male				**Peto test**	**Female**				**Peto test**
Dose (mg/m^3)	**0**	**0.02**	**0.2**	**2**		**0**	**0.02**	**0.2**	**2**	
No. of animals examined	**50**	**50**	**50**	**50**		**50**	**50**	**50**	**50**	
Neoplastic lesions										
Lung										
Bronchiolo-alveolar carcinoma	1	1	8*	10**	↑ ↑	0	1	0	5**	↑ ↑
Adenosquamous carcinoma	0	0	0	1		0	0	0	1	
Poorly differentiated adenocarcinoma	0	0	0	0		0	0	0	1	
Squamous carcinoma	0	0	0	0		0	0	0	1	
Total carcinoma	1	1	8*	11**	↑ ↑	0	1	0	8**	↑ ↑
Bronchiolo-alveolar cell adenoma	1	1	7*	5		3	1	4	3	
Total adenoma and/or carcinoma	2	2	13**	16**	↑ ↑	3	2	4	11**	↑ ↑
Peritoneum										
Malignant mesothelioma	0	3	1	1		0	0	0	0	

2. Sargent *et al*. (2014) is a tumour promotion study in B6C3F1 hybrid mice. Mice were pre-treated with the tumour initiator methylcholanthrene (MCA, 10 µg/g bw by single intraperitoneal injection) and exposed one week later by inhalation to MWCNT-

7 (5 mg/m^3, 5 hours/day, 5 days/week) for 15 days. After 17 months post-exposure, they were sacrificed and examined for tumour formation. As can be seen from table 2, 90.5 % of MCA+MWCNT-7 -exposed mice developed one or the other tumour type, compared to 51.9% of MCA-only treated mice or 26.5% of MWCNT-7-only mice. Bronchio-alveolar adenocarcinomas developed in 14 % of mice without MCA pre-treatment, which was close to the incidence in the air-control group (13 %), 22% without MWCNT-7 treatment and in 62% of mice treated both with MCA and MWCNT-7. Several pre-treated mice also developed malignant serosal tumours consistent with sarcomatous mesothelioma.

***Table**: Tumour findings in the study by Sargent et al. (2014).*

	Air	**MCA**	**MWCNT**	**MCA + MWCNT**
No. of animals	56	54	49	42
No. of animals with focal adenomatous alveolar hyperplasia	7	8	14*	26*
No. of bronchiolo-alveolar adenoma	6	18*	9	32*
% of mice with one or more of bronchiolo-alveolar adenoma	11 %	33 %*	18 %	76 %*
No. of bronchiolo-alveolar adenocarcinomas	7	12*	7	26*
% of mice with one or more of bronchiolo-alveolar adenocarcinomas	13 %	22 %*	14 %	62 %*
No. of bronchiolo-alveolar adenoma and/or adenocarcinomas	13	28*	13	38*
% of mice with lung tumours	23.2 %	51.9 %*	26.5 %	90.5 %*

3. The third set of evidence comes from IP studies (and from one intra-scrotal study) showing mesothelioma induction with MWCNT-7 (Mitsui). As described in section 'RAC general comment' in this opinion, the IP test is commonly used test to evaluate fibres in relation to their ability to cause pathogenicity specifically by a fibre -related mechanism.
    a. Huaux *et al*. (2016) injected Wistar rats intraperitoneally with MWCNT-7 (NRCWE-006; median length: 7.1 µm) or a ground short fibre fraction thereof as a single dose of 6 mg (= 2 x 10^9 WHO fibres and 0.36 x 10^9 WHO fibres, respectively). Crocidolite served as a positive control. Both CNT-7 and short CNT-7 induced mesothelioma, the latter to a lesser extent. The majority of animals developed tumours after a latency of 12 months, the first tumours occurring after 6 months (no exact figures provided). Only one animal developed mesothelioma 12 months after crocidolite injection.
    b. Takagi *et al*. (2008) used a p53 heterozygous asbestos-sensitive mouse model to explore the mesotheliomagenic potential of MWCNT-7. IP injection of MWCNT-7 (3 mg/mouse) resulted in a rapid induction of mesotheliomas, which were invasive to the abdominal wall, diaphragm, liver parenchyma and pancreas, and in some case involving the thoracic cavity. Distant metastasis was not observed (day 172 after injection). The overall mesothelioma incidence at day 84 post-treatment was even higher for MWCNT (87.5 %) compared to Crocidolite (77.8 %).

c. Takagi *et al*. (2012) used the same p53 heterozygous asbestos-sensitive mouse model as in their previous study. IP injection of MWCNT-7 at doses 3, 30 and 300 µg/mouse resulted in a dose dependent increase in mesotheliomas (0/20, 5/20, 17/20, 19/20 mesotheliomas for controls and three dose groups, respectively). Most mesothelioma were lethal. The 15 surviving mice at low dose treatment showed focal mesothelial atypical hyperplasia. No mesothelioma was observed in the vehicle control group.
d. Nagai *et al*. (2011) treated rats intraperitoneally with three different types of MWCNT ("NT50a": D:50 nm, L: 5.3 µm, long-crystalline fibres = MWCNT-7, "NT145": D: 145 nm, L: ~4.3 µm, thick tubes or NTtngl: D: 15 nm, L: 3 µm tangled tubes). In addition, a sub-fraction of non-aggregated NT50a fibres was tested at a number concentration equivalent to 1 mg NT145 ("NT50a-agg*). Injections with NT50a(-agg*) or 1 mg of NT50a (= MWCNT-7) induced malignant mesothelioma with a higher frequency and earlier progression than injections with 1 mg of NT145. No mesotheliomas were observed with 10 mg of NTtngl.

4. Sakamoto *et al*. (2009) administered 1 mg/kg bw MWCNT-7 to Fischer rats by intrascrotal injection. MWCNT treatment induced mesotheliomas in 6 of 7 treated rats that died prior to the end of the 52 week observation period. Apart from these mesothelial proliferative lesions, granulomas with high cellularity, including macrophages and multinucleated giant cells were observed. Rats treated similarly with 2 mg/kg bw crocidolite also developed granulomas but no mesotheliomas. The authors explained the absence of mesotheliomas with the low particle number concentration of crocidolite in their study. Further evidence on the fibre-like pathogenicity of MWCNTs comes from short term studies providing information on the pleural penetration and inflammatory effects. Most relevant are the studies by Xu *et al*. (2012 and 2014). Increased cellularity in pleural fluid and mesothelial proliferation were demonstrated in rats after transtracheal intrapulmonary spraying with MWCNT-7 (Mitsui), MWCNT (Nikkiso), lab synthesized 150 nm (in diameter) NTs but not with tangled 15 nm NTs (Xu *et al*., 2012 and 2014). This means that CNTs, and the positive control, crocidolite, were able to enter the pleural fluid at high enough levels to cause inflammation, resulting in mesothelial proliferation. The mesothelial proliferation can be seen as an early precursor event to mesotheliomas.

