National Research Centre
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Scientific basis for setting a health-based occupational exposure limit

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FOREWORD

In 2015, the Danish Working Environment Council made 22 recommendations to promote safe handling of nanomaterials in the working environment, which were enforced by the Minister of Employment. One of these recommendations was 'That the Danish Working Environment Authority in cooperation with relevant scientific experts assesses whether adequate scientific documentation can be provided to use the scientific quality committee for an assessment of the scientific evidence to determine limit values for specific nanomaterials in the work environment.' (https://www.amr.dk/nano.aspx).

On this background, The Danish Working Environment Authority asked the National Research Centre for the Working Environment to review the scientific evidence with the aim of clarifying the possibilities for suggesting nanospecific occupational exposure limits for three different nanomaterials (titanium dioxide, carbon black and carbon nanotubes).

The purpose of the present report is to suggest a health-based occupational exposure limit for carbon nanotubes.

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Copenhagen, August 2018

EXECUTIVE SUMMARY

In this report, a working group at the National Research Centre for the Working Environment reviews data relevant to assessing the hazard of carbon nanotubes (CNTs), i.e. human studies (Chapter 2), toxicokinetics (Chapter 3), animal studies (Chapter 4), mechanisms of toxicity (Chapter 5), previous risk assessments of CNTs (Chapter 6), scientific basis for setting an OEL (Chapter 7) and finally summarize and suggest a health-based occupational exposure limit for CNTs (Chapter 8). The focus of this report is only occupational exposure by inhalation.

Carbon nanotubes are a very diverse class of nanomaterials with large variation in physico-chemical properties including diameter, length, specific surface area, level and type of contaminations, and surface modifications. Variation in toxicity potential based on different physico-chemical properties has been reported, but at the time of this report, the relationship between physico-chemical properties of CNTs and their inhalation toxicity is not fully clarified. Furthermore, most commercially available CNT preparations are very heterogeneous. Therefore, the present working group considers toxicity data from all types of CNTs in order to obtain a precautious approach and derivation of an OEL value that protects against as many different types of CNTs as possible.

Assessments of human occupational exposure to CNTs at settings such as laboratories and production sites have reported personal breathing zone concentration levels ranging from non-detectable to ca. 80 $\mu g/m^3$. This demonstrates that human occupational exposure does occur during handling of CNTs. However, at present almost no human data on toxicity and epidemiological studies is available. The current working group therefore used studies in mice and rats to assess potential human hazard. Inhalation studies were prioritized and risk assessments were solely based on these. However, for the description of toxicological endpoints and mechanism of toxicity, studies using pulmonary deposition from intratracheal instillation exposure and pharyngeal aspiration exposure were included when no quality inhalation studies were available.

Pulmonary inflammation, and inflammatory-related changes, was the most commonly reported adverse effect of pulmonary exposure to CNTs. Four sub-chronic and one chronic study inhalation study in rats were identified as suitable for identification if relevant NOAECs/ LOAECs and determining a derived-no-effect level (DNEL) for pulmonary inflammation. In general, these studies identified ”no observed adverse effect concentrations”(NOAECs) ranging from 0.05 mg/m^3 to 1 mg/m^3 and ”lowest observed adverse effect concentrations” (LOAECs) ranging from 0.25 mg/m^3 to 5 mg/m^3. The deposited surface area of the CNTs was identified as a predictor of pulmonary inflammation (neutrophil influx in the broncho alveolar lavage fluid). As dose-dependency was identified for inflammation and as it was possible to detect a NOAEC, inflammation was considered a threshold effect.

The genotoxic and carcinogenic potential of CNTs were investigated in several studies. CNT-induced genotoxicity was reported; however, although diameter thickness was suggested as a driver of CNT-induced genotoxicity, no clear coupling to physico-

chemical properties could be identified. In addition, no dose response relationship was identified regarding the genotoxic properties. One chronic cancer study in rats was identified as suitable for risk assessment. This 2 year inhalation study investigated pulmonary pathological changes after exposure to the long and thick MWCNT type called MWNT-7/XNRI-7, and the authors reported lung adenomas and carcinomas at the middle and high dose (0.2 and 2 mg/m^3, 6 h/day, 5 days/week for 104 weeks), whereas 0.02 mg/m^3 was found as a NOAEC. The same MWCNT type (MWNT-7/XNRI-7) had previously been classified as possibly carcinogenic by IARC. Dose-response relationships have been identified for MWCNT-induced carcinogenic effects in several independent studies. The present working group found that the mechanism of action of CNT-induced carcinogenic effect has not been fully clarified. CNTs have reported to induce ROS generation similar to carbon black. CNTs may also induce genotoxicity through they fibrous shape, both in regards to diameter thickness and length. In addition, secondary genotoxicity due to CNT-induced inflammation has been recognized as an important and well-documented mechanism of action for the development of lung cancer. Based on the lack of dose-response relationship for genotoxicity and the unclear mode of action for cancer, the current working group did not find sufficient evidence for a threshold mechanism for CNT-induced carcinogenicity and decided to consider it as non-threshold effect.

CNT-induced cardiovascular effects were reported in several animal studies. Both primary changes, such as accelerated plaque progression, and changes related to/or leading to cardiovascular effects, such as the acute phase response, were identified. Dose-response relationships have only sparsely been reported for CNT-induced increased plaque progression, whereas dose-response relationships have been established between CNT exposure and increased levels of acute phase response proteins. CNT-induced atherosclerotic effects have solely used pulmonary deposition as exposure method, and thus, the studies cannot be used to establish OELs. Due to the close interplay between inflammation, acute phase response and plaque progression, the current working group regards inflammation as a proxy for cardiovascular effects. Cardiovascular effects are considered a threshold effects that is regulated in parallel to inflammation.

The present working group regards inflammation and carcinogenicity as the critical adverse effects of CNT exposure by inhalation and the subsequent risk assessments are conducted based on studies reporting these effects. Based on dose-response relationships and mode of action for these effects, the current working group decided to perform the risk assessment based on both a threshold and a non-threshold mechanism of action. Four sub-chronic and one chronic inhalation study in rats were identified as suitable for determining a DNEL for pulmonary inflammation. A conservation approach was selected and the DNEL was calculated based on the study using the CNT with the largest specific surface area and reporting the lowest NOAEC estimate. The suggested exposure limit based on inflammation was 1 μg/m^3.

For the non-threshold approach on carcinogenic effects, the 2 year inhalation study in rats were identified as suitable and excess cancer risks at the levels of 1:1,000, 1:10,000 and 1 in 100,000 were calculated based this study using two approaches (please see the

accompanying table). In the first approach, lung burden was used to estimate the exposure levels. In the second approach, air concentrations were used directly. Independently of the applied method for risk assessment, the acceptable exposure levels were all very low. These levels are all more than 5 magnitudes lower than the present Danish occupational exposure limit for bulk carbon black of 3.5 mg/m^3.

Suggestion for an OEL for CNTs				
Mechanism of action		Inflammation	Lung cancer (Method I)	Lung cancer (Method II)
Threshold based	DNEL	1 μg/m^3		
Non-threshold based	Excess cancer risk:			
	1:1,000		0.03 μg/m^3	0.043 μg/m^3
	1:10,000		0.003 μg/m^3	0.0043 μg/m^3
	1:100,000		0.0003 μg/m^3	0.00043 μg/m^3

Table showing overview of DNEL based on a threshold based mechanism of action and exposure levels resulting in excess cancer risk levels at 1:1000, 1:10 000 and 1: 100 000 based on a non-threshold based mechanism of action.

The present working group regards cancer as the most critical adverse effect of CNT inhalation and recommends the approach using the excess lung cancer risk estimates based on lung burden, since this approach takes the retained pulmonary dose into account. Thus, the expected excess lung cancer risk based on lung burden approach is 1:1,000 at 0.03 μg/m^3, 1:10,000 at 0.003 μg/m^3 and 1:100,000 at 0.0003 μg/m^3.

DANSK SAMMENFATNING

I denne rapport vurderer en arbejdsgruppe ved Det Nationale Forskningscenter for Arbejdsmiljø data, der er relevante for at vurdere faren ved eksponering for kulstofnanorør (CNTer), dvs. humane studier (kapitel 2), toksikokinetik (kapitel 3), dyreforsøg (kapitel 4), toksicitetsmekanismer (kapitel 5), tidligere risikovurderinger af CNTer (kapitel 6), videnskabeligt grundlag for fastlæggelse af en grænseværdi i arbejdsmiljøet (kapitel 7) og endelig opsummeres og foreslås en helbredsbaseret grænseværdi for CNTer i arbejdsmiljøet (kapitel 8). Fokus i denne rapport er alene på erhvervsmæssig eksponering ved indånding.

Kulstofnanorør er en meget forskelligartet gruppe af nanomaterialer med store variationer i de fysisk-kemiske egenskaber. Disse inkluderer diameter, størrelse, længde, specifikt overfladeareal, mængde og type af metalurenheder og overflademodifikationer. Variationer i CNTers fysisk-kemiske egenskaber har vist sig at kunne påvirke det toksiske potentiale, men på nuværende tidspunkt er sammenhænge mellem CNTers fysisk-kemiske egenskaber og deres toksicitet efter indånding endnu ikke fuldstændigt klarlagt. Derudover er de fleste kommercielt tilgængelige CNT præparationer meget heterogene af natur. Derfor anser den nærværende arbejdsgruppe alle CNT typer som farlige ved indånding og foreslår at regulere alle CNTer som én gruppe.

Eksponeringsmålinger på arbejdspladser, såsom laboratorier og produktionsvirksomheder, har påvist CNT koncentrationer i den personlige indåndingszone som spænder fra under detektionsgrænsen til ca. 80 $\mu g/m^3$. Dette viser, at der er erhvervsmæssig eksponering for CNT ved håndteringen af CNT på arbejdspladser. Men på nuværende tidspunkt forefindes der stort set ingen data på toksicitet i mennesker eller epidemiologiske studier. Den nærværende arbejdsgruppe har derfor brugt studier i mus og rotter til at vurdere den potentielle menneskelige helbredsrisiko. Subkroniske og kroniske inhalationsstudier blev prioriteret og risikovurderingerne blev udelukkende foretaget på baggrund af disse. Dog blev studier, der anvendte lungedeponering ved intratracheal instillation, inkluderet til beskrivelse af toksikologiske endepunkter og toksisitetsmekanismer, når der ikke forelå inhalationsstudier af tilstrækkelig kvalitet.

Lungeinflammation, og inflammationsrelaterede ændringer var de hyppigst rapporterede helbredseffekter efter lungeeksponering af CNTer. Fire sub-kroniske og et kronisk inhalationsstudie i rotter blev fundet egnede til at bestemme en "derived-no-effect level" (DNEL) for lungeinflammation. Disse studier rapporterede generelt "no observed adverse effect concentrations" (NOAECs)mellem 0.05 mg/m^3 og 1 mg/m^3 og "lowest observed adverse effect concentrations" (LOAECs) mellem 0.25 mg/m^3 og 5 mg/m^3. Det deponerede overfladeareal af CNTer blev identificeret som en prædiktor for lungeinflammation (neutrofilt influx i lungeskyllevæsken). Der var dosisafhængighed for CNT-induceret lungeinflammation, og da det var muligt at bestemme en NOAEC, blev inflammation anset for at være en tærskeleffekt.

CNTers genotoksiske og kræftfremkaldende potentialer er blevet undersøgt i flere studier. CNT-induceret genotoksicitet er blevet rapporteret, men selvom

diametertykkelse er blevet forslået som prædiktor for CNT-induceret genotoksicitet, er der endnu ikke fundet en klar sammenhæng mellem genotoksicitet og de fysisk-kemiske egenskaber for CNTer. Derudover blev der ikke fundet dosis-respons sammenhæng. Et kronisk kræftstudie i rotter blev identificeret som egnet til risikovurdering. I dette 2 års inhalationsstudie blev patologiske ændringer i lungen efter eksponering for den lange og tykke CNT type kaldet MWNT-7/XNRI-7 undersøgt, og forfatterne rapporterede lungekræft ved den midterste og højeste dosis (0,2 og 2 mg/m^3, 6 timer/dag, 5 dage/uge i 104 uger). Denne type CNT (MWNT-7/XNRI-7) er blevet klassificeret som muligvis kræftfremkaldende (2b) af IARC. Der blev identificeret dosisafhængighed for CNT-induceret kræft i flere uafhængige studier. Den nærværende arbejdsgruppe fandt ikke, at mekanismen for CNT-induceret kræft er fuldstændig klarlagt. Studier har vist, at CNTer kan inducere reaktive oxygen-forbindelser på samme måde som carbon black. De kan muligvis også inducere genotoksicitet via deres fiberform, både i forhold til diameter og til længde. Derudover er det velkendt at sekundær genotoksicitet, pga. CNT-induceret inflammation, er en mekanisme for udviklingen af kræft. På baggrund af den manglende dosisafhængighed for genotoksicitet og en uklar virkningsmekanisme for kræft, fandt den nærværende arbejdsgruppe ikke tilstrækkelig bevis for at CNT-induceret kræft er en tærskeleffekt. CNT-induceret kræft blev derfor anset som en ikke-tærskeleffekt.

