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Carbon black nanomaterials:

Scientific basis for setting a health-based occupational exposure limit

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CARBON BLACK NANOMATERIALS: 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 (NFA) 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 nanosized carbon black.

The working group wishes to thank Chief Toxicologist Poul Bo Larsen, DHI, Denmark, for reviewing the report.

Copenhagen, November 2018

EXECUTIVE SUMMARY

Carbon black (CB) is the black dye of the world and millions of tonnes are used each year. It is a solid inorganic and poorly soluble compound which differs between products in size and surface area but also the levels of impurities such as polycyclic aromatic hydrocarbons.

In this report, a working group at the NFA reviews scientific data relevant to assessing the hazard of CB nanomaterials (CB NMs), i.e. human studies, toxicokinetics, animal studies, mechanisms of toxicity, previous hazard and risk assessments of CB NMs, scientific basis for setting an occupational exposure limit (OEL) and finally we summarise and suggest a health-based OEL for CB NM. The focus of this report is only occupational exposure by inhalation. The present working group evaluated the relevant literature on CB NM from both epidemiological studies and pulmonary exposure in animal studies. Cell culture studies were only used for the description and clarification of mechanisms and modes of action.

Epidemiological data were inconclusive. Two European CB production cohorts show evidence of excess cancer incidence; especially in the British cohort where a high prevalence for all causes mortality and lung cancer was observed. This was mainly driven by a large increase at two (out of five) facilities. Some indications of increased risk were observed in a German cohort, but this was unrelated to years of exposure/employment. In contrast, American CB employees demonstrated no excess occurrence of cancer mortality. Actually, a decreased mortality was observed in spite of some high estimated CB exposure levels. The latter result was explained by a strong healthy worker effect. Also, smoking frequency over time and other work-related exposures, in the general and in the CB worker populations may be a possible strong confounder and was not controlled for in any of the studies. However, other cigarette smoke induced diseases was not increased in UK cohort indicating that this was not the cause for the observed excess lung cancer cases. None of the studies provided information on the particle size range of the exposure. The present working group found that the available epidemiological studies cannot be used for risk assessment of CB NM and it was decided to base the suggested health-based OEL on data from experimental animal studies.

Pulmonary inflammation and carcinogenicity was observed in inhalation studies in rats. The present working group regards inflammation and carcinogenicity as the main adverse effects and the subsequent hazard assessments are conducted based on sub-chronic and chronic inhalation studies reporting these effects. The present working group found a dose response relationship for neutrophil influx as a marker of pulmonary inflammation. Neutrophil influx correlated with deposited surface area and was inversely correlated with particle size. The working group considers inflammation as a threshold effect.

The present working group concludes that there is substantial evidence for genotoxicity of CB NM. The literature shows that CB NM can induce mutations, oxidative damage to deoxyribonucleic acid (DNA) as well as DNA strand breaks in rats and mice. It is known

that inflammation and associated cellular production of reactive oxygen species is closely linked to genotoxicity. In addition, genotoxicity due to particle-induced inflammation is an important and well- documented mechanism of action for the development of lung cancer. However, the present working group found evidence for a non-threshold mechanism-of-action for carcinogenicity. Therefore, the present working group followed the European Chemicals Agency (ECHA) guidelines suggesting a precautionary non-threshold approach. Consequently, the present working group decided to perform the hazard assessment based on both a threshold mechanism for inflammation and a non-threshold mechanism for cancer.

For an OEL based on threshold effects, the following traditional approach suggested by Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is utilized: 1) identification of the critical effect, 2) identification of the no observed adverse effect concentration (NOAEC), 3) calculation of the OEL using assessment factors adjusting for inter and intra species differences and the duration of the study. For non-threshold effects, the current working group uses two approaches. The first, Method I, 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 II, suggested by ECHA, uses air mass concentrations directly.