It is noted that the study by Saleh (2020, submitted during the consultation) showed only non-significant increases in combined lung adenoma and carcinoma after once-a-week intra-tracheal intra-pulmonary spraying (TIPS) of a total dose of 0.5-1.0 mg of MWCNT-7. Also, the positive control crocidolite (total dose 1.0 mg) remained negative in this study. However, the study included only 20 animals per dose group.

Although the majority of studies have been performed with MWCNT-7 (Mitsui) type nanotubes, the mesotheliomagenic potential is not restricted to MWCNT-7. There are several studies showing that also other, rigid MWCNTs fulfilling WHO fibre criteria are also able to induce mesothelioma. These include the following studies:

1) Rittinghausen *et al*. (2014) tested several different synthesized MWCNTs with a diameter between 37 nm-85 nm and a length between 7.91-10.24 µm. They all resulted in high incidences of peritoneal mesotheliomas after IP injection in rats. This study also provides the lowest diameter (37 nm) shown so far to cause mesothelioma in an IP test.

2) Nagai *et al*. (2011, see more detailed description above) evaluated four different types of MWCNTs including MWCNT-7 (Mitsui), Shova Denko NT-145 (145 nm), NT-50 and tangled (D: 15 nm; L: 3 µm) nanotubes. All caused mesothelioma after IP administration except the tangled CNTs. The potency of 145 nm nanotubes was, however, lower compared to MWCNT-7 and NT-50.
3) Suzui *et al*. (2016) performed an intrapulmonary spraying study in rats using three different sieve fractions of MWCNT (Nikkiso), which has dimensions similar to MWCNT-7 but with 10-times lower iron content. The diameter range of these nanofibers was 30-80 nm, an "unfiltered" fraction had a mean tube length of 4.2 µm and a "flow-through" fraction had a mean tube length of 2.6 µm. The "retained" fraction contained agglomerates, which precluded length determination. Exposure to a total dose of 1 mg/animal of all these three fractions resulted in the induction of lung tumours and pleural malignant mesothelioma, with a combined incidence of 52.6% (control incidence 0%).
4) Murphy *et al*. (2011) showed induction of asbestos-like inflammation, pleural proliferation and fibrosis after intrapleural administration of MWCNT-7 (Mitsui), long straight MWCNTs (mean diameter 165 nm, Univ of Manchester) in mice. No similar effect was observed with short (length 0.5–2 µm) or two tangled (D: 15 nm, L:1-5 µm or 5-20 µm) MWCNTs.
5) Xu *et al*. (2012) and (2014) studies (see above) showed increased cellularity in pleural fluid and mesothelial proliferation in rats after transtracheal intrapulmonary spraying with MWCNT-7 (Mitsui), MWCNT (Nikkiso), lab synthesized 150 nm (in diameter) NTs but not with tangled 15 nm NTs.

All these studies together with evidence indicating high biopersistence and associated non-neoplastic findings provide strong evidence for a fibre-like pathogenicity similar to asbestos. The DS has also summarised several *in vitro* studies trying to further elucidate the mechanisms of the induction of cancers by the MWCNTs. These are, however, of less importance for this classification proposal.

As discussed in the 'RAC general comments'-section, the fibre paradigm is not affected by the chemical composition of the fibre unless this affects biopersistence. Therefore, RAC agrees with the DS that the chemical composition and other material specific factors are in general secondary to fibre dimensions and of lesser importance.

It is important to note that the available data on mesotheliomagenic potential comes from the fibres of which the mean diameter is 37 nm or above. Fibres with a diameter of 15 nm or below has not caused mesothelioma when tested using IP exposure (Muller *et al*., 2009, Nagai *et al*., 2011, 2013), intrapleural injection (Murphy *et al*. 2011) or transtracheal intrapulmonary spraying (Xu *et al*., 2014) for mesothelioma induction. There is a lack of data on fibres between 15-30 nm. Although thin, tangled fibres do not cause mesothelioma and other effects via the mechanism related to fibre paradigm, they may cause cancer by other mechanisms. Evidence for this is provided by the recent study (Saleh *et al*., 2020), submitted during consultation of the CLH report. This study was a 2-year comparative carcinogenicity study with either straight-type MWCNT (approximately 150 nm in diameter) or tangled-type MWCNT (7.4 nm in diameter) administered via intra-Tracheal Intra-Pulmonary Spraying (TIPS) to rats (once a week over a 7 week period, followed by a 2-year observation period). Crocidolite asbestos was used as the reference material. The rats administered straight type MWCNT or asbestos did not have a significant increase in bronchiolo-alveolar hyperplasia or tumours in the lung. However, tangled MWCNT did have significantly elevated incidences of bronchioloalveolar hyperplasia and tumours in the lung (the incidence of adenoma and

adenocarcinoma combined being 1/19, 5/20, and 7/20 in the control, low dose and high dose groups, respectively). Malignant pleural mesothelioma was not induced in any of the groups. Overall, the results of this initial study suggests that also tangled-type MWCNT are carcinogenic to the rat lung when administered via the airway but probably via a different mechanism.

Comparison with the criteria

Since classification in Carc. 1A category requires human evidence, this category is not applicable in this case.

Carc. 1B applies, if the substance is presumed to have carcinogenic potential for humans and classification is largely based on animal evidence. A basic requirement is an increased incidence in malignant neoplasms in at least two species of animals, or at least two independent studies in one species. In this case, the key study is the study by Kasai *et al* 2016, showing a clear induction of lung tumours in rats after long term inhalation exposure both in males and females. Further evidence is provided by a number of supporting studies; the tumour promotion study by Sargent *et al*. (2014) in mice and studies demonstrating the mesotheliomagenic potential of MWCNTs in rats or mice after IP administration (Huaux *et al*. 2016, Takagi *et al*., 2008, 2012, Nagai *et al* 2011, Rittinghausen *et al*., 2016), after intra-scrotal administration (Sakamoto *et al*. (2009) or after transtracheal intrapulmonary spraying (Suzui *et al*., 2016). Also studies by Xu *et al*. (2012, 2014) and Murphy *et al*., (2011) showing pleural inflammation and mesothelial proliferation similar to asbestos support the fibre like carcinogenic potential of MWC(N)Ts.