Resultaterne fra flere dyrestudier viste CNT-inducerede effekter på hjerte-karsystemet. Dette gjaldt både primære effekter, som åreforkalkning, og ændringer relateret til eller førende til hjerte-kareffekter, så som akutfaseresponset. Dosisafhængighed er kun i begrænset omfang beskrevet for CNT-induceret øget åreforkalkning, hvorimod dosisafhængighed er veldokumenteret for sammenhængen mellem CNT eksponering og akutfaseproteiner. Men da de studier, hvor resultaterne viser CNT-inducerede effekter på hjerte-karsystemet, udelukkende har brugt lungedeponering som eksponeringsmetode, kan de ikke bruges til risikovurdering af CNTer. På grund af den tætte sammenhæng mellem inflammation, akutfaseresponset og åreforkalkning har den nærværende arbejdsgruppe valgt at anse inflammation som en proxy for effekter på hjerte-karsystemet. Hjerte-kareffekter blev derfor anset som en tærskeleffekt på lige fod med inflammation.

Den nærværende arbejdsgruppe anser inflammation og carcinogenicitet som de vigtigste negative helbredseffekter forårsaget af indånding af CNT, og de efterfølgende risikovurderinger er baseret på studier, der rapporterer disse effekter. Baseret på viden om dosis-respons sammenhæng og underliggende biologiske mekanismer har den nærværende arbejdsgruppe besluttet både at foretage risikovurderinger baseret på en tærskeleffekt og på en ikke-tærskeleffekt. Fire subkroniske og et kronisk inhalationsstudie i rotter blev identificeret som velegnede til fastlæggelse af DNEL for lungeinflammation. Den nærværende arbejdsgruppe valgte en konservativ tilgang, og derfor blev DNEL beregnet med udgangspunkt i det studie, som anvendte den CNT, der havde det største specifikke overfladeareal, og som rapporterede det laveste NOAEC. Den foreslåede grænseværdi for kulstofnanorør baseret på inflammation er 1 μg/m^3.

Den nærværende arbejdsgruppe valgte at anvende ikke-tærskeleffekt tilgangen til beregning af grænseværdi baseret på kræftrisiko, og et 2-års inhalationsstudie i rotter

blev identificeret som velegnet. De eksponeringsniveauer, som resulterer i overskydende kræftrisiko hos 1:1.000, 1:10.000 og 1 ud af 100.000 udsatte, blev beregnet på baggrund af dette studie på to forskellige måder og er vist i tabellen nedenfor. Ved den første beregningsmetode bruges den lunge-deponerede dosis til at estimere det tilsvarende eksponeringsniveau. Ved den anden beregningsmetode blev luftkoncentrationer anvendt direkte. Uafhængigt af metodevalg er alle de beregnede grænseværdier for kulstofnanorør meget lave. Grænseværdierne er alle mere end 5 000 gange lavere end den nuværende danske grænseværdi for carbon black (som er 3,5 mg/m^3).

Forslag til grænseværdi for kulstofnanorør				
Virkningsmekanisme		Inflammation	Lungekræft (Metode I)	Lungekræft (Metode II)
Tærskeleffekt-baseret	DNEL	1 μg/m^3		
Ikke-tærskeleffekt-baseret	Overskydende lungekræft:			
	1:1.000		0,03 μg/m^3	0,043 μg/m^3
	1:10.000		0,003 μg/m^3	0,0043 μg/m^3
	1:100.000		0,0003 μg/m^3	0,00043 μg/m^3

Tabellen viser en oversigt over DNEL baseret på tærskeleffekt som virkningsmekanisme for lungeinflammation og de eksponeringsniveauer, som resulterer i overskydende kræftrisiko hos 1:1.000, 1:10.000 og 1 ud af 100.000 baseret på en ikke-tærskeleffekt-baseret biologisk virkningsmekanisme for lungekræft.

Den nærværende arbejdsgruppe anser kræft for at være den vigtigste helbredseffekt ved indånding af kulstofnanorør og anbefaler ydermere at bruge beregningsmetode I fordi denne beregningsmetode tager udgangspunkt i den faktiske lungedeponerede dosis.

Det estimeres derfor at 0.03 μg/m^3 kulstofnanorør vil forårsage 1:1.000 overskydende lungekræfttilfælde ved indånding i arbejdsmiljøet, mens 0,003 μg/m^3 kulstofnanorør vil forårsage 1:10.000 overskydende lungekræfttilfælde og 0,0003 μg/m^3 forventes at forårsage 1:100,000 overskydende lungekræfttilfælde.

CONTENTS

ABBREVIATIONS

ALP	Alkaline phosphatase
BAL	Broncho alveolar lavage
BALF	Broncho alveolar lavage fluid
BET	Brunauer–Emmett–Teller
CVD	Cardiovascular disease
CNT	Carbon nanotube
CRP	C-reactive protein
DNEL	Derived-no-Effect Level
DSP	Daily sperm production
DWCNT	Double-walled carbon nanotubes
EC	Elemental carbon
ECHA	European Chemicals Agency
ENRHES	Engineered Nanoparticles: Review of Health and Environmental Safety
EM	Electron microscopy
GI	Gastrointestinal
IARC	The International Agency for Research on Cancer
ICAM	Intercellular Adhesion Molecule
INEL	Human indicative no-effect levels
IP	Intraperitoneal
HARN	High aspect ratio nanomaterials
HDL	High-density lipoproteins
HiPCO	High-pressure carbon monoxide method
LDH	Lactate dehydrogenase
LDL	Low-density lipoproteins
LOAEC	Lowest observed adverse effect concentration
MWCNT	Multi-walled carbon nanotube
NIOSH	National Institute for Occupational Safety and Health
NM	Nanomaterial
NOAEC	No observed adverse effect concentration
OEL	Occupational exposure limit
OECD	Organisation for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
REL	Recommended exposure limit
SAA	Serum amyloid A
SWCNT	Single-walled carbon nanotube
TEM	Transmission electron microscopy
TWA	Time-weighted average
VCAM	Vascular cell adhesion molecule

INTRODUCTION

Carbon nanotubes (CNTs) comprise a group of poorly soluble, highly variable, cylindrical, hollow, fibrous nanomaterials. They are grouped according to their number of side-walls and generally divided into three categories: Single-walled (SW), double-walled (DW), or multi-walled (MW) CNTs. Their side-walls are made of carbon atoms, mainly in sp^2 configuration, arranged in inter-connected hexagon similar to that of graphene sheets. As the name implies, SWCNTs consist of one rolled-up graphene layer, whereas DW- and MWCNTs consist of two or more layers. The difference in wall numbers affects the diameter and rigidity of the CNTs; whereas some are entangled, others are fiber-like. Correspondingly, the diameters can vary from around 1 nm (most SWCNTs) to up to 150 μm (some MWCNTs) (Jensen et al. 2015). Because of their long lengths (up to several mm), CNTs are high aspect ratio (length:diameter) nanomaterials (HARN). Long CNTs (over 15 μm in length) comply with the WHO fiber paradigm, which states that fibers with long lengths, small diameters and high biopersistence display increased toxicity, as they reach the alveoli region of the lungs, retain their structure, and are difficult for the macrophages to phagocytize (Donaldson et al. 2010).

The physical appearance of CNTs can vary greatly from one type of CNT to another. The rigidity of the CNT, and thus its level of fiber-like appearance, relies prominently on its number of graphene walls. However, MWCNTs with few walls resembles SWCNT physically more than fiber-like MWNCTs with several walls (Figure 1). It is therefore difficult to address specific SW- or MWCNT effects. Instead of separating CNTs based on wall numbers, the current working group has chosen to separating based on physico-chemical properties as surface area, length, diameter, chemical composition etc. This report therefore does not distinguish between SW- or MWCNT effects, and CNTs data from both SWCNT and MWCNT were evaluated. Based on this, the current working group has selected the most adequate data for OEL derivation.

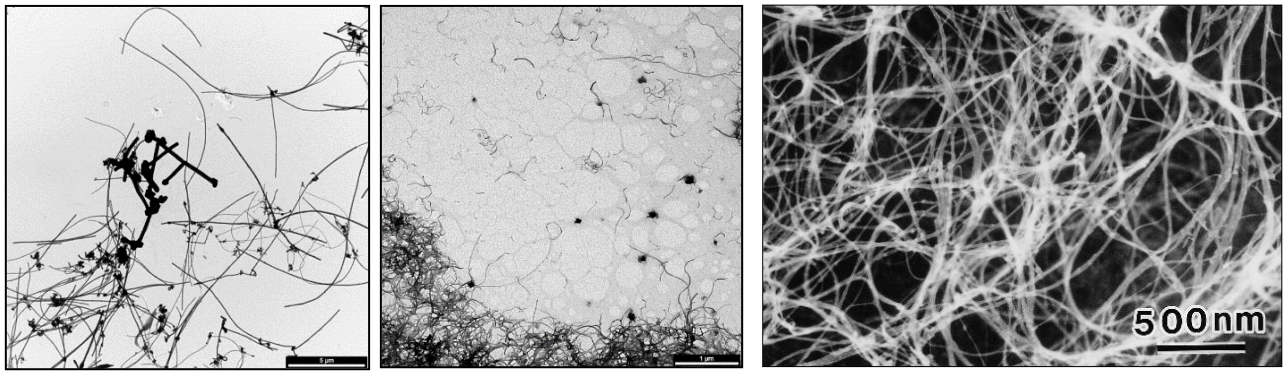

Figure 1. Different CNTs. Left: Transmission electron microscope (TEM) image of fiber-like MWCNTs (Poulsen et al. 2015b). Bar size: 1 μm. Middle: TEM image of flexible MWCNTs (Poulsen et al. 2015b). Bar size: 1 μm. Right: TEM image of SWCNTs (Balarak et al. 2016).

CNTs can be synthesized though different processes and the method chosen affect the level of impurities and structural defects. In general, most synthesis processes take place in vacuum, on a catalyst (often a metal such as Fe, Ni or Co), with controlled process gasses and controlled temperature (Ismail et al. 2018). The most commonly used method for commercially available CNTs is chemical vapor deposition (CVD). In this process, the type of CNT, its structure and its size can be controlled by the size and type of catalyst, the choice of gas and the temperature. This means that physical dimensions are well controlled although it may result in many structural defects on the CNTs, especially MWCNTs. Another commonly used method for large-scale production is arc discharge, which is a fairly easy and inexpensive method. The disadvantage to this method is that carbon black, fullerenes and soot are generated as byproducts and that the raw CNTs formed often contain a lot of metal catalyst residues. More precise, but also more expensive, CNT synthesis processes, as laser ablation, also exist.

The most common structural defects introduced during the synthesis process are missing carbon atoms or replacement of hexagons by pentagons or heptagons. Such defects may increase the curvature of the CNTs due to elongation or compression of one side of the CNT (Zhang and Li 2006). It is also possible that structural defects could render the graphene sheets and thereby the CNTs more susceptible to biological degradation, which would change their toxic potential. Equally, the presence of bioavailable metal impurities from catalysts on the surface of the CNT could have influence their toxic potential. Some metals, especially iron, are known to induce reactive oxygen species (ROS) (Knaapen et al. 2004). ROS may induce damage to cellular components such as DNA, lipids and proteins, thereby causing genotoxicity.

Pure, graphitized CNTs are very hydrophobic in nature. Functionalization of CNTs is therefore an important tool for increasing their solubility in aqueous solutions or their chemical binding in solid composites. This is also important for the potential use of CNTs in biological applications. By changing their polarity, the dispersion and the biological interactions in the lung milieu, as well as the fate of the CNTs, may change dramatically. This could ultimately change the toxic potential of the CNTs. Several studies have reported altered toxicity of functionalized CNTs compared to pristine CNTs (Hamilton, Jr. et al. 2013;Jain et al. 2011;Poulsen et al. 2016;Sager et al. 2014;Sayes et al. 2006).

The overall structure and composition of CNTs facilitate excellent electric and thermal conductivity, high tensile strength, and good chemical stability (Dresselhaus et al. 2004). Due to these abilities, CNTs are desirable for use in a variety of products, including composite materials, electronics, plastics and rubbers, coatings, insulation and in biomedical applications (De Volder et al. 2013;Jensen et al. 2015). As an example, the high-aspect ratio and high tensile strength of CNTs makes them ideal for low weight materials, e.g. sports equipment and wind mill wings. Due to their many possible applications, CNTs are already produced and utilized at commercial scale, and they are available worldwide. The global market for CNT products is expected to grow from an estimated USD 3.43 Billion in 2016 to USD 8.70 billion in 2022 (Markets and Markets 2017). Thus with increased production, the potential exposure risks for both workers and consumers have also increased.

To our knowledge, there are no legally enforced occupational exposure limits (OEL) for CNTs. In Denmark, the approximate OEL is the current for bulk carbon black, which has the same chemical composition as CNTs. The limit is 3.5 mg/m^3 and is regulated by the Danish Working Environment Authority. The aim of the present report is to investigate if the present knowledge allows for a suggestion of a health-based, OEL for CNTs. This document will therefore review the relevant literature on the adverse effects of CNTs. As suggested in the guidelines from REACH (ECHA 2012), the risk assessment methodology in this report will be divided into threshold or non-threshold effects. The threshold effect approach relies on the assumption that the organism can withstand a certain dose before adverse effects occur, whereas non-threshold effects assume that any exposure to the substance can result in adverse effects. For an OEL based on threshold effects, the following traditional approach is utilized (ECHA 2012): 1) identification of critical effect, 2) identification of the NOAEC, 3) calculation of OEL using assessment factors adjusting for inter and intra species differences. For non-threshold effects, the current working group will use two approaches. The first method, used by Kasai et al. 2016 and Erdely et al. 2013, uses the measured lung burden in rats exposed by inhalation and the alveolar surface area of rats and humans to estimate the human equivalent lung burden. The second method, suggested by (ECHA 2012;SCHER/SCCP/SCENIHR 2009), uses air concentrations directly. Conclusively, the calculated OELs will be compared and lastly, a recommended OEL for CNT exposure will be proposed.