The working group considered that data from five rodent inhalation studies were the best basis for the hazard assessment. The following studies were selected to be used for calculation of derived no effect level (DNEL) and excess cancer risk, respectively: DNEL studies were: A 12-month chronic inhalation study in rats (mass concentrations: 0, 2.5, and 6.5 mg/m^3), a 13-week sub-chronic inhalation study in mice, rats, and hamsters (0, 1, 7, and 50 mg/m^3), and a 13-week sub-chronic inhalation study in rats (0, 1, 7, and 53 mg/m^3). Cancer studies were: a 2-year chronic cancer inhalation study in rats (0 and 12 mg/m^3) and a 2-year chronic cancer inhalation study in rats (0, 2.5 and, 6.5 mg/m^3).

The table below shows the DNEL for pulmonary inflammation, and excess lung cancer risk at 1 in 1 000, 1 in 10 000 and 1 in 100 000 derived using the above-mentioned two different approaches. Independently of the applied method for hazard assessment, the resulting OEL suggestions were all low compared to the current Danish OEL for CB of 3.5 mg/m^3.

Overview of threshold-based DNEL and non-threshold-based exposure levels leading to excess cancer risk using two different approaches.

		Suggestion of a health based OEL for CB NM		
Mechanism of action		Inflammation	Lung cancer (method I)	Lung cancer (method II)
Threshold based	DNEL	20 μg/m^3 #		
Non-threshold based	Excess cancer risk			
	1: 1 000		3 μg/m^3	45 μg/m^3
	1: 10 000		0.3 μg/m^3	4.5 μg/m^3
	1: 100 000		0.03 μg/m^3	0.45 μg/m^3

[#]Based on NOAEC values in 2 sub-chronic inhalation studies.

The present working group recommends the hazard assessment approach estimating the excess lung cancer risk based on lung burden (Method I), since this approach takes the retained pulmonary dose into account. Thus, the expected excess lung cancer risk in relation to occupational exposure to CB NMs is 1: 1 000 at 3 μg/m^3, 1: 10 000 at 0.3 μg/m^3 and 1: 100 000 at 0.03 μg/m^3 CB NM.

DANSK SAMMENFATNING

Carbon black (CB) er verdens sorte farvestof og der bruges millioner af tons om året. CB er et fast uorganisk materiale med lav opløselighed, og forskellige CB-produkter varierer i størrelse, overfladeareal samt mængden af urenheder som fx polyaromatiske hydrocarboner.

I denne rapport vurderer en arbejdsgruppe ved Det Nationale Forskningscenter for Arbejdsmiljø (NFA) data, der er relevante for at vurdere faren ved udsættelse for CB-nanomaterialer (CB NM), dvs. humane studier, toksikokinetik, dyreforsøg, toksicitetsmekanismer, tidligere fare og risikovurderinger af CB NM samt det videnskabelige grundlag for fastlæggelse af en grænseværdi. Endeligt opsummeres og foreslås en helbredsbaseret grænseværdi for CB NM i arbejdsmiljøet. Fokus i denne rapport er alene på erhvervsmæssig eksponering ved indånding. Den nærværende arbejdsgruppe evaluerede den relevante litteratur om CB NM fra både epidemiologiske undersøgelser og inhalationsforsøg med dyr. Celleforsøg er kun blevet evalueret hvor de var nødvendige for at afklare og beskrive CB NMs virkningsmekanismer.

Der kunne ikke konkluderes endeligt på de epidemiologiske data omhandlende human CB-eksponering. To europæiske kohorter med arbejdere fra CB produktionsfaciliteter har vist, at arbejde med CB øger risikoen for at udvikle kræft. Specielt i en britisk kohorte var der en høj prævalens af død generelt og død forårsaget af lungekræft. Dette blev overvejende drevet af en stor effekt i arbejdere fra to ud af fem britiske produktionsfaciliteter. Der var desuden indikationer på en øget risiko for lungekræft i en tysk kohorte. Men dette var ikke korreleret til antallet af eksponeringsår. Der blev ikke set øget forekomst af kræft i amerikanske CB-arbejdere. Faktisk blev der her set en nedsat forekomst, og det på trods af, at de estimerede eksponeringsdoser var høje. Dette blev forklaret som en stærk *healthy worker effect*.