RAC agrees with the DS that these data provide sufficient evidence for the **classification of MWCNTs specified in the proposal in category 1B for carcinogenicity.**

Although the key findings come from MWCNT-7 (Mitsui) type tubes, RAC agrees with the DS that there is sufficient evidence that the carcinogenicity is not limited only to MWCNT-7 (Mitsui) but also other, rigid MWC(N)Ts fulfilling WHO fibre dimensions can cause cancer by the same fibre pathogenicity paradigm related mechanism. This has been demonstrated by the studies by Rittinghausen *et al*. (2016), Nagai *et al*. (2011), Murphy *et al*. (2011) and Suzui *et al*. (2016), and are supported by the toxicokinetic and mechanistic evidence (e.g. studies by Xu *et al*., 2012 and 2014).

RAC agrees with the DS that the classification for carcinogenicity should be restricted to the inhalation route, resulting in hazard statement **H350i** (May cause cancer by inhalation). Considering the MoA related to the fibre pathogenicity paradigm, which requires high biopersistence resulting in a high long-term deposition of MWC(N)T in the lung, it is highly unlikely that exposure by the dermal or even oral route would lead to a carcinogenic response. This is also in accordance with our knowledge on the carcinogenicity of other carcinogenic fibres meeting the WHO fibre definition and in line with classification of other fibres encompassing WHO fibre dimensions.

RAC notes that the doses at which induction of lung tumours was observed in the study by Kasai *et al* (2016) are very low and in some IP studies, MWCNTs showed even higher mesotheliomagenic potency than crocidolite. This suggests a high carcinogenic potency of MWCNTs, which could justify the setting of an SCL (0.01%) instead of applying the generic concentration limit of 0.1%. However, RAC notes that the current guidance for the potency evaluation for the setting of SCL is designed primarily for systemic, non-threshold genotoxic carcinogens and may not be fully applicable to local carcinogens – and especially for fibres - with multiple modes of action playing a role in the carcinogenicity. The guidance for the setting

SCLs for substances which are carcinogenic via inhalation is currently under development but may not be applicable to fibres in any case. Additionally, in the case of MWC(N)Ts, the verification that the concentration limit of fibres >30 nm in diameter is not exceeded is likely to require electron microscope (SEM/TEM) evaluation and it is not known how achievable percentages below 0.01% are in practice.

Finally, RAC notes that there is a gap in knowledge concerning fibres with a diameter between 15-30 nm. Thus, there is an apparent need to study the ability of fibres to cause cancer. On the other hand, RAC further notes that even though MWCNTs <30 nm in diameter have not been included in the current CLH proposal, MWCNTs with a mean diameter <30 nm are still subject for classification if they contain ≥0.1% (GCL) of individual MWCNTs with the dimensions specified in this proposal. Although fibres of 15 nm or below have not caused mesothelioma, they may cause lung cancer (via other mechanisms) as suggested by the study of Saleh *et al* (2020). Therefore, RAC identifies a need to evaluate the available data on MWCNTs with a diameter of <30 nm for their possible classification according to CLP.

10.10 Reproductive toxicity

Hazard class not assessed in this dossier.