HUMAN STUDIES

Exposure

CNTs are synthesized as powders and inhalation is therefore considered the main route of exposure in humans, although dermal exposure is also possible. Exposure though inhalation may occur during the entire CNT lifecycle: Manufacturing, storage, transportation, product application, and end-of-life processes. However, in their final applications CNTs rarely exist as free fibers. When bound to a surface or suspended in either liquids or solids, the individual CNTs are surrounded and more or less bound in the matrix. It has been reported that this diminishes their toxic potential substantially (Saber et al. 2016). Therefore the greatest risk of human exposure is in the working environment, especially during production and handling of large quantities of free CNTs. The nano-sized properties of CNTs are important for scenarios involving human exposure in the occupational settings:

- More individual CNT per mass unit compared to larger fibers.
- Larger surface area per mass unit compared to larger fibers.
- More dusty and therefore stays in the air longer compared to larger fibers.

Measurement of personal exposure levels to CNTs in occupational settings has been reported in several studies (Table 1). Whereas the earlier exposure assessment studies measured total inhaled mass or total gravimetric mass in the personal breathing zone (Han et al. 2008;Lee et al. 2010;Maynard et al. 2004;Methner et al. 2010), the more recent studies measured elemental carbon (EC) concentrations in personal breathing zone samples (Birch et al. 2011;Dahm et al. 2012;Dahm et al. 2015;Lee et al. 2015;Methner et al. 2012;Ono-Ogasawara et al. 2015;Shvedova et al. 2016;Takaya et al. 2012;Erdely et al. 2013;Hedmer et al. 2014), which is more specific for carbon-based particles, including CNTs. As a possible consequence of this, the older studies tended to report higher exposure levels compared to newer studies. In general, the newer occupational exposure assessment studies found personal breathing zone concentration levels ranging from non-detectable to ca. 80 $\mu g/m^3$. The differences in personal breathing zone concentration levels are primarily attributed to different worker exposure scenarios. These studies demonstrate that human exposures to CNTs occur in occupational settings.

Table 1. Measured personal occupational exposure levels of CNTs.

Type of samples collected	Work place	Work process	Personal breathing zone mass concentrations (μg/m^3)	Reference
Estimated inhalable mass	Four facilities producing SWCNTs	HiPCO or laser ablation production	0.7 - 53	Maynard et al. 2004
Total gravimetric mass	MWCNT research facility	Handling of MWCNTs: Blending, weighing, spraying, miling, etc..	N.D. - 331.7	Han et al. 2008
Total gravimetric mass	Two research institutes, two labs and three industrial facilities	Production and handling of MWCNTs	7.8 - 320.8	Lee et al. 2010
Total carbon-inhalable size fraction	Five research and development labs, and one manufacturer	Handling of CNTs: Weighing, mixing, wet sawing, processing, drying, etc.	64-1094	Methner et al. 2010
Elemental carbon-respirable size fraction	A facility manufacturing and processes vapor-grown carbon nanofibers	Carbon nanofiber handling	45 - 80	Birch et al. 2011
Elemental carbon-inhalable size fraction	Five facilities producing CNTs and one developer of semiconductor	Production and handling of CNTs: Weighing, mixing, sonicating, milling, etc.	N.D. - 7.86	Dahm et al. 2012
Elemental carbon-inhalable size fraction	Two facilities producing CNTs and two facilities producing carbon nanofibers	Production and handling of CNTs and carbon nanofibers: Weighing, spraying, filtration, cleaning, harvesting	N.D. - 38	Methner et al. 2012
Respirable elemental carbon mass concentrations	Factory creating fabric from yarns covered with MWCNTs	Weaving	3.5 - 4.8	Takaya et al. 2012
Elemental carbon-inhalable size fraction	Eight facilities producing or using MWCNT	Production and handling of CNTs: Weighing, mixing, sonicating, milling, etc.	N.D - 79.6	Erdely et al. 2013

Elemental carbon-respirable size fraction	Small-scale producer of MWCNTs by arc discharge	Production and handling of MWCNTs: Cleaving, sieving, cleaning, harvesting, grinding, etc.	<0.08 - 7.4	Hedmer et al. 2014
Inhalable elemental carbon mass concentrations	A MWCNT manufacturing company	Manufacturing of MWCNTs	5.5 - 9.3	Lee et al. 2015
Respirable elemental carbon mass concentrations	Primary and secondary manufacturers of CNT or carbon nanofibers	Production and handling of CNTs: Weighing, mixing, sonicating, milling, etc.	0.02 - 2.94	Dahm et al. 2015
Respirable elemental carbon concentrations	A MWCNT manufacturing facility	Production and handling of MWCNTs: Harvesting, disintegration, packaging, laboratory handling, etc.	0.54 - 6.11	Shvedova et al. 2016

N.D: Not detected, i.e. below detection limit.

Biomonitoring

Although carbon nanotubes have been known since the mid 50's, they did not receive much attention from the scientific community as a whole until Sumio Iijima described MWCNTs in his Science article from 1991 (Iijima 1991). CNTs are therefore a relatively new material, and large-scale productions have only started within the last decade. Consequently, studies reporting toxicological effects after both occupational and non-occupational human exposure are very scarce. At present date, only one study is available in the literature.

Shvedova and colleagues investigated biomarkers in induced sputum and blood of workers exposed to MWCNTs in a manufacturing facility in Tambov, Russia (Nanotech Center Ltd.) (Shvedova et al. 2016;Fatkhutdinova et al. 2016). They recruited 8 workers exposed to MWCNTs (as having direct contact with MWCNT aerosol for at least 6 months) and 7 non-exposed controls from the same facility. Further, exposure assessment of the personal breathing zone at different tasks in the facility was also conducted. The 8-h, TWA elemental carbon concentrations in respirable size fractions were measured at different workstations and were in the range of 0.7–2.8 $\mu g/m^3$ (Fatkhutdinova et al. 2016). The control group, who did not handle MWCNTs, was not exposed to MWCNTs. Exposed workers had more than 2-fold increased serum levels of IL1B, TNF, IL4 and IL10 and increased sputum levels of Il1B, TNF, IL6, IL4, IL5, IL8 all indicative of systemic inflammation. Acute phase response proteins C-reactive protein (CRP) and serum amyloid A (SAA) were not assessed. No no-observed-adverse-effect concentration (NOAEC) or lowest-observed-adverse-effect concentration (LOAEC) was calculated, but the authors of the current report note that the exposure levels were relatively low at 0.7–2.8 $\mu g/m^3$, indicating that even this low level was sufficient to induce markers for systemic inflammation in humans.

TOXICOKINETICS

The behavior and distribution of CNTs after exposure is of great importance for how and where they affect the organism. The main entry way, which in the case of occupational exposure is the lung and to some extent the skin, will undoubtedly receive the greatest load and experience the greatest changes. However, extra-pulmonary alterations have previously been reported after pulmonary exposure to CNTs (Poulsen et al. 2015a;Poulsen et al. 2017;Kim et al. 2015). Translocation of CNTs is a likely explanation for these distal changes, although it is possible that secondary exposure through inflammation plays a significant role. In order to understand potential target effects, it is important to identify the tissues where CNTs accumulate.

Umeda and colleagues evaluated deposition pattern of MWCNTs MWNT-7 (D: 88±5 nm, L: 5.0±4.5 μm) after inhalation exposure in F344 rats of both sexes (doses 0.2, 1 or 5 mg/m^3 MWCNT, 6 h/day, 5 days/week for 2 weeks)(Umeda et al. 2013). MWCNT deposition was observed in the entire lung (bronchiolar space, alveolar space, alveolar walls) and in the nasal cavity immediately after exposure. The MWCNTs were primarily detected within alveolar macrophages with, a few free MWCNT fibers found in the bronchi and alveolar space. The quantity of MWCNTs was higher in the rats exposed to 5 mg/m^3 MWCNTs compared to the lower doses. Deposited CNTs have been reported to reach the sub-pleural region after pulmonary exposure in rodents (Ryman-Rasmussen et al. 2009;Mercer et al. 2010). Inhaled MWCNTs (L:0.5–40 μm, D:10–50 nm, 1 or 30 mg/m^3 for 6 h) translocated rapidly to the sub-pleura region and were detected until the end of the experiment 14 weeks post-exposure. The authors suggested that macrophages facilitated the transport by engulfing the MWCNTs (Ryman-Rasmussen et al. 2009). In concordance with this, Mercer et al. showed that 0.6% of the pulmonary deposited dose had translocated to the sub-pleural region one day after pharyngeal aspiration of the MWCNT MWNT-7 (L:3.9 μm, D: 49 nm)(Mercer et al. 2010). The translocation to the sub-pleural region may suggest a similar mode of action as asbestos.
(Mercer et al. 2013) exposed male mice to MWCNTs MWNT-7 (aerodynamic diameter of 1.3 μm) by inhalation (5 mg/m^3 MWCNT aerosol for 5 hours/day for 12 days, 4 times/week for 3 weeks, estimated lung burden of 28.1 μg/lung). At 1 day and 336 days after the exposure period they assessed the biodistribution of MWCNTs by darkfield microscopy. They estimated that 7.3% of the lung burden measured at post-exposure day 1 was cleared from the lung at post-exposure day 336. The vast majority of this had translocated to the lymph nodes, however 0.03% of the initial MWCNT lung burden was found in the liver and lesser percentages was found in the kidney, heart, brain and diaphragm.

Lung clearance and translocation to liver and spleen was assessed by (Czarny et al. 2014), who exposed female Balb/c mice to 20 μg ^{14}C skeleton-labelled MWCNTs by pharyngeal aspiration. The MWCNTs had an average diameter of 41 nm, were 3.9 μm long and appeared as straight single fibers in EM pictures. The limit of detection of the radioactive labelling was determined to be in the order of 0.2 pg or 22 CNT fibers. Half the dosed MWCNTs were cleared from the lung within 1 day. Of the remaining 10 μg, 10% was detected in the lung tissue 3 and 12 months post-exposure. At 12 months post-exposure, 0.75% was found in liver and 0.20% was found in spleen. MWCNT

translocation to liver and spleen was confirmed with EM. Thus, this study shows that rigid MWCNTs was cleared from the lung and to some extent translocated to the liver and spleen. 10% of the dosed MWCNTs remained in the lung and no further clearance was detected the period between 3 and 12 months post-exposure. Based on the data provided in the paper, lung clearance of the alveolar deposited dose is estimated to occur with a half-life of 30 day.

In a two-year inhalation study, rats were exposed MWNT-7 at doses 0.02, 0.2 and 2 mg/m^3 (Kasai et al. 2016). The MWCNTs had diameters of 92.9-98.2 nm and mean lengths of 5.8-5.9 um, and were mainly found as single fibers. Lung burden increased linearly over time in a dose-dependent manner. Deposited dose was estimated to be 1.5-2.7% of the inhaled dose. Deposited dose per lung differed between sexes, but no difference was observed when normalized to lung weight. In addition to the lung, the MWCNTs were observed as single or aggregated fibers in nasal cavity, larynx, trachea, lungs, lymph nodes, spleen, liver, kidneys, olfactory bulb, and brain. In the kidney, olfactory bulb and brain, the MWCNTs were only observed as single fibers.

Pauluhn performed a 13-week inhalation study in rats using Baytubes, which were 10 nm in diameter and 200-300 nm long (Pauluhn 2010b). The MWCNTs appeared curved on EM pictures. The aerosol consisted mainly of CNT agglomerates. Pulmonary clearance in the rats was estimated by assessing Co content in the lung. Half-lives for clearance were 150-375 days. The highest clearance rate was observed for the highest air concentration. The current working group considers the highest dose to give the best estimate, as the Co content was close to the limit of detection.

Taken together, the data suggest that agglomerated CNTs are cleared away from the lung slower than CNTs that are dispersed as single fibers. In agreement with this, Pauluhn and Rosenbruch have shown that CNT clearance following inhalation exposure in rats occurs faster with well-dispersed Baytubes CNTs compared to aggregates of the same CNT (Pauluhn and Rosenbruch 2015). Half-lives for pulmonary clearance were estimated to be 87 and 46 days for aggregated and dispersed CNTs, respectively.

Translocation can in principle occur either by translocation from lung to blood or by secondary uptake via the GI tract following pulmonary clearance by mucociliary transport. However, no uptake was detected following oral dosing of the radioactively labelled MWCNTs suggesting that translocation occurred from lung to systemic circulation (Czarny et al. 2014).

ANIMAL STUDIES

Rodent versus human response

As almost no human data on toxicity and epidemiological studies is available, inhalation studies in mice and rats are used to assess potential human hazard.
One human study reported MWCNT-induced systemic inflammation following exposure to relatively low air concentrations of MWCNT (Shvedova et al. 2016;Fatkhutdinova et al. 2016). This finding is in overall agreement with strong inflammatory potential of several different CNTs in sub-chronic inhalation studies in rats (Pauluhn 2010b;Ma-Hock et al. 2009;Kasai et al. 2016;Kasai et al. 2015), although no quantitative comparison of the responses has been performed.