Evaluering af resultaterne kompliceres yderligere af, at det er svært at justere for rygning samt andre eksponeringer i den generelle befolkning såvel som hos CB-arbejdere. Fx korrigerede ingen studier for cigaretrygning. Det bemærkes dog, at andre sygdomme forårsaget af cigaretrygning ikke var øget i den britiske kohorte, hvilket indikerer, at dette ikke er baggrunden for det øgede antal dødsfald forårsaget af lungekræft. Ingen af studierne beskrev størrelsesfordelingen eller renheden af den producerede CB. Arbejdsgruppen fandt, at de tilgængelige epidemiologiske studier ikke kan bruges til farevurdering, og det blev derfor besluttet at basere de foreslåede grænseværdier på dyrestudier.

Der blev observeret lungeinflammation og lungekræft i inhalationsundersøgelser af rotter. Arbejdsgruppen betragter inflammation og kræft som de vigtigste skadelige effekter. Derfor baseres de efterfølgende farevurderinger på undersøgelser, der rapporterer om disse effekter. Tydelig dosis-respons-sammenhæng for tilgang af neutrofile celler til lungen som markør for lungeinflammation blev observeret. Det doserede specifikke CB overfladeareal prædikterede tilgangen af neutrofile celler som igen var omvendt korreleret til partikelstørrelse. Arbejdsgruppen anser inflammation for at være en tærskeleffekt.

Arbejdsgruppen konkluderer, at der er betydelig evidens for en DNA skadende effekt af CB NM. Litteraturen viser, at CB NM kan forårsage mutationer, oksidative DNA-skader samt DNA strengbrud i dyrestudier. DNA skadende effekter forårsaget af partikelinduceret inflammation er en vigtig og veldokumenteret virkningsmekanisme for udvikling af lungekræft. Også for CB NM er der betydelig evidens for denne virkningsmekanisme. Med de tilgængelige data kan en direkte og kræftfremkaldende mutagen mekanisme dog ikke afvises. Arbejdsgruppen fulgte derfor anbefalinger fra ECHA som foreslår en tilgang baseret på forsigtighedsprincippet. Farevurderingen af CB NM blev derfor udført både baseret på en tærskeleffekt for inflammation og en ikke-tærskeleffekt for kræft. Arbejdsgruppen fandt evidens for en ikke-tærskel baseret virkningsmekanisme for kræft. ECHA's anbefalinger om forsigtighedsprincippet og brugen af en ikke-tærskel-værdi blev derfor fulgt. Arbejdsgruppen foretog derfor en farevurdering baseret på både en tærskel-mekanisme for inflammation og en ikke-tærskel-mekanisme for kræft.

For en grænseværdi i arbejdsmiljøet baseret på tærskelværdier anvendes følgende traditionelle tilgang, som anbefalet af REACH: 1) identifikation af kritisk effekt, 2) identifikation af *no observed adverse effect concentration* (NOAEC), og 3) beregning af grænseværdi ved anvendelse af vurderingsfaktorer, der justerer for inter- og intraspecifikke forskelle. For effekter uden tærskelværdi anvender den nærværende arbejdsgruppe to metoder. Ved den første, Metode I, anvendes den målte lungedeponerede dosis hos rotter til at estimere den tilsvarende eksponering i arbejdsmiljøet. Ved den anden, Metode II, anvendes de direkte luftkoncentrationer.

Arbejdsgruppen fandt, at data fra fem inhalationsundersøgelser i rotter var det bedste grundlag for farevurderingen. Følgende undersøgelser blev udvalgt til beregning af henholdsvis *derived no effect level* (DNEL) og kræftrisiko: For DNEL var der en 12 måneders kronisk inhalationsundersøgelse af rotter (massekoncentrationer: 0; 2,5; og 6,5 mg/m^3), en 13-ugers subkronisk inhalationsundersøgelse af mus, rotter og hamstere (0; 1; 7; og 50 mg/m^3), og en 13-ugers subkronisk inhalationsundersøgelse af rotter (0; 1; 7; og 53 mg/m^3). Kræftstudier var: To 2-årige kroniske kræftinhalationsundersøgelser af rotter (0 og 12 mg/m^3, henholdsvis: 0; 2,5 og; 6,5 mg/m^3).