10.11 Specific target organ toxicity-single exposure

Hazard class not assessed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Table 14: Summary table of animal studies on STOT RE (see also Annex I, 3.12.1.1- 3.12.1.5)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance,	Route of exposure, dose levels, duration of exposure	Results	Reference
Inhalation Subchronic study (90d) OECD TG 413 Rat F344 DuCrlCrlj, m/f n=10/group	MWNT-7 (Hodogaya, Lot No. 071223, 080126) D: 90.7 nm (mean) L: 5.7 µm (mean; 48.7 % > 5 µm) Purity: > 99.5 % MMAD = 1.4-1.6 µm (~80 % mass as inhalable fraction)	Whole-body exposure to 0, 0.2, 1 or 5 mg/m^3 MWCNT aerosol for 6 h/day, 5 days/week for 13 weeks MWCNT mass doses corresponded to number concentrations of about 115,000, 577,0000, and 2,933,000 cpm (particles > 0.3 µm in length), respectively	Lung weights were significantly increased at 1 and 5 mg/m^3. Concentration-dependent increase in inflammatory BAL parameters (PMN and lymphocyte numbers, LDH, ALP and TP) in both sexes from 0.2 mg/m^3 and above. Inflammation-related histopathologic lesions included foreign body granulomas with increased numbers of associated multinucleated MWCNT-laden macrophages in the lung at 1 and 5 mg/m^3 in females and males and even at 0.2 mg/m^3 in males. Increased numbers of neutrophils and lymphocytes in the lungs, and hyperplasia of goblet cells in the nasal cavity and nasopharynx were observed.	Kasai et al. (2015)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance,	Route of exposure, dose levels, duration of exposure	Results	Reference
	Dry-aerosol generation		Multifocal fibrosis of the alveolar wall was observed at 1 mg/m³ and above in both sexes. The incidence and severity of fibrosis and granuloma formation increased at higher exposure concentrations. Inflammatory infiltration in the visceral pleural and subpleural areas was induced at 5 mg/m^3. MWCNT were primarily found within alveolar macrophages (longer fibres only partially phagocytosed in AM with foamy cytoplasm), but few single MWCNT were also found in bronchiolar and alveolar spaces. Occasionally, single MWCNTs were detected in the visceral subpleural areas and in the parietal pleura at the diaphragm. The lung burden of males at the highest exposure concentration was estimated as 120 µg, that one of females was about 80 µg left lung (accounting for the lower respiratory frequency of female rats). Accordingly, 13.7 µg per left lung resulted in granulomatous changes in 40 % of the animals after an additional 4 week recovery period, granulomatous changes were observed in 4/5 rats). ~2.3 µg/left lung did not cause granulomatous changes. The authors derived a LOAEC of 0.2 mg/m3 based on granulomatous changes and BALF parameters.	
Inhalation Subacute study (14d) No standard test guideline followed Rat F344 DuCrlCrlj rats, m/f n=10/group	MWNT-7 (Hodogaya, Lot No. 080126) D: 88 nm (mean) L: 5.0 µm (mean, 38.9 % > 5 µm) Purity: 99.8 % MMAD = 1.2-1.4 µm (~80 % mass as inhalable fraction) Dry-aerosol generation	Whole-body exposure to 0, 0.2, 1 or 5 mg/m^3 MWCNT aerosol for 6 h/day, 5 days/week 5 animals of each group sacrificed immediately after exposure, remaining rats necropsied at the end of a 4-week post-exposure period.	Persistent deposition of MWCNTs in the lungs of all MWCNT-exposed groups was observed. MWCNT deposited in bronchus-associated lymphoid tissue (BALT) and peritracheal lymph nodes were found after exposure and at 1 and 5 mg/m3 after p.e. Numbers of neutrophils and lymphocytes in BALF tended to remain elevated in the 5 mg/m^3 dose group, the numbers of multinucleated macrophages even increased at 5 mg/m^3 after post-exposure. Albumin and TP were still elevated.in the 1 and 5 mg/m^3 groups, ALP in the 5 mg/m^3. In histopathology, granulomatous changes and slight alveolar fibrosis occurred in the lung at the highest dose. Granulomatous changes slightly increased at the end of the p.e. At 1 and 5 mg/m^3, goblet cell hyperplasia in the nasal cavity and nasopharynx were observed, which largely regressed at the end of p.e. The total amounts of MWCNTs in the 5 mg/m^3 group was approximately 43.4 µg/lung at the end of the 2-week exposure period and approximately 41.2 µg/lung at the end of the 4-week p.e. period. A NOAEC of 0.2 mg/m^3 was derived by the study authors based on BAL parameters and histopathologic findings:	Umeda et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance,	Route of exposure, dose levels, duration of exposure	Results	Reference
Inhalation Short-term study (4d) No standard test guideline followed (lung only organ investigated) Mouse C57BL/6, BALB/c, f Kit^{Wsh}/HNihrJaeBsmJ (mast-cell deficient), f n=10-20 (7-9)/group	1. "rCNT": XNRI MWNT-7 (Mitsui) D: > 50 nm L: ~13 µm Purity: 99.79 % (rigid, rod-like structure) 2. "tCNT" MWCNTs 8-15 nm (CheapTubes) D: 8-15 nm L: 10-50 nm Purity: 99.76 % (flexible tangled structure)	Whole body exposure for 4 days (4h/d) at 6.2-8.2 mg/m^3 for rCNT and 17.5-18.5 mg/m^3 for tCNT Mice were sacrificed immediately or 24 h after last exposure	Both tests induced the recruitment of inflammatory cells, especially eosinophils. In the lung, rCNT fibres were detected in alveolar macrophages in interstitial areas. Macrophages were found to undergo "frustrated phagocytosis" and form foreign-body giant cells. Evidence was presented that inhaled fibre-like MWCNT (rCNT) can induce immunity-mediated allergic-like airway inflammation in healthy mice, including effects such as marked eosinophilia accompanied by mucus hypersecretion, airway hyper responsiveness, and the expression of Th2-type cytokines and eosinophil chemoattractants.	Rydman et al. (2014)
Inhalation Short-term study (12d) No standard test guideline followed (examination limited to the respiratory tract; histopathology included nose, lung and tracheobronchial lymph nodes) Mouse C57BL/6J, m n=7-9/group	MWNT-7 (Hodogaya, Lot No. 061220-31) Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm. 1.32 % metal contamination (1.06 % iron)	Whole-body exposure (0 or 10 mg/m^3) for 2, 4, 8, or 12 days	Dose-dependent increase in whole lung lavage (WLL) markers for lung inflammation (PMN), lung cytotoxicity (LDH) and alveolar air-blood barrier integrity (albumin) over controls. Lung histopathology revealed bronchiolocentric inflammation, bronchiolar epithelial hyperplasia and hypertrophy, minimum to mild bronchiolocentric fibrosis, vascular changes (medial hypertrophy and contraction, mural neutrophil infiltrates and rare mural MWCNT) and rare pleural penetration. Translocation of MWCNT to the lymph nodes was observed, accompanied by lymph node enlargement with paracortical hyperplasia (the authors speculated that these lymph nodes are a major site of lung clearance and that activated macrophages containing MWCNT that reach the pleura travel through these lymphatics). A linear increase in lung burden ranging from 6.6 and 30.6 µg after 2 and 12 days, respectively, which the authors deemed occupationally relevant equivalent doses.	Porter et al. (2013)
Inhalation Short-term study (12d) No standard test guideline followed Mouse C57BL/6J, m	MWNT-7 (Hodogaya, Lot No. 061220-31) L: 4.3 µm (mean) Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm.	Whole-body exposure (0 or 5 mg/m^3) for 12 days Post- observation time points: 1, 14, 84, 168, and 336 days	Progressive alveolar fibrosis was observed and persisted over the whole postexposure period (increase of connective tissue thickness in the alveolar region by 70 % 336 days after exposure). This was in line with measurements of inflammatory BAL parameters (PMN, LDH, and albumin), which increased rapidly (day 1) and declined slowly over postexposure time (still significantly increased on day 168).	Mercer et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance,	Route of exposure, dose levels, duration of exposure	Results	Reference
n=7-9/group	1.32 % metal contamination (1.06 % iron) Stable, acoustical-based generated aerosol		Granulomatous lesions as foreign body response were not observed (in contrast to bolus aspiration experiments), possibly due to the absence of larger agglomerates. Smaller MWCNT structures were rapidly incorporated into the alveolar interstitium. The initial p.e. lung burden was 21.8 µg, 84 % of which deposited in the alveolar region, mainly in alveolar macrophages (56 %). Clearance reduced the alveolar macrophage burden of MWCNT by 35 percent between 1 and 168 days p.e., while the content of MWCNTs in the alveolar tissue increased by 63 percent.	

Human data

No human data available.