Rats are the preferred animal model in particle toxicology and more sensitive than mice to particle-induced lung cancer and fibrosis. However, rats do not express the acute phase proteins serum amyloid a isoforms Saa1, Saa2 and Saa3, which are expressed by humans and mice (Saa3 in mice only)(Cray et al. 2009). Serum amyloid is causally related to plaque formation (Thompson et al. 2018). Since rats do not express SAA, a key acute phase protein, they may be less well suited as model of human hazard assessment of atherosclerotic effects. In this case, mice would be a more accurate model animal. Like mice, humans also express SAA in lung tissue (Calero et al. 2014). Particle-induced acute phase response in terms of increased SAA and CRP levels in blood was recently shown in human volunteers following inhalation of ZnO nanoparticles (Monse et al. 2018).

Intratracheal instillation versus inhalation

Inhalation studies are the gold standard of toxicity testing, as this exposure route is the closest surrogate to human exposure. For practical reasons, pulmonary deposition by intratracheal instillation is widely used in screening studies (Bourdon et al. 2012b;Husain et al. 2013;Poulsen et al. 2015b;Saber et al. 2012b;Saber et al. 2012a) and has been proposed as an alternative to inhalation exposure. This exposure method ensures that the same dose is delivered to the lung for all nanomaterial exposures, demands less material and is more user-friendly. Intratracheal installation has previously been shown to give widespread distribution of particles throughout the lung (Mikkelsen et al. 2011), also for MWCNT (Poulsen et al. 2016).

A number of studies have compared the toxicological response following inhalation and instillation of nanomaterials. Two studies have compared the global transcriptional profiles as a means to investigate the pulmonary biological response after inhalation compared to instilled or aspirated nanomaterials. Inhalation and intratracheal instillation of a surface modified TiO_2 NP resulted in similar transcriptional changes, with the acute phase response and inflammation as the most important pulmonary responses to inhaled and instilled TiO_2 (Halappanavar et al. 2011;Husain et al. 2013). Similarly, (Kinaret et al. 2017) compared the global transcriptomic profiles of lung tissue from mice exposed to a straight and long MWCNT by inhalation or aspiration. The authors concluded that the perturbed pathways were very overlapping, suggesting that the transcriptiomic response to MWCNT exposure was very similar for inhaled and pulmonary dosed MWCNTs.

Other studies compared levels of pulmonary inflammation, measured as neutrophil influx, after exposure by inhalation or intratracheal instillation in rodents. Two studies using MWCNT reported that both methods resulted in pulmonary inflammation, with inhalation being more potent at inducing inflammation (Morimoto et al. 2012b;Porter et al. 2013). Baisch et al. reported that instillation of a high dose of TiO_2 NPs induced greater inflammation compared to low dose rate delivery through inhalation, even though the same pulmonary deposited dose were delivered. The authors concluded that intratracheal instillation is useful for quantitative ranking of NP hazards, but not for quantitative risk assessment (Baisch et al. 2014).

Selection of studies and endpoints

In the present report inhalation studies will be prioritized. For the description of toxicological endpoints and mechanism of toxicity, studies using pulmonary deposition as intratracheal instillation will be included where no quality inhalation studies are available. Risk assessments, however, are solely conducted based on inhalation studies.

Hazard endpoints were evaluated based on reported adverse effects of CNT exposure in reports and in the scientific literature. Previous assessments on CNTs have mainly focused on inflammation as critical effect. However, these evaluations were conducted prior to the pivotal, long term inhalation study in rats investigating the carcinogenic potential of the long, thick MWCNT called MWNT-7 (Kasai et al. 2016) and the IARC classification of MWNT-7 as possibly carcinogenic (2B) (Grosse et al. 2014). This report will therefore include both endpoints. In addition, cancer and cardiovascular disease have been identified as two of the main mortality causing diseases in the world, with a combined estimate of approximately 26 million annual deaths worldwide (World Health Organization 2018;Cancer Risks UK 2018). Both diseases are potentially initiated by inflammation, as described in *Mechanism of toxicity*. In conclusion, the critical endpoints were chosen based on literature review, mechanistic understanding and general importance in regards to worldwide mortality rates.

Pulmonary inflammation

Pulmonary inflammation, and inflammatory-related changes, is the most commonly reported adverse effect of CNT exposure. However, the inflammogenic potential of CNTs varies and is largely dependent on deposited dose and their physico-chemical properties. The influx of neutrophilic cells into the BAL fluid is a commonly used and reliable marker of pulmonary inflammation (Ma-Hock et al. 2009;Morimoto et al. 2012a;Erdely et al. 2009). Studies have shown that neutrophil influx correlates with increases in both pro-inflammatory cytokine and acute phase response mRNA, and protein levels (Bourdon et al. 2012b;Bourdon et al. 2012a;Bourdon et al. 2013;Husain et al. 2013;Jackson et al. 2013;Poulsen et al. 2013;Poulsen et al. 2015b). In this report, the current working group therefore chose pulmonary neutrophil influx in BAL fluid as the marker of pulmonary inflammation.

Due to the large number of studies reporting pulmonary inflammation after exposure to CNTs, the current working group chose to highlight quality chronic and sub-chronic inhalation studies (Table 2).

Chronic inhalation studies

One inhalation study was identified (Kasai et al. 2016). Here, rats were exposed to MWCNTs MWNT-7 6h/day, 5 days/week for 2 years (D: 92.9-98.2 nm, L: 5.8-5.9 μm). Statistically significantly increased neutrophil influx was observed at 2 mg/m^3, but not at 0.2 or 0.02 mg/m^3 (Kasai et al. 2016). LOAEC for MWNT-7 in this study was therefore 2 mg/m^3 and NOAEC 0.2 mg/m^3. The LOAEC and NOAEC levels for neutrophil influx were lower in this chronic study, compared to the levels in the sub-chronic study of 5 mg/m^3 and 1 mg/m^3, respectively, from the same authors (Kasai et al. 2015). This indicates that the severity of pulmonary inflammation increases with exposure time and thereby with increasing deposited dose.

Sub-chronic inhalation studies

Four different 13-week inhalation studies performed according to the OECD guidelines have been published using 4 different MWCNTs (Ma-Hock et al. 2009;Pauluhn 2010b;Pothmann et al. 2015;Kasai et al. 2015). In all studies, rats were exposed 6h/day, 5 days/week for 13 weeks (Table 2).

Ma-Hock and colleagues exposed Wistar rats to aerosols of a short and thin MWCNT type (D: 5-15 nm, L: 0.1-10 μm) at doses 0.1, 0.5, 2.5 mg/m^3 (Ma-Hock et al. 2009). The authors reported no histopathological changes in any organ, except the lung, after the 13 weeks of exposure. Lung findings were dose-dependent and included increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis at doses at 0.5 and 2.5 mg/m^3. The investigators did not observe pulmonary fibrotic effects in the exposed rats. At the lowest exposure level, 0.1 mg/m^3, the authors reported minimal granulomatous-type inflammation in the lungs and lung-associated lymph nodes, which were considered sub-clinical and unlikely to be associated with functional effects. Based on the observations reported by the authors, a NOAEC of 0.1 mg/m^3 and LOAEC of 0.5 mg/m^3 were established for this MWCNT type.

In the second sub-chronic inhalation study, Pauluhn exposed Wistar rats in nose-only chambers to a short and thin MWCNT type (D: 10 nm, L: 0.2-0.3 μm) at doses 0.1, 0.4, 1.5, and 6 mg/m^3 (Pauluhn 2010b). Pulmonary effects were examined up to 6 months post exposure. Sustained pulmonary inflammation, in terms of increased neutrophil influx in BAL fluid, was observed at doses 0.4 mg/m^3 and up. At 0.4 mg/m^3 and above, exposure-related lesions in the upper and the lower respiratory tracts were revealed by histopathology. Focally increased interstitial collagen staining, indicative of fibrosis, was observed at doses 1.5, and 6 mg/m^3, with borderline effects at dose 0.4 mg/m^3. All endpoints increased in intensity from exposure weeks 8 to 13, followed by a time-dependent decrease in severity for all exposure groups. Based on the observations of the study, a NOAEC of 0.1 mg/m^3 and LOAEC of 0.4 mg/m^3 were established for this MWCNT type.

Pothmann and colleagues exposed Wistar rats to a short and thin MWCNT type (D: 12.1 nm, L: 1.07 ± 1.1 μm) by nose-only exposure at doses 0.05, 0.25 and 5.0 mg/m^3 (Pothmann et al. 2015). The animals were euthanized 1 or 90 days after the last exposure. Significantly increased neutrophil levels were observed after exposure to the highest dose (5.0 mg/m^3) immediately after last exposure and at doses 0.25 and 5.0 mg/m^3 90 days after exposure. These increases were accompanied by increased levels of cytokines IL-1β, IL-5, TNF-α, and IL-1 α. Histopathological examination revealed focal/multifocal collagen depositions after exposure to 5.0 mg/m^3. Based on the observations of the study, a NOAEC of 0.05 mg/m^3 and LOAEC of 0.25 mg/m^3 were established for this MWCNT type.

In the last sub-chronic inhalation study, Kasai and colleagues exposed F344 rats by whole-body inhalation to a long and thick MWCNT type (MWNT-7)(D: 94.1-98.0 nm, L: 5.53-6.19 μm) at doses 0, 0.2, 1 and 5 mg/m^3 (Kasai et al. 2015). In contrast to the previous sub-chronic inhalation studies, the animals were exposed to aerosolized single fibers, instead of agglomerates. The authors reported increased neutrophil influx in BAL fluid at dose 1 mg/m^3 and granulomatous changes in the lung at dose 0.2 mg/m^3 and up. Histopathological examination revealed focal fibrosis of the alveolar wall at dose 1 mg/m^3 and up. Based on the observations of this study, a NOAEC of 0.2 mg/m^3 and LOAEC of 1 mg/m^3 for inflammation were established for this MWCNT type. However, lactate dehydrogenase and alkaline phosphatase activities, and total protein levels were all increased after exposure to 0.2 mg/m^3 MWCNTs.

Three of the four MWCNTs were thin (5-12 nm in diameter) and were aerosolized as dense agglomerates. The BET surface area of these MWCNTs was approximately 10 times larger than the BET surface area of the fourth MWCNT, MWNT-7. The observed NOAEC based on neutrophil influx was 0.05-0.1 mg/m^3 for the thin MWCNTs and 1 mg/m^3 for the thick MWNT-7, and thus appeared proportional to the BET.

Conclusion

In general, the chronic and sub-chronic inhalation studies identified NOAECs ranging from 0.05 mg/m^3 to 1 mg/m^3 and LOAECs ranging from 0.25 mg/m^3 to 5 mg/m^3 (Table 2). As dose-dependency was identified for inflammation and as it was possible to detect a NOAEC, inflammation is considered a threshold effect.

Table 2. Overview of sub-chronic and chronic inhalation studies in rats with inflammation as an endpoint

	Sub-chronic studies				**Chronic study**
	Ma-Hock et al. 2009	**Pauluhn 2010b**	**Pothmann et al. 2015**	**Kasai et al. 2015**	**Kasai et al. 2016**
Rat strain	Male and female Wistar rats (N=10/group)	Male and female Wistar rats (N=6-10 per group)	Male and female rats, RccHan©: WIST(SPF) (N=10/group)	Male and female F344 rats (N=10/group)	Male and female F344/DuCrlCrlj rats (N=50/group)
MWCNT	Nanocyl NC 7000	Baytubes	Graphistrength© C100 (NM-402)	MWNT-7	MWNT-7
MWCNT Dimensions	D: 5-15 nm L: 0.1-10 µm BET: 250-300 m^2/g Aerosolized as respirable agglomerates	D: 10 nm L: 0.2-0.3 µm BET: 253 m^2/g Aerosolized as respirable agglomerates	D: 12.1 nm L: 1.07 ± 1.1 µm BET: 225.6 m^2/g Aerosolized as respirable agglomerates	D: 94.1-98.0 nm L: 5.53-6.19 µm BET: 24-28 m^2/g Aerosolized as single fibers	D: 92.9-98.2 nm L: 5.8-5.9 µm BET: 24-28 m^2/g Aerosolized as single fibers
Air concentrations (mg/m^3)	0.1, 0.5, 2.5 mg/m^3	0.1, 0.4, 1.5, 6 mg/m^3	0.05, 0.25 and 5.0 mg/m^3	0.2, 1, 5 mg/m^3	0, 0.02, 0.2, and 2 mg/m^3
Exposure setup	6h/day, 5 days/week for 13 weeks	6h/day, 5 days/week for 13 weeks	6h/day, 5 days/week for 13 weeks	6h/day, 5 days/week for 13 weeks	6h/day, 5 days/week for 104 weeks
Post exposure time points	At the end of exposure	1 day, 28 days, 13 w, 26 w	1 and 90 days	After overnight fastening	At the end of exposure
NOAEC neutrophil influx 1 day post-exposure	0.1 mg/m^3	0.1 mg/m^3	0.05 mg/m^3	1 mg/m^3	0.2 mg/m^3
LOAEC neutrophil influx 1 day post-exposure	0.5 mg/m^3	0.4 mg/m^3	0.25 mg/m^3	5 mg/m^3	2 mg/m^3
NOAEC fibrosis 1 day post-exposure	No fibrosis reported	0.1 mg/m^3	0.25 mg/m^3	0.2 mg/m^3 based on LDH, ALP and total protein in BALF	0.02 mg/m^3
LOAEC fibrosis 1 day post-exposure	No fibrosis reported	0.4 mg/m^3 (females not assessed)	5 mg/m^3	1 mg/m^3	0.2 mg/m^3

Genotoxicity and cancer

Genotoxicity and cancer are well studied, possible adverse effects of exposure to CNTs. Genotoxicity often occurs relative rapidly after exposure, whereas cancer is a more complex pathological endpoint that requires longer time to develop. In this report the current working group therefore chose to differentiate between genotoxicity in shorter-term studies and cancer in long-term studies.