Tabellen nedenfor viser den beregnede DNEL for lungeinflammation, og overskydende lungekræftrisiko hos 1 ud af 1.000, 1 ud af 10.000 og 1 ud af 100.000 beregnet på to forskellige måder. Uafhængigt af den anvendte metode til farevurderingen, er de beregnede forslag til grænseværdier alle lave sammenlignet med den nuværende danske grænseværdi på 3,5 mg/m^3 for CB i arbejdsmiljøet.

Oversigt over tærskelbaseret DNEL og ikke-tærskelbaseret eksponeringsniveauer, der resulterer i overskydende kræftrisikoniveauer. Beregnet på ved to forskellige metoder.

		Forslag til grænseværdi for CB NM		
Virkningsmekanisme		Inflammation	Lungekræft (metode I)[1]	Lungekræft (metode II)[2]
Tærskel-baseret	DNEL	20 μg/m³ [#]	¤	¤
Ikke tærskel-baseret	Overskydende kræftrisiko			
	1: 1 000		3 μg/m³	45 μg/m³
	1: 10 000		0,3 μg/m³	4,5 μg/m³
	1: 100 000		0,03 μg/m³	0,45 μg/m³

[#]Baseret op NOAEC værdier fra to subkroniske inhalationsstudier.

Arbejdsgruppen anbefaler metoden, hvor den overskydende risiko for lungekræft baseres på lungebyrde, da denne tilgang tager højde for den faktiske lungedeponerede dosis. Således er den forventede overskydende lungekræftrisiko i forbindelse med erhvervsmæssig udsættelse for CB NM 1: 1 000 ved 3 μg/m³, 1: 10 000 at 0,3 μg/m³ and 1: 100 000 at 0,03 μg/m³ CB NM.

[1] Metode I er baseret på CB NM luftkoncentrationer som giver forhøjede antal af lungekræft tilfælde. Udregnet med en deponeringsfraktion på 8,6 %.

[2] Metode II er baseret på *unit-risk*-metoden som er beskrevet af det Europæiske Kemikalieagentur

CONTENTS

ABBREVIATIONS

8-oxo-dGua	8-Oxo-2'-deoxyguanosine
ApoE$^{-/-}$	Apolipoprotein E knockout mice
BAL	Broncho-alveolar lavage
BET	Brunauer–Emmett–Teller
Bw	Body weight
CB	Carbon black
CRP	C reactive protein
DNA	Deoxyribonucleic acid
DNEL	Derived no effect level
ECHA	European Chemicals Agency
H	Hour
HDL	High density lipoprotein
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
IARC	International Agency for Research on Cancer
ICAM-1	Intercellular adhesion molecule-1
LDL	Low-density lipoproteins
LOAEC	Lowest observed adverse effect concentration
LOAEL	Lowest observed adverse effect level
mRNA	Messenger ribonucleic acid
MWCNT	Multi-walled carbon nanotube
NFA	National Research Centre for the Working Environment
NIOSH	National Institute for Occupational Safety and Health
SRM	Standard reference material
NM	Nanomaterial
NOAEC	No observed adverse effect concentration
NOAEL	No observed adverse effect level
OEL	Occupational exposure limit
PAH	Polyaromatic hydrocarbon
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RR	Relative risk
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCCS	Scientific Committee on Consumer Safety
SMR	Standardised mortality ratio
TiO_2	Titanium dioxide
UK	United Kingdom
USA	United States of America
VCAM-1	Vascular cell adhesion molecule 1

Some carbon black nanomaterials will be frequently mentioned through this report as they have been included in numerous studies. These are listed below, for easy access to important physical and chemical parameters.

Product	**Manufacturer**	**Size (diameter)**	**Surface area**	**PAH content**
Printex 90	Degussa-Hüls, Germany	14 nm	337 m^2/g	0.123 ppm[α]
Monarch 880	Cabot Corp., MA, USA	16 nm	220 m^2/g	<0.1%[β]
Elftex-12	Cabot Corp., MA, USA	37 nm	43 m^2/g	0.012 %[γ]
Sterling V	Cabot Corp., MA, USA	70 nm	37 m^2/g	329.7 ppm[α]

[α]Summarised content of; phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)pyrene, and benzo (ghi) perylene (Borm et al., 2005). [β]From Cabot Corp. technical data sheet; toluene extract according to ISO 6209. [γ]The authors state that the extractable fraction of the CB was 68-times less than that for their tested diesel exhaust soot.