Table 15: Summary table of other studies relevant for STOT RE

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Intratracheal instillation Experimental study No standard test guideline followed Mouse C57BL/6, f n=6/dose group	1. NM-401 ("CNT_{Large}") (physicochemically similar to MWNT-7 but more flexible) (CP0006-SG; IO-LE-TECNanomaterials,) D: 67 ± 26.2 nm L: 4.05 ± 2.40 µm 2. NRCWE-026 ("CNT_{Small}") (NC-7000, Nanocyl) D: 11 ± 4.5 nm L: 0.85 ± 0.457 µm	Single administration (18, 54, 162 µg/animal). Lung tissues were harvested 24 h, 3 days and 36 28 days post-exposure	Both MWCNT elicited strong acute phase and inflammatory responses that persisted up to 28 days: increased cellular influx in bronchioalveolar lavage fluid, interstitial pneumonia and gene expression changes in lung tissue. In contrast to "CNT_{Small}", "CNT_{Large}" elicited an earlier onset of inflammation and induced more fibrosis and a unique fibrotic gene expression signature at day 28, compared to the tangled Nanocyl NC7000.	Poulsen et al. (2015)
Pharyngeal aspiration Experimental study (study report) No standard test guideline followed Mouse	1. Long needle-like MWCNT (Mitsui-7) D: > 50 nm L: ~13 µm Purity: 99.79 % 2. Long tangled MWCNT 8-15 nm (CheapTubes)	10 µg/mouse or 10 and 40 µg/mouse Mice were sacrificed 4, 16 or 28 days after exposure	Long, needle-like MWCNT caused an inflammatory response with PMN influx, certain cytokines and chemokines as well as granuloma formation, which was even stronger than for asbestos. Tangled MWCNT induced only slight pulmonary neutrophilia.	Rydman et al. (2013)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
C57BI/6J, f n = 6-8/group	D: 8-15 nm L: 10-50 nm	Positive control crocidolite asbestos (concentration unknown)		
Pharyngeal aspiration Experimental study No standard test guideline followed Mouse C57BL/6 mice, f n=3/group	MWCNT of various geometry: - Tangled forms: 1. D: ~15 nm, L: 1-5 µm [NanoLab] 2. D: ~10 nm, L: 5-20 µm [NanoLab] - Short straight form: 3. D: 20-30 nm, L: 0.5-2 µm [Nanostructured & Amorphous Materials, Inc.] - Long straight forms: 4. D: 84 nm, L: 13 µm (12 % > 20 µm; 24 % > 15 µm) [Mitsui, MWNT-7?] 5. D: 165 nm, L: 56 µm (76 % > 20 µm; 84 % > 15 µm) [Univ. Manchester]	Bolus administration of dose of 25 µg MWCNT/animal Examination 1 and 6 weeks after exposure.	Long straight MWCNT (nos. 4 and 5) but not tangles or short MWCNT caused acute (transient) neutrophilic inflammation in BAL at 1 week and progressive thickening of the alveolar septa. After 6 weeks, the long straight MWCNT also induced an inflammatory response in the pleural cavity and lesions along the chest wall and diaphragm.	Murphy et al. (2013)
Pharyngeal aspiration Experimental study No standard test guideline followed Mouse C57BL/6J mice, m n=4/group	MWNT-7 (Mitsui, Lot No. 05072001K28) D: 49 nm	Single dose administration of 10, 20, 40 or 80µg Examination 1, 7, 28, or 56 days p. e.	Dose-dependent increase of BAL markers for lung inflammation (PMN) and lung damage (LDH, albumin), peaking at 7 d p. e. but declining to control levels thereafter, except at highest dose, which showed elevated BAL values above vehicle controls also at 56 d p. e. Histopathology revealed rapid pulmonary fibrosis (7d p. e.) and persisting (56 d p. e.) granulomatous inflammation.	Porter et al. (2010)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			Fibrosis, granuloma, proliferative bronchointerstitial pneumonia and pleuropneumonia, hypertrophy and hyperplasia of bronchiolar epithelium, mucous metaplasia of the bronchiolar epithelium became manifest at equivalent occupationally relevant doses. Evidence was also provided for MWCNT reaching and penetrating the pleura, for incomplete phagocytosis by alveolar macrophages and possibly disturbed lymphatic clearance from the lung caused by lymphangiectasia.	

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Human data on STOT-RE is not available.

A number of animal studies tested the toxicity of inhaled MWCNT in rats and mice. A highly reliable 90 d inhalation study according to OECD TG 413 is available, which tested dry-generated aerosol of MWNT-7 in rats (Kasai et al., 2015). This study, which derived a LOAEC as low as 0.0002 mg/L based on inflammatory BAL parameters and granulomatous changes is rated as key as it fulfils the criteria for classification in STOT RE Cat.1, CLP (with the lung as primary target organ). Even more severe lung damage of multifocal fibrosis was first observed at a dose of 0.001 mg/L in both sexes. This would still result in classification into category 1. This classification is confirmed by extrapolation of 14 d inhalation study using the same test material, which reported persisting inflammation and aggravating granulomatous changes 4 weeks after exposure as well slight alveolar fibrosis (Umeda et al., 2013). See Table 14 for further study details.

Three other inhalation studies (Table 14) of shorter duration (4-12 d) which tested MWNT-7 would also justify STOT RE classification when applying extrapolation according to Haber's rule, though not into Cat.1 (Table 16). However, extrapolation has to be taken with caution. All of these studies (Porter et al., 2013, Mercer et al., 2013, Rydman et al., 2014) tested only a single dose. Furthermore, extrapolation from a 4 d exposure study (Rydman et al., 2014) yields highly uncertain effect values. Accordingly, the studies should be regarded as supportive, demonstrating that inflammation and fibrosis in the lung becomes manifest already at shorter exposure durations than 90 days.

In addition, Mercer et al. (2013) showed that MWNT-7 retained in the lung produced a progressive and persistent fibrotic response up to 336 d post-exposure and Porter et al. (2013) provided evidence for extrapulmonary translocation and tissue interaction of inhaled MWNT-7, as indicated by LALN hyperplasia and pleural penetration. The focus of the 4d inhalation study by Rydman et al. (2014) was to demonstrate that the early inflammatory process to MWNT-7 involves an allergic response in the lung, indicated by marked eosinophilia and airway hyper responsiveness. Thus, this is the first and only study providing evidence for a respiratory sensitisation activity elicited by MWCNT, which, however, is beyond the scope of this dossier and deemed insufficient for corresponding classification.

An earlier study applying pharyngeal aspiration with an up to 28 d post-observation period (Rydman et al., 2013) supports a more strict classification, as it revealed that MWNT-7 induced a granulomatous response similar to but even more marked than the one resulting from asbestos exposure.

Other studies are available supporting classification (summarised in table 15). All of these studies applied non-physiological pulmonary exposure conditions but essentially confirm that MWNT-7 induces persisting (or progressive) inflammation, granulomatosis and/or fibrosis in the lung similar to asbestos (Poulsen et al., 2015; Rydman et al. 2013, Murphy et al. 2013; Porter et al., 2010). Interestingly, several of these studies compared different types of high diameter (> 30 nm) MWCNTof WHO fibre length. Collectively, they show that longer, rigid MWCNT induce a more severe lung response than shorter or tangled MWCNT types under the conditions used.

The aspiration study by Murphy et al. (2013) is particularly relevant as it provides evidence of an asbestos-like pathology (including alveolar as well as pleural fibrosis) not only for MWNT-7 but also for another long and straight MWCNT with similar fibre-like characteristics, as well as its absence in the case of tangled or short MWCNT types. This observation, together with findings from carcinogenicity studies, which clearly indicate that progressive inflammatory fibrotic lesions in the lung and in the pleura are a prerequisite for tumour formation (see section 10.9 on carcinogenicity), supports the assumption of a generic asbestos-related pathomechanism for inhaled MWCNT types of WHO fibre length and rigidity-promoting diameter. Altogether, the available information from repeated dose inhalation or other lung toxicity studies, carcinogenicity studies and toxicokinetic studies on the distribution and transport of lung-deposited rigid WHO fibre-like MWCNT (see section 9 on toxicokinetics), justifies the same classification of MWNT-7 and other MWCNT with similar dimensions by the weight of evidence.