Genotoxicity

The genotoxic potential, and thus the ability to induce cancerous changes, is likely depended on the physico-chemical properties of the CNTs. Indeed, studies have highlighted differences in CNTs' ability to induce genotoxicity. Pulmonary exposure by intratracheal instillation to 10 different MWCNTs (diameters 13-32 nm) in mice (doses: 6-54 μg) increased DNA strand break levels in both BAL fluid and lung tissue for some of the MWCNTs (Poulsen et al. 2016). No dose-response was observed. Using linear regression analysis with independent physico-chemical parameters, the authors identified diameter thickness as a possible predictor for DNA strand break levels in BAL cells and lung tissue, even for these relatively thin MWCNTs. In concordance with this, another study reported increased DNA strand breaks following pulmonary dosing of the long and straight MWCNT MWNT-7 (1–200 μg/mouse by pharyngeal aspiration and 8.2–10.8 mg/m^3 for 4 days, 4 h/day by inhalation), but not a thin and entangled CNT (diameter 8-15 nm) (Catalan et al. 2016). This could indicate that needle-like CNTs are more genotoxic than thinner and often more entangled CNTs.

However, pulmonary exposure by intratracheal instillation of straight, thick and long MWCNTs (D: 67 nm, L: 4 μm), and thin and short MWCNTs (D: 10 nm, L: 1.5 μm) in mice both increased DNA strand break levels in lung tissue across doses (18-162 μg) and time points (1-28 days) (Poulsen et al. 2015b). Similarly, two sub-chronic inhalation studies in rats using thin CNTs (D: 10-15 nm and 12.1 nm, respectively) at comparable doses reported opposing genotoxic potential. The first study showed increased levels of DNA strand breaks in lung tissue from male and female rats following 28 days inhalation (0.17-0.96 mg/m^3, 6 h/day, 5 days per week) (Kim et al. 2014), whereas the other reported no effect on DNA strand break levels, oxidative DNA damage or micronuclei formation after 90 days inhalation (0.05-5.0 mg/m^3, 6 h/day, 5 days per week) (Pothmann et al. 2015). This indicates that other factors than diameter thickness play a part in CNT-induced genotoxicity.

Cancer

The MWCNT MWNT-7 (D: 92.9-98.2 nm, L: 5.8-5.9 μm) was reported to cause peritoneal mesotheliomas up to one year after non-pulmonary deposition in two studies (3-300 μg in p53 heterozygous mice, or 0.5 or 5 mg MWCNT in rats twice with a 1-week interval, respectively) (Takagi et al. 2012;Nagai et al. 2011). The same MWCNT also promoted bronchioloalveolar adenoma and carcinoma in male mice after inhalation (5 mg/m^3, 5 h/day, 5 days/week, 3 weeks) (Sargent et al. 2014). Based on these studies IARC classified MWNT-7 as possibly carcinogenic (2B) (Grosse et al. 2014). In contrast, intraperitonal injection of 2 or 20 mg short and thin (11 nm diameter, length ca 0.7 um) with or without

structural defects in rats did not induce abdominal tumors up to 2 years after exposure, whereas the positive control crocidolite asbestos (2 mg) did (Muller et al. 2009).

After publication of the IARC evaluation, more studies investigating the carcinogenic potential of CNTs were published. (Rittinghausen et al. 2014) showed that IP injection of 4 different long (>7.9 um) and relatively thick (diameter: 37-85 um) MWCNTs at dose levels of 1 and $5x10^9$ fibers induced malignant mesotheliomas in a dose-dependent manner for each of the studied MWCNTs. As the MWCNTs were dosed as 1 or $5x10^9$ fibers, the dosed mass of the MWCNTs differed significantly between the 4 MWCNTs.

(Suzui et al. 2016) exposed rats to 1 mg NIKKISO MWCNTs by trans-tracheal intrapulmonary spraying into the lung. MWCNTs were fractionized by size by filtering through a 25 μm pore size sieve. The mean lengths of the MWCNT fractions were: Unfiltered: 4.2 +/- 2.9 μm, flow-through: 2.6 +/- 1.6 μm, and retained > 2.6 μm. The physico-chemical properties of NIKKISO were very similar to MWNT-7, except that the flow-through fraction was shorter. The rats were exposed to the MWCNTs fractions 8 times during a 2 week period with a total dose of 1 mg/rat. The groups consisted of 12-15 rats and they were followed for 109 weeks post-exposure. Lung tumors were observed in the combined three groups (37%) with no statistically significant differences between groups. Mesotheliomas were statistically significantly increased in the combined group, and were observed in the unfiltered and flow-through fractions although not statistically significant. No mesotheliomas were observed in the retained fraction. NIKKISO were carcinogenic even though the fibers were relatively short compared to MWNT-7, and no difference in lung cancer incidence was observed across the different fractions. This indicates that the length of MWCNTs is of less importance for their ability to promote cancer.

A pivotal 2 year inhalation study investigated pulmonary pathological changes after exposure to MWCNTs MWNT-7. F344 male and female rats (N=50 per exposure group) were exposed to MWNT-7 for 6 h/day, 5 days/week for 104 weeks at concentrations of 0, 0.02, 0.2, and 2 mg/m^3 (Kasai et al. 2016). Scanning electron microscope of MWNT-7 demonstrated that most MWCNTs were aerosolized as single straight fibers with a mean length of 5.8-5.9 μm.
The incidences of bronchiolo-alveolar carcinomas, total carcinomas (bronchiolo-alveolar carcinomas, adenosquamous carcinoma, adenocarcinoma and squamous cell carcinoma), and total carcinomas and/or adenomas were significantly increased in males exposed to 0.2 and 2 mg/m^3 MWNT-7 and females exposed to 2 mg/m^3 MWNT-7 compared with their respective control groups (Table 3)(Kasai et al. 2016). The incidence of malignant mesothelioma was not increased.

Table 3. Total incidence of adenoma and/or carcinoma in the lungs of rats in the two year inhalation study by (Kasai et al. 2016).

MWNT-7 concentration	0 mg/m^3	0.02 mg/m^3	0.2 mg/m^3	2 mg/m^3
Female rats	3/50	2/50	4/50	11/50*
Male Rats	2/50	2/50	13/50**	16/50**

*) $p<0.05$ **) $p<0.01$ by Fischer's exact test

The total deposited MWCNT dose was calculated based on the number of fibers observed and was concentration dependent. At 2 mg/m^3, deposited dose was approximately 1.8 mg/lung and 1.2 mg/lung for male and female rats, respectively, and at 0.2 mg/m^3 it was approximately 0.15 and 0.12 mg/lung for male and female rats, respectively. The number of fibers per gram body weight that induced lung carcinoma was calculated to be 3.92×10^6 MWNT-7 fibers/gram body weight in males and 42.5×10^6 MWNT-7 fibers/gram body weight in females (Kasai et al. 2016).

In addition to the cancerous effects, exposure to MWNT-7 MWCNTs also induced increased lung weight, pulmonary hyperplasia, granuloma formation and focal fibrosis, and increased levels of total protein, lactate dehydrogenase, and alkaline phosphatase in the BAL fluid at doses 0.2, and 2 mg/m^3 (Kasai et al. 2016). The dose of 2 mg/m^3 also induced focal fibrosis in the pleura.

Conclusion

One specific thick and long carbon nanotube, MWNT-7/Mitsui-7/XNRI-7, has been classified as possibly carcinogenic by IARC. After publication of the IARC evaluation, the same MWCNT has been shown to induce lung adenomas and carcinomas by inhalation in male (at 0.2 and 2 mg/m^3) and female rats (at 2 mg/m^3). Pulmonary dosing of 1 mg/rat NIKKISO MWCNTs, which were shorter than MWNT-7, but otherwise with similar physico-chemical properties, caused lung cancer. Other thick (>37 nm) and long (>7.9 μm) have been shown to cause cancer following IP injection, whereas IP injection of high doses (2 and 20 mg) of a thin (diameter 11 nm) and short (0.7 um) MWCNT did not cause cancer in a 2-year study in rats. It is therefore very probable that other thick and straight MWCNTs are equally carcinogenic by inhalation. No firm conclusions can be reached regarding length because of the large heterogenicity of the tested samples.

Dose-response relationships have been identified for MWCNT-induced carcinogenic effects in several independent studies (Kasai et al. 2016;Rittinghausen et al. 2014;Takagi et al. 2012). However, in the study by (Rittinghausen et al. 2014) several different MWCNTs were compared, and the carcinogenic potential could not be explained by mass or by the number of CNT fibers. In contrast to the cancer studies, no consistent dose-response relationship has been observed for CNT-induced DNA strand breaks in the comet assay (Poulsen et al. 2016;Catalan et al. 2016;Poulsen et al. 2015b). Due to the lack of dose-response relationship and the severity of cancer as a disease, the current working group has therefore decided to consider genotoxicity and cancer as non-threshold effects, as this is the most conservative approach.

Cardiovascular effects

The term cardiovascular effects cover pathological changes in the entire cardiovascular system. Atherosclerosis is a central cardiovascular disease, which is manifested as increased plaque deposition or build-up in the arteries. In the later stages this can lead to various other cardiovascular diseases, including coronary artery disease and stroke. For CNT-induced cardiovascular effects, the present working group will focus on studies using intratracheal instillation as pulmonary exposure route, as these adverse effects primarily have been investigated in such studies. Differentiation between studies reporting primary changes, such as accelerated plaque progression, and studies reporting changes related to/or leading to cardiovascular effects, such as the acute phase response, will be conducted.

Accelerated plaque progression

The lipid profile of mice differs significantly from that of humans. In mice, rapid clearance of hepatic low-density lipoproteins (LDL) results in low and rather stabile total serum cholesterol levels, even after increased cholesterol intake and synthesis. Atherosclerotic changes are therefore mainly investigated in ApoE -/- mice, which are deficient in apolipoprotein E (apoE), a glycoprotein associated with all lipoproteins except LDL. ApoE -/- mice develop spontaneous atherosclerosis as early as 3–4 months of age when fed normal chow (Nakashima et al. 1994). This makes them suitable models for investigating cardiovascular effects.

Several studies have reported CNT-induced accelerated plaque progression. Intrapharyngeal instillation of 20 μg SWCNTs every other week for 8 weeks (total dose: 80 μg) in ApoE -/- mice fed either regular chow or high fat chow, revealed that SWCNT induced accelerated plaque formation in mice fed on high fat chow (Li et al. 2007) Similar increase was also observed in ApoE -/- mice exposed to SWCNT or DWCNT once weekly for 10 weeks to 10 or 40 μg by pharyngeal aspiration (total dose 100 and 400 μg/mouse)(Suzuki et al. 2016). The authors reported accelerated plaque formation at the high dose of 10 times 40 μg for both SWCNT and DWCNT. Two different short and thin (D: 10-11 nm, L: 1.5 μm) MWCNT (NM-400 and NM-402) also induced accelerated plaque formation in female ApoE -/- mice fed western diet after intratracheal instillation once a week for 5 weeks to 25.6 μg/mouse (total dose 128 μg)(Cao et al. 2014).

In contrast, exposure to 4 or 40 μg of the thick and long MWCNT MWNT-7 (L: 3.86-5.7 μm, D: 49–74 nm) once a week for 10 weeks (total dose 40 or 400 μg) by intratracheal instillation did not affect plaque formation in female ApoE -/- mice fed western diet (Christophersen et al. 2016). The results indicate that accelerated plaque formation is observed following pulmonary exposure to thin (SWCNT, DWCNT, NM-400 and NM-402) but not thick (MWNT-7) MWCNT.

Acute phase response

The acute phase response is induced in humans in response to infection, infarction and trauma, and it is defined by increases in acute phase response proteins with the most predominant being C-reactive protein (CRP), Serum amyloid A (SAA), and fibrinogen. During an acute phase response these proteins can increase thousand fold (Gabay and

Kushner 1999). Elevated plasma levels of CRP and SAA have been reported as a risk factor for CVD in humans (Johnson et al. 2004;Lowe 2001;Lowe 2001;Mezaki et al. 2003;Ridker et al. 2000). In mice, the SAA isoforms are the main acute phase response proteins, while CRP is only moderately induced by inflammatory stimuli (Whitehead et al. 1990;Pepys and Hirschfield 2003). SAA (SAA1-4) is a highly conserved family of apolipoproteins associated with high density lipoproteins (HDL).