INTRODUCTION

Carbon black (CB), CAS number 1333-86-4, is a black solid inorganic and poorly soluble compound. The fine black powder is produced by finely controlled incomplete combustion of various carbonaceous (primarily petroleum) gases or liquid products. It is produced by a variety of methods with furnace black being the far most common product accounting for approximately 95% of the total produced CB (IARC, 2010). Other methods yielding products such as thermal black, lampblack, acetylene black and channel black are much less frequently used. CB products are typically fluffy powders of very low density. It is aggregates or agglomerates of primary particles within a size range of 10-100 nm; although larger and smaller products do exist. All products have a very high surface area to mass ratio mainly between 30-1000 m^2/g.

Global production exceeded 10 million tonnes in 2005 (IARC, 2010), and 13 million tonnes in 2015 and are expected to reach 19.2 million tonnes in 2022 (Industry_Experts, 2012); thus, CB is a major industrial chemical, and the most used nanomaterial (NM) in the world. Most CB products are almost pure elemental carbon with low amounts of impurities of solvent-extractable organic compounds like polyaromatic hydrocarbons (PAHs) (Borm et al., 2005; Jacobsen et al., 2008b). The level of such impurities varies significantly with certain CB products, such as Sterling V, containing at least three orders of magnitude more than e.g. CB Printex 90 (Borm et al., 2005). CB products at the high end such as Sterling V approaches similar PAH-concentrations as found in e.g. diesel exhaust particles (Jacobsen et al., 2008a; Wise and Watters, 2006).

The by far largest application for CB is as a reinforcing agent in rubber products. This accounted for 93% of total CB use in 2013. Used as a reinforcement agent in automobile tires accounted for 73%, whereas 20% were use in rubber hoses, rubber belts and similar. Other use of CB includes printing inks, paints, cosmetics and plastic products and as conductive fillers in batteries, which combined accounted for the remaining 7% (Auchter, 2005; IHS_Markit, 2017). In general, rubber products contain the cheap furnace blacks, whereas the more expensive high specialty CBs are used in the other products. The high price for specialty CBs has, in spite of the smaller volume, propelled this class to the forefront of CB research and development (IHS_Markit, 2017).

The International Agency for Research on Carcinogenicity (IARC) reclassified CB in 1996 to *possibly carcinogenic to humans* (group 2B). This classification was based on inadequate evidence for carcinogenicity in humans, but sufficient evidence of carcinogenicity in experimental animals of both CB and CB extracts. This IARC classification (volume 65) was confirmed when CB was revisited in volume 93 (IARC, 2010).

To our knowledge, there exists no legally binding nano-specific occupational exposure limit for CB NMs. The present Danish occupational exposure limit for CB is 3.5 mg/m^3 and is regulated by the Danish Working Environment Authority. CB has the annotation K, meaning that the substance is regarded as carcinogenic (Arbejdstilsynet, 2007).

The aim of the present report is to investigate if the present knowledge allows for a suggestion of a health-based nano-specific occupational exposure limit (OEL) for CB

NM. In this document, we review the relevant literature on the adverse effects of CB. The hazard assessment methodology of this report will follow the guidelines suggested by Registration, Evaluation, Authorisation and Restriction of Chemicals (ECHA, 2012). First, threshold and non-threshold effects are determined. Threshold effect assumes 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. A part of the current work is to evaluate whether there is substantial evidence for the involvement of a non-threshold mechanism in CB NM induced carcinogenicity. For an OEL based on threshold effects, the following traditional approach is utilized: 1) identification of the critical effect, 2) identification of the no observed adverse effect concentration (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 different approaches for calculating excess lung cancer risk. In the first approach lung burden will be used to estimate the exposure levels. In the second approach, air concentrations were used directly. Conclusively, the calculated OELs will be compared and lastly, a recommended OEL for CB NM exposure will be proposed.