It should be noted that in Tables 14 and 15 studies which tested tangled MWCNT only were not included. There is a number of subchronic test guideline studies available for commercial tangled MWCNT types such as "Nanocyl", "Baytubes" or "Graphistrength" (Ma-Hock et al., 2009, Pauluhn et al., 2010, Pothmann et al.,

2015). Non-consideration in the context of this CLH dossier, which focuses on rigid MWCNT, does not preclude a proposal for classification of tangled or other MWCNT types at a later time point.

Table 16: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser duration than 90 days

Study reference	Effective dose (mg/L)	Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
Kasai et al. (2015)	LOAEC: 0.0002 mg/L	90 days	No extrapolation	STOT RE 1
Umeda et al. (2013)	LOAEC: 0.001 mg/L	14 days	0.0064 mg/L	STOT RE 1
Porter et al. (2013)	LOAEC: 0.01 mg/L (only dose tested)	12 days	0.075 mg/L	STOT RE 2
Mercer et al. (2013)	LOAEC: 0.005 mg/L (only dose tested)	12 days	0.0375 mg/L	STOT RE 2
Rydman et al. (2014)	LOAEC: 0.0072 mg/L (average value of only dose tested)	4 days	0.162 mg/L	STOT RE 2

10.12.2 Comparison with the CLP criteria

The available rat and mouse studies (one subchronic guideline study, several short-term inhalation studies and non-physiological lung exposure studies) provide sufficient evidence for STOT-RE classification of rigid WHO fibre-like MWCNT, which induce significant and severe toxic effects in the rat lung at concentrations below the guidance value for classification in cat. 1 (0.02 mg/L/6h/day) after 90 days of exposure, relevant to human health. Most notably, multifocal fibrosis in the alveolar region (occasionally also in the pleura) and progressive granuloma formation is frequently observed. Persisting inflammation of the lung (according to BAL cell and biochemical parameters) of up to one year cannot be seen as mere adaptive foreign-body responses but are severe adverse effects in this vital organ (altogether ultimately leading to tumour development). Taken together the effects are considered severe with profound impact on health. Even in case of recovery after transient exposure, fibrotic lesions would most likely lead to scarring of lung tissue and subsequent to impaired lung function, thus affecting health significantly.

The rationale for applying Category 1 related to STOT RE according to CLP regulation is based on human or animal data (Guidance on the Application of the CLP Criteria):

Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.

Category 2 applies, if:

Substances that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

Guidance value for classification by dust inhalation (rat) according to Regulation (EC) No. 1272/2008:

STOT RE 1: $C \leq 0.02$ *mg/L/6h/day*

STOT RE 2: $0.02 < C \leq 0.2$ *mg/L/6h/day*

10.12.3 Conclusion on classification and labelling for STOT RE

With regard to the classification of MWCT/MWCNT, there is no human data available.

A key animal study (Kasai et al., 2015) derived a LOAEC for MWNT-7 of 0.0002 mg/L. Therefore MWNT-7 meets the criteria for classification as STOT RE 1 (guidance value: ≤ 0.02 mg/L/6h/d).

MWCT/MWCNT with dimensions in a WHO rigid fibre-like morphology prone to an asbestos-related pathology, are also proposed to be classified as STOT RE 1 substances based on supporting lung toxicity and exposure data using a weight of evidence approach. Similarity to MWNT-7 is given by a tube length of ≥ 5 µm (corresponding to WHO fibre length) and a tube diameter > 30 nm (as a proxy for rigidity).

It is therefore proposed that Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T be classified as STOT RE 1, with the lung specified as the target organ.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The STOT RE 1 classification proposal is based on the available data for the cut off levels as chosen by the DS and a weight of evidence approach for rigid WHO fibre-like MWC(N)T.

A key study was a 90d-inhalation study which gives a LOAEC of 0.0002 mg/L for MWCNT-7. Concentration-dependent increases in inflammatory broncho-alveolar lavage (BAL) parameters (polymorphonuclear (PMN) cells and lymphocyte numbers, lactate dehydrogenase (LDH), alkaline phosphatase and total protein) were seen in both sexes starting from this dose level (the lowest dose level tested). Chronic retention of inhaled fibre-like MWCNT was associated with granulomatous lesions in the lungs starting from the lowest dose in males. Multifocal fibrosis of the alveolar wall was observed at 0.001 mg/L and above in both sexes.

This study was supported by shorter duration inhalation studies (Umeda *et al*., 2013; Porter *et al*., 2013; Mercer *et al*., 2013; Rydman *et al*., 2014) and studies applying intratracheal administration or pharyngeal aspiration. These have been listed as tables 14 (inhalation studies) and 15 (other studies) in the CLH report. The majority of the studies were performed using MWCNT-7 (Mitsui) with a diameter typically between 30-90 nm and length > 5 µm. This was considered to represent a prototype for fibre-like MWCNT of high diameter. Studies by Poulsen *et al*. (2015); Rydman *et al*. (2013), Murphy *et al*. (2013); Porter *et al*. (2010) compared different types of MWCNTs, including MWCNT-7 and tangled MWCNTs with a diameter ≤15 nm. The study by Murphy *et al*. (2013) was considered particularly relevant as it provides evidence of an asbestos-like pathology (including alveolar as well as pleural fibrosis) for a long and straight MWCNT, other than MWCNT-7 with similar fibre-like characteristics, as well as its absence in the case of tangled or short MWCNT types. This observation was considered to justify the same classification for MWCNT-7 and other MWCNT with similar dimensions based on a weight of evidence assessment.

Comments received during consultation

There were no comments related specifically to the STOT RE classification proposal. However, general comments regarding the fibre dimensions defined in the CLH proposal are relevant also for STOT RE classification. Industry criticised the extension of the scope to all MWC(N)Ts with geometric tube diameter range $\geq$ 30 nm to < 3 µm and a length $\geq$ 5 µm and aspect ratio $\geq$ 3:1 and proposed to follow the IARC approach and classify only MWCNT-7 type tubes, since the majority of the data comes from this type of MWCNTs. It was also pointed out that not only do fibre dimensions define the morphology but also the production method and high diameter fibres may also be tangled. Two commenting Member States pointed out that a diameter of 30 nm should not be seen as a limit for hazardous vs non-hazardous materials. One MSCA emphasised the lack of data on fibres with a diameter of 15-30 nm and suggested to extend the lower limit to 15 nm. Another MSCA referred to the recent study by Saleh *et al*. (2020), which showed lung inflammation and carcinogenicity after Intra-Tracheal Intra-Pulmonary Spraying (TIPS) of MWCNTs with a diameter of 7.4 nm and asked to consider also these effects in CLH proposal.