Several studies have reported changes in *Saa* expression or increased SAA protein levels after pulmonary exposure to CNTs. Intratracheal instillation of both the thick MWCNT MWNT-7 (D: 40–50 nm, L: 1– 4 μm) and two SWCNTs (D: 0.8–1.7nm, L: <1 μm) in female C57BL/6 mice strongly increased *Saa3* mRNA levels in lung tissue (Saber et al. 2013). The authors also observed elevated SAA3 protein levels in broncheoalveolar lavage fluid and plasma after exposure to MWNT-7, whereas no changes in hepatic *Saa3* levels were reported. Global gene expression analysis of pulmonary changes after exposure to a small (L: 847 ±102 nm, D: 11 (6–17) nm) and a large MWCNT (L: 4048 ± 366 nm, D: 67 (24–138) nm) revealed that all *Saa* isoforms were among the most differentially regulated genes 1, 3 and 28 days post-exposure (Poulsen et al. 2015b). Interestingly, both MWCNT types also induced dose- and time-dependent increases in plasma protein SAA3 levels. Increased SAA3 plasma protein levels have been reported in male mice exposed to a pristine MWCNT (L: 15 ±5.0 μm and 13.5 ±1.50 nm) up to 1 year after intratracheal exposure (Kim et al. 2015). This highlights the impact of pulmonary exposure to CNTs on long-term vascular changes.

Changes in plasma SAA1/2 and SAA3 protein levels were investigated 1, 28 and 90 days after pulmonary exposure to 14 commercial, well-characterized MWCNTs in female C57BL/6J mice (Poulsen et al. 2017). In general, the majority of the MWCNTs induced increases in both SAA1/2 and SAA3 plasma protein levels. No correlation was observed between SAA1/2 and SAA3 plasma protein levels, which indicated that the changes are controlled by different mechanisms. However, a very close correlation between neutrophil influx and *Saa3* mRNA levels in lung tissue and SAA3 protein levels in blood in mice have previously been reported (Poulsen et al. 2016;Saber et al. 2014), linking inflammation and cardiovascular effects.

Conclusion

CNTs may promote atherosclerosis directly, by inducing accelerated plaque progression, or indirectly, by inducing increased blood levels of acute phase response proteins. Dose-response relationships have only sparsely been reported for CNT-induced increased plaque progression, in part due to the low dynamic range of the assay, but also due to study designs. In contrast, dose-response relationships have been established between CNT exposure and increased levels of acute phase response proteins. However, the studies reporting CNT-induced atherosclerotic effects have solely used pulmonary deposition as exposure method, and thus, the studies cannot be used to establish OELs. Due to the close interplay between inflammation, acute phase response and plaque progression, inflammation as neutrophil influx can be used as a proxy for the acute phase response. The current working group therefore considers cardiovascular effects as a threshold effect that is regulated in parallel to inflammation.

Reprotoxicity

A recent review summarized several studies of the potential for reproductive toxicity of CNTs. Very few studies used the airway route of exposure (Ema et al. 2016). The review concluded that there is potential for placental transfer of single- and multi-walled CNTs, as reported in mouse studies following intravenous injection. Both SWCNTs and MWCNTs were toxic to mouse fetuses when administered to the pregnant dam by injection (intraperitoneal as well as intravenous). Oral gavage of MWCNTs induced no developmental toxicity in mice and rats. In the following, only studies using airway exposure to carbon nanotubes are reviewed. Sexually mature female mice received one intratracheal instillation of 67 μg/mouse (~3.3 mg/kg) of MWCNTs (NM-400), one day prior to cohabitation with a mature male. Pathological changes in lungs and liver were observed in dams 6 weeks after instillation of the MWCNTs. The delivery of the first litter was delayed by an average of 5 days in dams instilled with MWCNTs. No effects were observed in gestation and litter parameters, offspring behavior in the open field or startle test, or daily sperm production in the male offspring (DSP) (Hougaard et al. 2013).

To further characterize the delay in delivery of the first litter, estrous cycle regularity was investigated by comparing vaginal smears before and after a single intratracheal instillation of 67 μg of NM-400 MWCNT or vehicle. Reproductive function was analyzed by measuring time to delivery of litters after instillation of 2, 18 or 67 μg of NM-400. Exposure to MWCNT significantly prolonged the estrous cycle during which MWCNT exposure took place compared to the pre-exposure estrous cycles. In contrast was the estrous cycle immediately after the exposed cycle significantly shortened in MWCNT exposed females. No consistent effects were seen on time to delivery of litter or other gestational parameters. Litter parameters, such as litter size, sex ratio, implantations, and implantation loss were not affected by exposure (Johansson et al. 2017).

MWCNTs suspended in 2% carboxymethyl cellulose were intratracheally instilled in pregnant mice at 3, 4, or 5 mg/kg (Fujitani et al. 2012). At 4 and 5 mg/kg, exposed females presented with reduced weight gain and significant increases in leucocyte counts in peripheral blood. Fetal growth was significantly inhibited at 5 mg/kg. Data analysis showed statistically significant increases in the number of litters with malformed fetuses, at 4 and 5 mg/kg, owing mainly to increased incidences of fetuses with fusion of vertebral bodies and arches. It should be noted that the vehicle used is unusual, and that compared to control animals, the exposed females presented with 25 and 45% increases in lung weight at 4 and 5 mg/kg, respectively. The finding warrants further investigation, preferably exposing the pregnant animals by inhalation.

Conclusion

The findings in these studies indicate that CNTs may interfere with reproduction. The applied route of instillation is intratracheal instillation in all three studies, and they can therefore not be used as basis for derivation of occupational limit values.

MECHANISMS OF TOXICITY

Pulmonary inflammation

Following pulmonary exposure, CNTs interact with the cellular membranes of resident cells and proteins in the lung environment. When sensing CNTs through scavenger receptors and/or toll-like receptors, macrophages and epithelial cells start secreting pro-inflammatory cytokines. Alternatively, macrophages can initiate the inflammatory response after sensing damage recognition-associated molecular patterns released from damaged epithelial cells interacting with CNTs.

The surface area of the deposited CNTs is of great important for their level of interaction with the resident pulmonary cell and thus their inflammogenic potential. In a study investigating neutrophil influx after intratracheal instillation to 10 different MWCNTs at three different time points (1, 28 and 92 days post-exposure), 83% of the observed variation in neutrophil influx was predicted by BET surface area of the delivered dose (r^2=0.69)(Poulsen et al. 2016). In multiple linear regression analysis with independent physico-chemical properties, dose and BET surface area were the most consistent and significant predictors of inflammation in terms of neutrophil influx)(Poulsen et al. 2016). In agreement with this, particle-induced pulmonary inflammation was similarly predicted by deposited surface area of low-toxicity-low solubility particles (Duffin et al. 2007;Schmid and Cassee 2017).

Identification of physico-chemical properties important for MWCNT-induced inflammation, also revealed that MWCNT length and oxygen content (interpreted as surface hydroxylation and carboxylation) affected the inflammatory potential of MWCNTs (Poulsen et al. 2016). Length has previously been proposed as a determinant of CNT-induced inflammation, albeit the strongest cases have been made after intraperitoneal or pleural deposition (Murphy et al. 2011;Poland et al. 2008;Yamashita et al. 2010). CNTs of a certain length (above 10 μm) can comply with the fiber paradigm (Donaldson et al. 2011) resulting in frustrated phagocytosis due to the inability of macrophages to completely engulf the long CNTs. This leads to lysosomal instability, activation of the NALP inflammasome, and chronic stimulation of the cell, which results in the macrophage releasing a range of pro-inflammatory molecules (Donaldson et al. 2011). Length could also be important for inflammogenicity of the vast types of CNTs with lengths lesser than 10 μm. Kobler et al. reported that a thick, fiber-like MWCNT appeared to escape vesicle enclosure more often compared to a smaller, entangled MWCNT, which could lead to increased intracellular damage (Kobler et al. 2015). However, the same two types of MWCNT induced comparable levels of neutrophil influx up to 28 days after exposure by intratracheal instillation (Poulsen et al. 2015b). In conclusion, the importance of length for CNT-induced inflammation is therefore largely uncertain for CNTs with lengths below 10 μm.

A higher surface density of oxidized groups on the CNTs could increase their hydrophilicity and dispersion in the lung. Supporting this, increased MWCNT oxygen content (surface hydroxylation and carboxylation) was identified as protective of inflammation 28 days after exposure to 10 different MWCNTs (Poulsen et al. 2016).

Similar lowered or eliminated toxicity has previously been reported in the literature (Jain et al. 2011;Sager et al. 2014). Hydrophilic CNTs may be cleared faster from the lung than hydrophobic CNTs, as they more easily disperse in the lung environment. In addition, the acidic treatment used to introduce the oxidized groups often introduces structural defects in the CNTs. This renders the CNT more susceptible for enzymatic and oxidative breakage, resulting in greater bio-degradability and thus more effective pulmonary clearance (Liu et al. 2010).

In conclusion, CNTs are in general inflammogenic upon pulmonary exposure and their surface area has been identified as important for the potency of the inflammatory response. Inflammation is considered a threshold effect.

Genotoxicity and cancer

CNT-induced genotoxicity may occur by several mechanisms. These are briefly described.

Surface-dependent ROS generation
Carbon-based nanomaterials such as carbon black are efficient generators of radicals *in vitro* and in acellular assays (Jacobsen et al. 2008). SWCNT and MWCNTs also generate ROS in acellular assays (Jackson et al. 2015;Jacobsen et al. 2008). ROS may result in oxidation of the DNA, which can result in several types of DNA damage, including base modifications, abasic sites, single-strand breaks, protein-DNA adducts, and DNA crosslinks (Waris and Ahsan 2006). SWCNT-induced ROS is linked to generation of fpg-sensitive sites *in vitro*, but did not affect the mutation frequency in the FE1 cell line (Jacobsen et al. 2008).

Fiber-related DNA damage
Poulsen et al. (Poulsen et al. 2016) searched for physico-chemical predictors of DNA strand break levels. Increasing MWCNT diameter was the most consistent and significant predictor of DNA strand break levels in the comet assay. This supports a mechanism related to the fibrous structure of thick MWCNTs. Similar to this, (Catalan et al. 2016) compared genotoxicity of one entangled MWCNT and one straight MWCNT (MWNT-7), and found that the straight MWCNT in contrast to the entangled MWCNT induced DNA stand breaks in bronchoalveolar lavage cells and in lung tissue. The straight MWCNT also induced micronuclei in alveolar type II cells, whereas this was not assessed for the entangled MWCNT. The authors concluded that both primary and secondary mechanisms may be involved in the observed genotoxicity of the straight MWCNT.

The fiber paradigm as mechanism of CNT-induced carcinogenicity
Long CNTs have been proposed to induce gentoxicity according to the fiber paradigm (Donaldson et al. 2011). Thus, macrophage interaction with long and straight MWCNTs results in frustrated phagocytosis due to the inability of macrophages to completely engulf the long CNTs. The slow clearance of insoluble MWCNT and frustrated phagocytosis are the essential elements of the fiber paradigm (Donaldson et al. 2011).

In conclusion, the mechanism of CNT-induced cancer has not been firmly established yet. With respect to DNA damage there is no evidence indicating for a threshold mode of action for CNTs. The current working group therefore assumes a non-threshold mode of action for CNT-induced carcinogenicity.

Cardiovascular effects

CNT exposure can lead to cardiovascular effects either: 1. Directly, by translocation of CNTs from the lung to the vascular system. 2. Indirectly, as a consequence of pulmonary inflammation. 3. Alterations in autonomic nervous system activity to elicit peripheral effects.

Atherosclerosis is a central cardiovascular effect, which is manifested as increased plaque deposition or build-up in the arteries. It is initiated by a biological, chemical or physical insult to the artery walls. Translocated CNTs could induce this insult by interacting directly with the endothelium. This leads to the expression of cell adhesion molecules (selectins, VCAM-1 and ICAM-1) on the endothelial lining of the arteries, which facilitates the activation, recruitment and migration of monocytes through the endothelial monolayer (Hansson and Libby 2006;Cybulsky et al. 2001). Inside the intima layer, the monocytes differentiate into macrophages and internalize fatty deposits (mainly oxidized low density lipoprotein), transforming them into foam cells, which is a major component of the atherosclerotic fatty streaks. The fatty streaks reduce the elasticity of the artery walls and the foam cells promote a pro-inflammatory environment by secretion of cytokines and ROS. In addition, foam cells also induce the recruitment of smooth muscle cells to the intima. Added together, these changes lead to the formation of plaques on the artery walls. A fibrous cap of collagen and vascular smooth muscle cells protects the necrotic core and stabilizes the plaque (Libby 2002;Virmani et al. 2005). Although initially asymptomatic, narrowing of the blood vessels can lead to other cardiovascular diseases, such as coronary artery disease or stroke. In addition, blood clots can be formed if the plaque ruptures. These may travel with the bloodstream and obstruct the blood flow of smaller vessels.

Pulmonary exposure to CNTs may also promote accelerated atherosclerosis indirectly through an induced pulmonary acute phase response. Introduction of CNTs to the lung promotes neutrophil influx and release of proinflammatory cytokines, which leads to increased production of SAA proteins. The SAAs are hydrophobic proteins that upon secretion in their target organs are able to translocate to the blood. A statistically significant correlation between Saa3 mRNA levels in the lung and SAA3 protein levels in the blood have previously been reported (Poulsen et al. 2015a), indicating that SAA3 produced in the target organ translocate to systemic circulation. SAA circulating in the blood becomes incorporated with HDL, thereby replacing Apolipoprotein A1 (Apo-A1) as the major HDL-associated protein and forming HDL-SAA. The formation of HDL-SAA has a double effect on plaque progression: 1. HDL is a major component of reverse cholesterol transport, a multi-stepped process resulting in the movement of cholesterol through the blood from peripheral tissues (including the artery walls) to the liver. The formation of SAA-HDL impairs the HDL-mediated reverse cholesterol transport, resulting in reduced cholesterol transport and an increased systemic total cholesterol

pool (Lindhorst et al. 1997;Steinmetz et al. 1989). 2. SAA and SAA-HDL have been shown to directly stimulate the transformation of macrophages into foam cells and to stimulate uptake of oxidized LDL in the macrophages (Lee et al. 2013). In addition, SAA-HDL has a lower capacity to promote cellular cholesterol efflux from macrophages than native HDL (Artl et al. 2000).