HUMAN STUDIES

Several epidemiological studies have investigated the potential for adverse effects of exposure to CB amongst workers at CB factories. The literature and previous evaluations especially focus on 3 large cohort studies relating to CB facilities in the United Kingdom (UK), Germany, and the United States of America (USA). These studies focus on causes of mortality, with special emphasis on lung cancer.

A few smaller studies on CB workers have also been published. These include chest radiographs amongst European CB workers; pulmonary function amongst Chinese and American CB workers and dockyard workers occupationally exposed to CB while unloading CB at the dock of Genova, Italy. These studies will be described towards the end of this section.

Additionally, a range of other studies have been performed on people occupationally exposed for CB in specific industries. These include workers in rubber/tire industry, printing industry and battery manufacture (Bulbulyan et al., 1999; Greene et al., 1979; Malker and Gemne, 1987; Oleru et al., 1983; Parent et al., 1996; Ramanakumar et al., 2008; Straif et al., 2000, 1999; Szeszenia-Dabrowska et al., 1991). Although carbon black may have been the dominating exposure in these industries, there are some indications that exposure for e.g. asbestos and talc is confounders in these industries (IARC 2010). Therefore, the present working group will not include these in the current evaluation.

IARC has previously evaluated the human carcinogenicity of CB and concluded *inadequate evidence* (IARC, 2010). IARC evaluated the available epidemiological studies and considered the 3 large studies of CB workers in the United Kingdom, the USA, and Germany to be the most informative when assessing cancer risk. The 2 European studies indicated an excess risk. Although smoking is a possible confounder and smoking status was unknown, it is unlikely to have explained the entire excess risk as there was no excess of other diseases known to be associated with smoking. However, links between increasing exposure and risk levels were equivocal for the UK study or not existing for the German study with a rather crude exposure assessment. In contrast, the US study did not suggest excess mortality for any reported cancer site but did not assess risk by level of exposure. This has since been updated (Dell et al., 2015). There was no indication that long-term employment for service workers had higher risks than short-term employed. Tobacco smoking habits were not evaluated. IARC found isolated results for excess risks for cancers of the urinary bladder, kidney, stomach and esophagus; but evaluated that these are not sufficient to support an evaluation of human carcinogenicity (IARC, 2010).

The present working group agrees with the above-mentioned IARC review and evaluation. Below is an update of the latest publication on epidemiological studies on CB exposure. Older publications within the 3 large cohorts, already evaluated by IARC are included for easier access and for a more complete story of these cohort studies.

UK

The cohort following male workers at 5 CB production plants (Merseyside, West Midlands, South West England, Scotland and Wales) consisted at initiation of 1422 male

process workers with at least 12 months of exposure between 1947 and 1974; with follow-up until 1980 (Hodgson and Jones, 1985). Dust exposure was estimated based on 47 personal samples as well as background dust samples taken by the Factory Inspectorate in 1976. Half of the samples had CB mass concentrations higher than the time weight average OEL of 3.5 mg/m^3; with highest levels observed for filter bag replacement crew (79 mg/m^3). The authors adjusted for regional variations in mortality by assigning regional specific male mortality rates to each factory. As two factories were in areas, Merseyside and West Midlands Conurbation were mortality rates were different compared to the region. These were adjusted for local mortality and age-specific factors. Increased risk of lung cancer (Standardised Mortality Ratio; SMR: 152) were observed at all 5 plants. Notably this increase was not statistically significant. When analysing the factories individually, the excess lung cancer deaths were statistically significant at 2 plants both showing ~2-fold increased number of deaths compared to what was expected for their individual region (12 cases were observed, and 5.8 cases were expected). Adjustment for malignancies observed in the first 10 working years (these are likely not related to exposure at work) did not change this (10 observed; 5.1 expected). The same 2 plants were also those with the lowest dust mass levels, although the levels were still very high. A non-statistically significant increase in bladder cancer (SMR: 250) deaths were also observed (5 plants combined). As the figures are too small, an excess risk cannot be concluded or excluded. The incomplete data makes the interpretation difficult, and as mentioned by the authors do not allow for a negative conclusion regarding CB exposure and lung and bladder cancer (Hodgson and Jones, 1985).