Assessment and comparison with the classification criteria

The key study is a 90 day inhalation study (6 h/day, 5 days per week) in rats using MWCNT-7 (Mitsui) type tubes with dose levels of 0.0002, 0.001 and 0.005 mg/L (Kasai *et al*., 2015). The effects observed included increases in inflammatory BAL parameters and granulomatous changes starting from the lowest dose of 0.0002 mg/L. Multifocal fibrosis was observed at a dose of 0.001 mg/L in both sexes.

The same group also performed a 14 d inhalation study (with an additional 4 week recovery period) using the same test material and the same dose levels (Umeda *et al*., 2013). Granulomatous changes and slight alveolar fibrosis occurred in the lung at the highest dose.

The chronic (carcinogenicity) study by the same research group (Kasai *et al*., 2016) showed a similar dose-dependent increase in non-cancer lung effects, including epithelial hyperplasia, granulomatous change, localized fibrosis, and alterations in BAL parameters starting from the lowest dose-level of 0.02 mg/m3 (i.e. 0.00002 mg/L) in rats (see the carcinogenicity section).

These three highly reliable studies were supported by three additional 4-12 day inhalation studies in mice.

Porter *et al* (2013) exposed mice to 0 or 0.010 mg/L of MWCNT-7 for 2, 4, 8, or 12 days and observed a dose-dependent increase in PMN leucocyte, LDH and albumin levels in whole lung lavage markers when compared to the controls. In lung histopathology bronchiolocentric inflammation, bronchiolar epithelial hyperplasia and hypertrophy, minimum to mild bronchiolocentric fibrosis, vascular changes and rare pleural penetration were seen. The effects were related to increasing cumulative dose when different exposure times were compared.

Mercer *et al* (2013) exposed mice to 0 or 0.005 mg/L of MWCNT-7 for 12 days with post-exposure observations at 1, 14, 84, 168, and 336 days. Inflammatory BAL parameters (PMN, LDH, and albumin), were increased at day 1 post-exposure and declined slowly over the post-exposure period, being still significantly increased on day 168. Progressive

alveolar fibrosis was also observed with an increase in connective tissue thickness in the alveolar region by 70 % 336 days after exposure.

Rydman *et al* (2014) exposed mice for 4 days (4h/d) to 0.0062-0.0082 mg/m^3 of rigid rod-like CNTs (rCNTs – MWCNT-7) and 0.0175-0.0185 mg/m^3 of flexible, tangled (tCNTs - MWCNTs 8-15 nm thick). rCNTs, but not tCNTs induced the recruitment of inflammatory cells, especially eosinophils accompanied by mucus hypersecretion, hyperresponsiveness and the expression of Th2-type cytokines, and up-regulation of genes involved in innate immunity and cytokine/chemokine pathways. Macrophages were found to undergo "frustrated phagocytosis" and form foreign-body giant cells. This was interpreted by the DS to suggest that MWCNTs may cause respiratory sensitization, which is, however, not the correct interpretation. This eosinophilic response, with eosinophilic crystals (similar to Charcot-Leyden crystals associated with chronic allergic asthma) and other features compatible with a Th2-type of inflammation, has so far been described for asbestos and for two high aspect ratio nanomaterials (HARNs), the MWCNT-7 and NM401 (Kobler *et al* 2015; Sabo-Attwood *et al* 2005; Rydman *et al* 2014; Rydman *et al* 2015). The response indicates a more persistent Th2 inflammation, which is a feature these types of CNTs share with the material originating the fibre paradigm, asbestos. It is, however, unclear if (and how) this is related to the long-term pathogenic processes caused by the fibres.

Although these studies mostly used the MWCNT-7 (Mitsui) type of nanotubes, there are additional studies using pharyngeal aspiration providing comparative data on other types of rigid nanotubes. These have been summarised in table 15 of the CLH report. The study by Murphy *et al*. (2013) provides comparative data on lung effects of various MWCNTs after bolus administration of a dose of 25 µg MWCNT/animal via pharyngeal aspiration with examination 1 and 6 weeks after exposure. MWCNTs included a tangled form with a diameter of 15 nm, one short straight form (length 0.5-2 µm, diameter 20-30 nm) and a MWCNT with a diameter of 165 nm and length 56 µm (76 % > 20 µm; 84 % > 15 µm) [*Note: in the CLH dossier it is stated that Murphy et al tested 5 different MWCNT but the original paper lists only three different types*]. Long straight MWCNT but not tangled or short MWCNT caused acute neutrophilic inflammation in BAL at 1 week and progressive thickening of the alveolar septa. An inflammatory response in the pleural lavage and lesions along the chest wall and diaphragm were seen after 6 weeks. The effects were comparable to those reported earlier after pharyngeal aspiration of 10, 20, 40 or 80 µg of MWCNT-7 in mice (Porter *et al*., 2010). Poulsen *et al* (2015) reported that also NM-401 (physicochemically similar to MWCNT-7 but more flexible) elicited an earlier onset of inflammation and induced more fibrosis and a unique fibrotic gene expression signature at day 28, compared to the tangled Nanocyl NC7000 (D: 11 ± 4.5 nm L: 0.85 ± 0.457 µm) in mice after intra-tracheal administration. However, both MWCNT elicited strong acute phase and inflammatory responses that persisted up to 28 days.

In addition, toxicokinetic data suggesting translocation of MWCNTs (both Mitsui type and other long rigid fibres, Kasai *et al*. 2015; Xu *et al*., 2012 and 2014) to the parietal pleura and induction of inflammation and mesothelial proliferation supports asbestos-like pleural pathogenicity of MWC(N)Ts. Parietal pleural penetration and related inflammatory and proliferative effects are generally considered to be a hallmark of fibre pathogenicity. No pleural penetration was observed in studies with MWCNTs with a diameter <15 nm but it needs to be noted that limited information is available on fibres between 15- 30 nm in diameter.

Comparison with the criteria

According to the CLH criteria, substances are classified in Category 1 for specific target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.

The guidance value for classification to STOT RE cat 1 by dust inhalation (in rats) is $\leq$ 0.02 mg/L/6h/day for a 90d study. If shorter duration studies (e.g. 4 wk study) are used for classification, Haber's rule can be applied (if considered applicable) and the cut off limit is elevated accordingly (e.g. the cat 1 limit is elevated to 0.06 mg/L in the case of a 4 week study). Comparison of the available inhalation data showing granuloma formation in 12-90 day studies with LOAECs between 0.0002- 0.01 mg/L, shows that STOT RE cat 1 criteria are clearly fulfilled for MWCNT-7 tested in these studies. Available evidence from the studies using intra-tracheal or pharyngeal aspiration shows inflammatory and fibrotic effects of similar magnitude also after exposure to other types of long, rigid carbon nanotubes. Toxicokinetic data showing translocation of both Mitsui type and other long rigid fibres to the parietal pleura also gives some support to the hypothesis that the fibre paradigm applies also to other fibres within the scope of the classification proposal and not only the Mitsui type MWCNTs.