Identification of physico-chemical properties important for changes in plasma SAA concentration indicated that increasing length predicts decreasing SAA1/2 plasma levels, whereas metal content is important for SAA3 plasma levels (Poulsen et al. 2017). The authors reported a significant correlation between plasma SAA3 levels and neutrophil influx in the lung. Interestingly, pulmonary neutrophil influx has previously been shown to correlate with deposited surface area of instilled MWCNT (Poulsen et al. 2016), which links deposited CNT surface area with increased risk of developing cardiovascular disease.

In conclusion, pulmonary exposure to CNTs can lead to accelerated plaque progression directly, through translocation, or indirectly, through an induced acute phase response. No single physico-chemical property has been identified as the driver of cardiovascular effects, but CNT surface area a likely important due to the close connection to pulmonary inflammation. As for inflammation, the current working group consider cardiovascular effects as a threshold effect. This is based on identified dose-response relationships between CNT exposure dose and induced acute phase response (Poulsen et al. 2015a;Saber et al. 2013), and the close interplay between inflammation, acute phase response and plaque progression. A very close correlation between neutrophil influx and *Saa3* mRNA levels in lung tissue and SAA3 protein levels in blood in mice were also observed (Poulsen et al. 2016;Saber et al. 2014). Based on this, the current working group suggests that inflammation in terms of neutrophil influx is used as biomarker of cardiovascular risk.

Dose-response relationships

In general, dose response relationship is observed for health effects following pulmonary exposure to CNTs.

Inflammation

Strong dose response relationships have consistently been observed for various markers of pulmonary inflammation (Pauluhn 2010b;Ma-Hock et al. 2009;Pothmann et al. 2015;Kasai et al. 2015) and acute phase response (Poulsen et al. 2017;Saber et al. 2014;Saber et al. 2013;Poulsen et al. 2015b;Poulsen et al. 2015a). The working group therefore considers inflammation as a threshold effect.

Cancer

Dose response relationship has been observed for carcinogenic effects of MWCNT in several independent studies (Kasai et al. 2016;Rittinghausen et al. 2014;Takagi et al. 2012). The dose-response relationship was observed on mass-base for the individual CNTs. However, in the study by (Rittinghausen et al. 2014) several different MWCNTs

were compared, and differences in the carcinogenic potential between the MWCNTs could not be explained by mass or by the number of CNT fibers.

Genotoxicity by comet assay

No consistent dose-response relationship has been observed for CNT-induced DNA strand breaks in the comet assay (Poulsen et al. 2016;Catalan et al. 2016;Poulsen et al. 2015b). However, the lack of dose-response relationship may reflect that the level of CNT-induced DNA strand breaks is low, leading to a low dynamic range of the comet assay for this application. The mechanisms of CNT-induced genotoxicity and carcinogenicity are not fully clarified. It is therefore not possible to conclude on a threshold mechanism and a non-threshold approach will be used as the most conservative approach.

Plaque progression

Dose-response relationships are rarely reported for CNT-induced increased plaque progression. This is in part due to the low dynamic range of the assay, but also due to the study designs, which often only include one dose. The exception is the study by Suzuki and colleagues, which reported that their high doses of SWCNT and DWCNT induced increased plaque progression after pharyngeal aspiration, whereas the low doses did not (Suzuki et al. 2016). Alternatively, the acute phase response proteins could be used as relevant biomarkers for plaque progression. Dose-response relationships are established between CNT exposure and increased levels of these proteins. The current working group considers cardiovascular effects as a threshold effect that should be regulated in parallel to inflammation.

PREVIOUS RISK ASSESSMENTS OF CARBON NANOTUBES

During the last couple of years, researchers, producers and organizations have proposed recommended exposure limits, indicative or derived no-effect-level (INEL/DNEL) and occupational exposure levels for CNTs. These have been set based on pulmonary inflammation or lung cancer identified as the most critical effects of CNT exposure. The previous recommendations of exposure limits are presented below and an overview can be found in Table 4.

- Aschberger et al. 2010, Critical Reviews in Toxicology (Aschberger et al. 2010).
- Pauluhn 2010, Regulatory Toxicology and Pharmacology (Pauluhn 2010a).
- ENRHES, Engineered Nanoparticles: Review of Health and Environmental Safety, Final report, 2009 (ENRHES 2009).
- NIOSH, 65, Occupational Exposure to Carbon Nanotubes and Nanofibers, 2013 (NIOSH 2013).
- IARC, Monograph no. 111 (Grosse et al. 2014).
- Ministry of Environment and Food of Denmark, Carbon nanotubes, Environmental project No. 1805, 2015 (Jensen et al. 2015).

Aschberger et al. 2010

As part of a comprehensive review on carbon nanotube toxicity and exposure, Aschberger and colleagues included an assessment of possible human exposure limits (Aschberger et al. 2010). INELs were derived using the methodology suggested in the REACH guidelines (chapter R.8 in ECHA, 2008), and were based on two sub-chronic inhalation studies by (Pauluhn 2010b) and (Ma-Hock et al. 2009). In these studies a NOAEC (Pauluhn 2010b) and a LOAEC (Ma-Hock et al. 2009) of 0.1 mg/m^3 were identified for pulmonary inflammation after end of exposure in rats exposed 6 hour/day, 5 days/week for 13 weeks to two different MWCNTs (Baytubes and Nanocyl, respectively). After applying default assessment factors, the authors estimated INELs of 2 μg/m^3 for Baytubes and 1 μg/m^3 for Nanocyl.

Pauluhn 2010

A study by Pauluhn from 2010 suggested an occupational exposure limit for Baytubes MWCNTs (a thin, flexible MWCNT type) at 0.05 mg/m^3 (time-weighted average) (Pauluhn 2010a). The author based his assessment on two previously published papers by the same author, investigating toxicity and agglomeration of inhaled Baytubes MWCNTs in rats (Pauluhn 2011;Pauluhn and Rosenbruch 2015). The author stated that, in the case of the Baytubes MWCNTs, overload could be the trigger of the cascade of events leading to lowered clearance and consequently increased MWCNT doses high enough to trigger sustained pulmonary inflammation. The occupational exposure limit in this study was calculated based on this mechanism. The author applied multiple interspecies adjustments, but did not follow the REACH guidelines.

ENRHES

Some of the first to establish derived-no-effect levels (DNEL) for CNTs was within the frames of the EU project Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES 2009). At the time of the report, no carcinogenicity studies were identified for CNT exposure. The derivation of a DNEL was therefore made under the assumption of a threshold driven mechanism of CNT toxicity. The calculated DNELs of 33.5 $\mu g/m^3$ for pulmonary inflammation and 0.67 $\mu g/m^3$ for systemic immune effects were derived based on a sub-acute study by (Mitchell et al. 2007). The calculations of DNELs followed the methodology suggested in the REACH guidelines.

NIOSH

In 2010, the National Institute for Occupational Safety and Health (NIOSH) proposed a recommended exposure limit (REL) of 7 $\mu g/m^3$ of CNTs in air as an eight-hour, time-weighted average, respirable mass concentration. However, this value was re-adjusted to be 1 μg carbon/m3 in April, 2013 (NIOSH 2013).This states: "The NIOSH REL is expected to reduce the risk for pulmonary inflammation and fibrosis. However, because of some residual risk at the REL and uncertainty concerning chronic health effects, including whether some types of CNTs may be carcinogenic, continued efforts should be made to reduce exposures as much as possible". The analysis conducted by NIOSH was focused on the types of CNT included in published research studies, and it primarily based is deriviation on the sub-chronic, rat inhalation studies (Ma-Hock et al. 2009;Pauluhn 2010b).

IARC

In 2014, the International Agency for Research on Carcinogenicity (IARC) classified the MWCNT MWNT-7 as possibly carcinogenic to humans (group 2B). In Denmark, substances classified as group 1, 2A and 2B by IARC are considered carcinogenic. MWCNTs other than MWNT-7 were not classifiable as to their carcinogenicity to humans (Group 3). SWCNTs were not classifiable as to their carcinogenicity to humans (Group 3). These classifications were based on: 1. Inadequate evidence in humans for the carcinogenicity of CNTs. 2. Sufficient evidence in experimental animals for the carcinogenicity of MWNT-7 MWCNTs. 3. Limited evidence in experimental animals for the carcinogenicity of two types of MWCNTs with dimensions similar to MWNT-7. 4. Inadequate evidence in experimental animals for the carcinogenicity of MWCNT other than MWNT-7. 5. Inadequate evidence in experimental animals for the carcinogenicity of SWCNTs.

Table 4. List of studies proposing occupational exposure limits based on LOAEC or NOAEC data in animal studies

Study		**Aschberger et al. 2010**		**Pauluhn 2010a**	**NIOSH 2013**	**ENRHES 2009**	
Critical effect		Pulmonary inflammation		Pulmonary inflammation	Pulmonary inflammation and fibrotic events	Pulmonary inflammation	Systemic immune effects
Duration		Acute	Sub-chronic	Sub-chronic	Sub-chronic	Sub-acute	Sub-acute
Key studies		Ellinger-Ziegelbauer and Pauluhn 2009	Pauluhn 2010b/ Ma-Hock et al. 2009	Pauluhn 2010b	Pauluhn 2010b/ Ma-Hock et al. 2009	Mitchell et al. 2007	Mitchell et al. 2007
CNT type		MWCNT, Baytubes	MWCNT, Baytubes and Nanocyl NC 7000	MWCNT, Baytubes	MWCNT, Baytubes and Nanocyl NC 7000	MWCNT, Shenzhen Nanotech	MWCNT, Shenzhen Nanotech
Risk determinant		LOAEC	NOAEC/LOAEC	NOAEC	NOAEC/LOAEC	NOAEC	LOAEC
Risk level in rodents		11 mg/m^3	0.1 mg/m^3	0.1 mg/m^3	0.1 mg/m^3	0.5 mg/m^3	0.3 mg/m^3
$NOAEC_{Corrected}$ Corrected starting point		5.5 mg/m^3 [a]	0.05 mg/m^3 [a]	0.05 mg/m^3	-	2.5 mg/m^3	0.15 mg/m^3
Uncertainty factors:	Default assessment factors	3[b]	-/2[b]	-	-	-	3 [f]
	Interspecies	2.5[c]	2.5[c]	-	-	2.5[c]	2.5[c]
	Intraspecies	5[c]	5[c]	-	-	5[c]	5[c]
	Sub-chronic or sub-acute to chronic	-	2[c]	-	-	6[e]	6[e]
	Dose-response issues: LOAEC/NOAEC extrapolation/ severity of effect	-	-	-	-	-	-
Overall uncertainty factor		37.5[d]	25[d]	-	-	75	75
Suggested OEL		150 µg/m^3	2 µg/m^3 / 1 µg/m^3	50 µg/m^3	1 µg/m^3	33.5 µg/m^3	0.67 µg/m^3

Table 4 description. a) Correction factors: 8 hour working hours and difference in respiratory volume between animals in rest and workers at light activity. Corrected L(N)OAEC$_{worker}$ for 8 working hours: (N(L)OAEC × 6 h/8 h). Correction for the difference in respiratory volume between animals at rest and workers at light activity (N(L)OAEC × 6.7 m^3/10 m^3). b) extrapolation from LOAEC to NOAEC. c) Interspecies default factor: 2.5. Intraspecies default factor: 5 for workers/10 for public. Sub-chronic to chronic default factor: 2. d) Altogether, overall assessment factors of 37.5 (3 × 2.5 × 5) for acute and of 25 (2.5 × 5 × 2) and 50 (2 × 2.5 × 5 × 2), respectively, for chronic exposure of workers. e) Sub-acute to chronic default factor: 6. f) Extrapolation from a LOAEC to a NAEC (extrapolation factor of 3).

SCIENTIFIC BASIS FOR AN OCCUPATIONAL EXPOSURE LIMIT

Different methods exist for calculating occupational exposure limits. The choice of method depends on the mode of action of the substance, and can fundamentally be split up in two approaches: Threshold effects or non-threshold effects. The threshold effect approach relies on the assumption that the organism can withstand a certain dose before adverse effects occur, whereas the non-threshold effects approach relies on the assumption that any exposure to the substance can result in adverse effects. In this report, the current working group will calculate proposed occupational exposure limits based both on threshold effects or non-threshold effects.

Calculations of exposure limits based on cancer as non-threshold effect

Carcinogenicity via genotoxic mechanisms is generally considered as a non-threshold effect. This applies for CNT-induced carcinogenicity, as no evidence for threshold effects are available for the mechanism of action for carbon nanotube-induced carcinogenicity.