The above study has since been followed up in 2001 and 2007, with certain adjustments (Sorahan et al., 2001; Sorahan and Harrington, 2007). SMRs were calculated based on both national and county-district specific mortality rates. The few workers not residing in the districts around the factories were assigned to the most common district of the factory at which they worked. Importantly, the longer follow-up time (up to an additional 24 years) of now 1147 CB workers allowed for a more precise calculation of risk estimates. Also, a more detailed retrospective exposure assessment was attempted. This included literature data on job categories and the exposure levels of CB at the factories (~15.000 measurements at 19 European factories between 1987-1995) (Gardiner et al., 1996, 1993, 1992), but also additional visits to the factories, interviews and additional collection of data. This enabled the generation of a job-exposure matrix with individual estimates of exposure by year and by job category. Overall, the data showed highly statistical significantly increased risk for death of lung cancer (2001; SMR: 173) (2007; SMR: 146). The data was in both cases driven by the previously mentioned 2 factories (2001; SMR: 278 and 315) (2007; SMR: 219 and 259). Mortality from all causes, when excluding lung cancer, was not significantly elevated (SMR: 106) (Sorahan et al., 2001; Sorahan and Harrington, 2007). In 2001 the authors did not find evidence for linking the excess lung cancer mortality to cumulative CB exposure

However, in 2007 the authors applied a ''lugged analysis'' to evaluate most recent 15 years of CB exposure. Notably the authors found that the risk of lung cancer mortality appeared linked to cumulative CB exposure in the most recent 15 years. This link was made for all 5 factories as well as the 2 with the highest risks. This is an important

finding because most epidemiological studies of occupational induced lung cancer focus on distant past (Sorahan and Harrington, 2007). The authors conclude that it is highly likely that occupational lung cancers, at least at 2 factories, were caused by CB or by CB production associated exposures; and that if truly based on a causal relationship: "*it is clear that current regulatory standards will only provide inadequate protection to workers at some carbon black production facilities*" (Sorahan and Harrington, 2007). The authors argue that smoking history and previous occupation involving exposure to lung carcinogens at the 2 factories cannot explain the difference to the other 3 factories. The 2 high risk plants were also those with the lowest mass dust levels (although still very high). As the authors also do, it is tempting to speculate in the importance of the size of the manufactured CB (Sorahan and Harrington, 2007) but also in the content of extractable organic material in the CB. However, as interesting as this hypothesis is, it should be emphasised that there was no information concerning size and purity of the CB produced at these plants.

Table 1. Epidemiological studies of plants in the UK

Reference	Location and exposure	Cohort	Endpoints/Results
(Hodgson and Jones, 1985)	5 CB plants in the UK. There is no direct information on CB exposure in mg/m^3	1422 male workers hired in the period of 1947 to 1975. Working at a facility for more than 1 year. Follow-up period up to 1980. Data on smoking habits were not available	Excess lung cancer deaths were observed at all 5 plants; although only statistically significant at 2 plants. The same two with the lowest mass dust levels. Incompleteness of data made interpretation of causes complicated.
(Sorahan et al., 2001)	5 CB plants in the UK Exposure level was between 0.5-30 mg/m^3 (1950s) with a decreasing trend to 0.5-5 mg/m^3 (1980s) for administrative (lowest) and cleaners (highest) respectively.	1147 male workers hired in the period of 1950 to 1975 and working at a facility for more than 1 year. Follow-up period till 1996. Exposure derived from a job-exposure matrix and records and interviews conducted at the 2 still functioning factories. Smoking history unknown	Mortality: All cause (SMR: 113*), Lung cancer (SMR: 173***). Highly elevated at 2 factories. No indication of risks increasing length of employment or estimated exposure.
(Sorahan and Harrington, 2007)	5 CB plants in the UK Exposure as above.	1147 male workers hired in the period of 1950 to 1975. Working at a facility for more than 1 year. Follow-up period till 2004. Exposure derived from a job-exposure matrix and records and interviews conducted at the 2 still functioning factories. Smoking history unknown	Mortality: All causes except lung cancer (SMR: 106) Lung cancer (SMR: 146**); highly elevated at 2 of the plants (SMR: 230***). CB (or associated chemicals) had effect on lung cancer with a clear dose response at these 2 plants but not the other 3 plants. The elevated lung cancer risk was limited to those with some employment in the most recent 15 years.