Therefore, it is reasonable to assume that repeated inhalation exposure to these MWCNT will result in similar lung effects as shown for MWCNT-7 in the study by Kasai *et al*. (2015). Therefore, RAC supports the DS proposal to classify MWC(N)Ts specified in this proposal as STOT RE 1. These lung effects are likely to occur only after inhalation exposure, resulting in high retention of MWCNTs in the lungs. Therefore, it can be specified that these effects occur only when inhaled. Therefore RAC concludes that classification is warranted as **STOT RE 1;H372, Causes damage to lungs through prolonged or repeated exposure via inhalation**.

The DS did not propose a specific concentration limit for the STOT RE classification. According to CLP guidance, specific concentration limits (SCLs) for STOT RE may be set for substances inducing target organ toxicity at a dose level or concentration clearly (more than one magnitude) below the guidance values. This is the case with MWCNTs. Taking a LOEAC of 0.0002 mg/m^3 from the 90 day study by Kasai *et al*. (2015) as a starting point and applying the formula given in Section 3.9.2.6 of the CLP guidance (2017) for the determination of the SCL, an SCL of 1% for the classification to STOT RE cat 1 is derived. **RAC proposes an SCL of 1 % for STOT RE 1 and an SCL of 0.1% for the STOT RE 2 to apply to the classification of MWC(N)Ts.**

As discussed also elsewhere in this opinion, it should be noted that the lower diameter limit of 30 nm given by DS in the classification proposal is chosen based on the availability of carcinogenicity data (on mesothelioma induction). There are subchronic studies available for commercial tangled MWCNT types with lower diameter focusing on the lung effects caused by these nanotubes (Ma-Hock *et al*., 2009; Pauluhn *et al*., 2010; Pothmann *et al*., 2015). These studies have not been evaluated in the classification proposal of the DS and classification of these MWCNTs falling outside of this classification proposal needs to be considered separately. RAC notes, however, that effects have been observed outside the size range, as also noted by Member State comments in the consultation, although the

mechanisms of pulmonary effects of thin, tangled fibres may differ from those of long rigid MWC(N)Ts (See also the related discussion under "carcinogenicity").

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Environmental hazards not assessed in this dossier.

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13 ANNEX

Abbreviations

µg	microgramme
µm	micrometer
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
ALB	Albumin
ALP	Alkaline Phosphatase
AM	Alveolar Macrophages
av.	average
BAL(F)	Bronchio-Alveolar Lavage (Fluid)
PCE	Polychromatic Erythrocytes
BALT	Bronchus-Associated Lymphoid Tissue
BET	Brunauer-Emmett- Teller
BSA	Bovine Serum Albumin
bw	body weight
Carc.	Carcinogenicity
CAS	Chemical Abstracts Service
Cat.	Category
CBMN	Cytokinesis-Block Micronucleus Assay
CHO	Chinese Hamster Ovary
CLP	Classification, Labelling and Packaging (Regulation EC No. 1272/2008)
CMAD	Count Median Aerodynamic Diameter
CMD	Count Median (Geometric) Diameter
CNT	Carbon Nanotube(s)
cpm	counts per minute
Cyt B	Cytochalasin B
d	days
D	Diameter
DCFH-DA	2′,7′-dichlorofluorescein diacetate (alt.: $DCFH_2$-DA)
EC	European Community
ESR	Electron Spin Resonance
Exp.	Experiment
f	female
FBGC	Foreign Body Giant Cells
FBS	Foetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
g	gramme
GD	Guidance Document
GLP	Good Laboratory Practice

gpt	Xanthine-guanine phosphoribosyltransferase (gene)
GSD	Geometric Standard Deviation
h	hours
Hb	Haemoglobin
hgprt	Hypoxanthin-Guanin-Phosphoribosyltransferase (gene)
IARC	International Agency for Research on Cancer
ICR	Institute for Cancer Research
IL	Interleukin
JRC	Joint Research Centre
L	Length
LALN	Lung-Associated Lymph Nodes
LC-MS/MS	Liquid Chromatography Coupled with Tandem Mass Spectrometry
LDH	Lactate Dehydrogenase
LOAEC	Lowest observed adverse effect concentration
LOAEL	Lowest observed adverse effect level
m	male
MARCO	Macrophage Receptor with Collagenous Structure
MCA	Methylcholanthrene
mg	milligramme
MMAD	Mass Median Aerodynamic Diameter
M-MDSC	Monocytic Myeloid Derived Suppressor Cells
MMVF	Man-Made Vitreous Fibres
MPPD	Multiple-Path Particle Dosimetry Model
MT	Micronucleus Test
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (dye)
MWCNT	Multi-Walled Carbon Nanotube(s)
n	Number of animal
n.a.	not applicable
NCE	Normochromatic Erythrocytes
ng	nanogramme
NM	Nanomaterial
nm	nanometer
NO	Nitric oxide
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
OD	Optical Diameter
OECD	Organization for Economic Co-operation and Development
p. e.	post exposure
PBMCs	Peripheral Blood Mononuclear Cells
PCNA	Proliferating Cell Nuclear Antigen (= CD68)

PF 68	Pluronic F 68 (a detergent)
PMN	Polymorphonuclear [leukocytes] (= Granulocytes)
ppm	parts per million
RI	Replication Index
ROS	Reactive Oxygen Species
SAXS	Small-Angle X-ray Scattering
SCE	Sister Chromatid Exchange
SPF	Specific Pathogen Free
SPI	Salmonella Pathogenicity Island (chromosome locus)
SSA	Specific Surface Area
STOT RE	Specific Target Organ Toxicity, Repeated Exposure
SWCNT	Single-Walled Carbon Nanotube(s)
Tf	Transferrin
TG	Test Guideline
TNF	Tumour Necrosis Factor
TP	Total Protein
TS	Test Substance
w/o	without
WHO	World Health Organisation
WLL	Whole Lung Lavage
WPMN	Working Party on Manufactured Nanomaterials
wt	weight
yr(s)	year(s)
γ-H2AX	Histon variant H2AX, phosphorylated

Chemical Element Symbols:

Ag	Silver	Mg	Magnesium
Al	Aluminium	Mo	Molybdenum
As	Arsenic	Na	Sodium
Be	Beryllium	Ni	Nickel
Bi	Bismuth	O	Oxygen
Ca	Calcium	Pb	Lead
Cd	Cadmium	S	Sulphur
Cl	Chlorine	Sb	Antimony
Co	Cobalt	Si	Silicon
Cr	Chromium	Ti	Titanium
Cu	Copper	V	Vanadium

Fe	Iron	Sr	Strontium
Li	Lithium	Zn	Zinc