Risk levels are calculated based on the 2 year inhalation study by (Kasai et al. 2016). In this study, MWNT-7 MWCNTs were used. MWNT-7 are relatively thick and long MWCNTs (D: 92.9-98.2 nm, L: 5.8-5.9 μm) and have very low levels of chemical contaminations compared to other MWCNTs. It is therefore unlikely that the observed effects are attributed to metal contaminations. The MWCNTs were aerosolized to generate single fibers.

Lung burden was measured in lung tissue. Tissue was digested and fibers were counted on SEM images of digested tissue. Time and air concentration-dependent accumulation of MWCNTs were observed during the entire 2-year study. Lung deposition was 1.5-2.7% (Kasai et al. 2016). Male rats were more sensitive compared to female rats, and thus the proposed occupational exposure limit was calculated based on cancer incidents in males. The authors reported statistically significantly increased lung cancer incidence in male rats at 0.2 mg/m^3. Both malignant and non-malignant tumors were included (Table 5).

Table 5. Cancer incidents and lung burden in male rats after exposure to different doses of MWCNT in (Kasai et al. 2016).

	0 mg/m^3	**0.02 mg/m^3**	**0.2 mg/m^3**	**2 mg/m^3**
Total cancer incidences	2/50	2/50	13/50	16/50
MWCNT lung burden (μg/lung)		10	152.4	1797.8

Method I

The current working group has chosen to use the approach used by (Kasai et al. 2016) and (Erdely et al. 2013), who use the measured lung burden in rats exposed by inhalation and the alveolar surface area of rats and humans to estimate the human equivalent lung burden:

Observed cancer incidence at 0.2 mg/m^3:

$$(13_{0.2\ mg} - 2_{control})/(50_{control} - 2_{control}) = 11/48 = 23\%$$

Lung deposited dose in male rats at 0.2 mg/m^3: 0.152 mg/lung.
The human equivalent dose is:

(Rat deposited dose) x (human alveolar surface area)/(rat alveolar surface area) =
0.152 mg x 102 m^2/0.4 m^2= 38.76 mg MWCNT per human lung.

The following standardized constants are assumed:
The standard value of human ventilation is 20 L/min during light work (1.2 m^3/h).
An average work day is 8h per day.
An average work week contains 5 days.
In Denmark, an average employee work 45 weeks per year.
An average working life is 45 years.

The pulmonary deposition rate was reported to be 1.4-2.7% in the study Kasai and colleagues (Kasai et al. 2016). However, in a 13 week inhalation study by Pauluhn, a pulmonary deposition rate of 5.7 % was reported for aerosolized agglomerated MWCNTs (Pauluhn 2010b). In order to take different kinds of MWCNTs as well as different states of agglomeration into account, the current working group use the pulmonary deposition rate of 5.7% as reported by (Pauluhn 2010b).

Using the values above, a lung burden of 38.8 mg in humans would require that workers are exposed to:

Air concentration =
38.76 mg/(8h/day x 5 day/week x 45 weeks/year x 45 years x 1.2 m^3/h x 0.057) =
0.00699 mg/m^3.

Thus, at an air concentration of 7 μg/m^3 during a 45 year work life, an excess lung cancer incidence of 23% is expected. Assuming a non-threshold linear dose-response relationship, then 1% excess lung cancer is expected at:

$$(7\ \mu g/m^3)/23 = 0.304\ \mu g/m^3$$

Table 6. Calculated excess lung cancer incidence at different MWCNT mass and fiber concentrations for 8 hour daily occupational exposure, Method I.

Excess lung cancer incidence	MWCNT Air concentration (μg/m^3)	Air concentration MWNT-7 fibers/cm^3 *
1:1,000	0.03	0.3
1: 10,000	0.003	0.03
1: 100,000	0.0003	0.003

***) The calculations of fiber concentrations were based on the following: 1 μg MWNT-7 MWCNT corresponds to 9.03 x 10^6 MWNT-7 fibers (Kasai et al. 2016).**

Only MWNT-7 has been shown to be carcinogenic by inhalation. One other type of MWCNT did not induce cancer in a 2 year study (Muller et al. 2009). However, several types of MWCNTs have been shown to be carcinogenic by other exposure routes (Rittinghausen et al. 2014) or genotoxic (Poulsen et al. 2016;Catalan et al. 2016;Poulsen et al. 2015b). Moreover, characterization of the physico-chemical properties of commercially available CNTs has shown large deviations between empirical data and data available from the supplier (Jackson et al. 2015). The present working group therefore assume that all CNTs are carcinogenic.

Method II

Calculation based on approach suggested by ECHA (ECHA 2012;SCHER/SCCP/SCENIHR 2009), calculated based on the one year MWCNT inhalation study in rats by (Kasai et al. 2016)(Table 5):

Excess cancer risk:

$$(13_{0.2\ mg} - 2_{control})/(50_{control} - 2_{control}) = 11/48 = 23\%$$

Correction of dose metric into a human dose situation (8h/d):

$$0.2\ mg/m^3 \times (6h/day)/(8h/day) \times (6.7\ m^2/10\ m^2) = 0.1\ mg/m^3 \text{ or } 100\ \mu g/m^3$$

Calculation of unit risk for cancer:

Risk level = exposure level x unit risk
0.23 = 100 μg/m^3 x unit risk
Unit risk = 2.3 x 10^{-3} per μg/m^3

At a dose of 1 μg/m^3, 2.3 x 10^{-3} excess cancers are expected.

Calculation of dose levels corresponding to risk level of 10^{-5} (and other risk levels)

10^{-5} risk level = exposure level x unit risk (2.3 x 10^{-3} per μg/m^3)
Exposure level (10^{-5}) = 4.3 x 10^{-3} μg/m^3

Table 7. Calculated excess lung cancer incidence at different MWCNT mass concentrations for 8 hour daily occupational exposure, Method II.

Excess lung cancer incidence	MWCNT Air concentration (μg/m^3)
1:1,000	0.043
1: 10,000	0.0043
1: 100,000	0.00043

Calculations of exposure limits based on inflammation as threshold effect

Several studies have reported dose-dependent inflammatory effects as well as dose-dependent acute phase response, a biomarker of risk of cardiovascular disease. Inflammation, acute phase response and cardiovascular effects are therefore considered threshold effects. However, at present only inflammogenicity has been investigated in inhalation studies of sufficient quality for risk assessment. The current working group therefore bases the risk assessment of threshold effects on the sub-chronic inhalation studies investigating inflammogenicity. The choice of using the sub-chronic studies (Ma-Hock et al. 2009;Pauluhn 2010b;Pothmann et al. 2015) , compared to the chronic one (Kasai et al. 2016) is based on the CNT type being investigated (Table 2). In the sub-chronic studies, the short, thin MWCNTs with large surface areas are used, whereas in the chronic study it is the long, thick MWCNT type with small surface area. As described in *Mechanism of toxicity*, CNT-induced inflammation has been shown to be predicted by the specific surface area. In order to take the variation specific surface area into account and as a precaution, the current working group has therefore decided to base the risk assessment on the data from the sub-chronic studies.

NOAECs were noted for the highest air concentrations, which do not induce statistically significantly increased levels of neutrophils (Table 2) (Ma-Hock et al. 2009;Pauluhn 2010a;Pauluhn 2010b;Pothmann et al. 2015). The lowest NOAEC for neutrophil influx was reported by (Pothmann et al. 2015) to be 0.05 mg/m^3. This is similar to the NOAECs reported in other studies, albeit approximately half the concentration (Table 2). As a precaution, the NOAEC of 0.05 mg/m^3 will be used for determining the proposed occupational exposure limits for threshold effects.

Calculation

The DNEL for CNT-induced pulmonary inflammation is established based on the methodology suggested in the REACH guidelines (ECHA 2012). Initially, a corrected NOAEC is made that takes into account that rats were exposed for 6 h/day, whereas humans are exposed for 8 h/day. In addition, exposed rats are at rest, whereas workers are considered to do light activity. A total breathing volume of 10 m^3 for an 8-hour shift at light activity is therefore assumed, compared to 6.7 m^3 at rest.

Corrected NOAEC:

$$0.05 \text{ mg/m}^3 \times (6\text{h/day})/(8\text{h/day}) \times (6.7 \text{ m}^2/10 \text{ m}^2) = 0.0251 \text{ mg/m}^3$$

Next, assessment factors are applied. As no valid argumentation for using alternative assessment factors could be found, the current working group uses the assessment factors as proposed by ECHA (ECHA 2012):

Extrapolation from sub-chronic to chronic exposure: 2

Neutrophil influx is considered an acute response and this would normally not warrant a duration factor from a sub-chronic study. However the accumulation of CNTs over time would continue beyond sub-chronic exposure and thus the default assessment factor of 2 to extrapolate from sub-chronic to chronic exposure were chosen.

Extrapolation from rats to humans: 2.5
Variation between workers: 5
Total assessment factor: 2 x 2.5 x 5 = 25

Suggested exposure limit based on inflammation:

$$0.0251\ mg/m^3/25\ (\text{total assessment factor}) = 0.001\ mg/m^3 = 1\ \mu g/m^3.$$

CONCLUSION

The present working group evaluated the relevant literature on CNT toxicity from both epidemiological and animal inhalation studies. However, as almost no human data on toxicity and epidemiological studies is available, inhalation studies in mice and rats were used to assess potential human hazard.

Carbon nanotubes are a very diverse class of nanomaterials with large variation in physico-chemical properties including diameter, length, specific surface area, level and type of contaminations, surface modifications. Therefore, as the relationship between physico-chemical properties of CNTs and their inhalation toxicity is not fully clarified, the present working group considers all types of CNTs a respiratory hazard and proposes to regulate all CNTs as one group.

The present working group regards inflammation and carcinogenicity as the main adverse effects of CNT exposure by inhalation and the subsequent risk assessments are conducted based on studies reporting these effects. CNT-induced cardiovascular effects were also identified in animal studies. But as none of these studies were sub-chronic or chronic inhalation studies, they were not suitable for risk assessment. However, the current working group regards the acute-phase response as a biomarker of cardiovascular effects. Due to the close association between pulmonary inflammation and the acute phase response, the current working group regards inflammation as a proxy for cardiovascular effects.

The present working group found that the mechanism of action of CNT-induced carcinogenic effect has not been fully clarified. CNTs have reported to induce ROS generation similar to carbon black. ROS may result in oxidation of the DNA, which can result in several types of DNA damage, including base modifications, abasic sites, single-strand breaks, protein-DNA adducts, and DNA crosslinks (Waris and Ahsan 2006). CNTs may also induce genotoxicity through they fibrous shape, both in regards to diameter thickness (Poulsen et al. 2016;Catalan et al. 2016) and length (Donaldson et al. 2011). In addition, secondary genotoxicity due to CNT-induced inflammation has been recognized as an important and well-documented mechanism of action for the development of lung cancer. Based on the above mentioned findings, the current working group did not find sufficient evidence for a threshold mechanism for CNT-induced carcinogenicity and decided to consider it as non-threshold effect.

In contrast to cancer, the mechanism of action for CNT-induced pulmonary inflammation is more established and the present working group found strong dose response relationships for various markers of pulmonary inflammation, including neutrophil influx (Kasai et al. 2015;Ma-Hock et al. 2009;Pauluhn 2010b;Pothmann et al. 2015). Neutrophil influx was predicted by deposited surface area. The working group considers inflammation as a threshold effect.

Based on the main adverse effects of pulmonary CNT exposure, inflammation and cancer, the working group decided to perform the risk assessment based on both a threshold and a non-threshold mechanism of action. Four sub-chronic and one chronic

study inhalation study in rats were identified as suitable for determining a DNEL for pulmonary inflammation. A conservation approach was selected and the DNEL was calculated based on the study using the CNT with the largest specific surface area and reporting the lowest NOAEC estimate (Pothmann et al. 2015)(Table 8). For the non-threshold approach on cancerous effects, a 2 year inhalation study in rats were identified as suitable (Kasai et al. 2016) and excess cancer risks at the levels of 1:1,000, 1:10,000 and 1 in 100,000 were calculated using two approaches (Table 8).

Table 8. Overview of DNEL based on a threshold based mechanism of action and exposure levels resulting in excess cancer risk levels at 1:1000, 1:10 000 and 1: 100 000 based on a non-threshold based mechanism of action.

Suggestion for an OEL for CNTs				
Mechanism of action		**Inflammation**	**Lung cancer (Method I)**	**Lung cancer (Method II)**
Threshold based	DNEL	1 μg/m^3		
Non-threshold based	Excess cancer risk:			
	1:1,000		0.03 μg/m^3	0.043 μg/m^3
	1:10,000		0.003 μg/m^3	0.0043 μg/m^3
	1:100,000		0.0003 μg/m^3	0.00043 μg/m^3

The present working group regards cancer as the most critical effect. Two different approaches were used for calculating excess lung cancer risk based on the same chronic inhalation study (Kasai et al. 2016). In the first approach, lung burden was used to estimate the exposure levels. In the second approach, air concentrations were used directly. Independently of the applied method for risk assessment, the acceptable exposure levels were all very low. These levels are all more than 5 magnitudes lower than the present Danish occupational exposure limit for bulk carbon black of 3.5 mg/m^3.

The present working group recommends the approach using the excess lung cancer risk estimates based on lung burden, since this approach takes the retained pulmonary dose into account. Thus, the expected excess lung cancer risk based on lung burden approach is 1:1,000 at 0.03 μg/m^3, 1:10,000 at 0.003 μg/m^3 and 1:100,000 at 0.0003 μg/m^3.

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