USA

The first publication on incidence of cancer amongst workers in the American CB industry dates to 1950. In this cohort study, excess morbidity and mortality was examined amongst 476 workers per year at one CB production facility in the years 1939-1949 (Ingalls, 1950). The follow-up period was later extended to 1949-1956 (Ingalls and Risquez-Iribarren, 1961), and both these studies found that CB workers have no excess risks of cancer mortality compared to other industrial groups or to National mortality data or New York State cancer data.

The cohort was enlarged to encompass 4 CB production facilities with an average annual number of employees of 1250. The workers were followed for 40 years between 1935 and 1974. No increased risks were found for all-cause-deaths, malignant neoplasms in the respiratory or digestive systems, or mortality due to heart disease or ischemic heart disease. Death rates were below expectations and sometimes significantly lowered. The authors suggest that these findings are explained by a "healthy worker effect"[3] (Robertson and Ingalls, 1980). The exposure was considered equal for all workers and evaluated as *person years of exposure*; i.e. there was no estimation of degree of exposure (Robertson and Ingalls, 1980).

In 2006 Linda Dell and co-workers published a comprehensive industry wide cohort study. It contained data from 18 CB production facilities and follow-up details of 5011 male and female workers hired for more than 1 year between the 1930s and 2004 (Dell et al., 2006). Results were presented using race, gender, state and year-specific mortality rates. No excess mortality caused by lung cancer was observed. The SMR was 89 (95% confidence interval: 0.75–1.06) or 97 (95% confidence interval: 0.82–1.15); if including 11 deaths of unknown causes (15% of 76 cases totally). Mortality from all causes or ischemic heart disease was 26% and 30% lower than expected. As no trends were observed between cause of death and duration of employment, the authors suggest that there is no increased mortality arising from employment in CB production (Dell et al., 2006). It should be mentioned that mortality from all causes, excluding lung cancer, was significantly reduced with an SMR of 72 (95% confidence interval: 68-76), suggesting that all deaths were not traceable, or the application of an incorrect measure of person-years working with CB production (IARC, 2009). Similar statistically significant reductions were observed for all cause deaths (SMR: 74) and all cause cancer deaths (SMR: 83). At least the data suggests a bias between the populations. A detailed exposure assessment was not attempted. This might have provided risks in relation to (semi)-quantitative estimates of CB exposure. Additionally, information on smoking or other possibly confounding exposures was not available (Dell et al., 2006).

Recently an updated analysis of the above-mentioned cohort was published (Dell et al., 2015). This study on the US cohort included a follow-up through 2011 of a full cohort of 6634 male and female workers CB workers and a smaller entry cohort of 3890 male

[3] A healthy worker effect arises when workers must meet a certain health level in order to function at the workplace. Workers of lower health will leave the job and thereby a difference in health between the workers and the public will arise.

hourly workers with an assumed larger potential for CB exposure. All workers were employed for more than 1 year at one of 18 US CB production facilities. The paper includes the new exposure metrics, and a job exposure matrix. The exposure matrix was completed on the basis of more than 8000 measurements (primarily of total dust but also of certain inhalable and respirable measurements) collected during 7 industry campaigns between 1979 and 2007 (Kerr et al., 2002; Muranko et al., 2001; Smith and Musch, 1982). Values were used for interpolation and backwards extrapolation before linkage to the work history data (title and dates) for each cohort member. Mortality from lung cancer was decreased for the full cohort but this was only borderline statistically significant for the entry cohort (SMR 77 and 87, respectively). The authors concluded that their data did not support an increased risk for lung cancer mortality for CB workers in this cohort, and that the exposure (up to 43 mg CB/m^3) experienced from the 1970s to the 1990s were not associated with increased risk of lung cancer. Risk for mortality caused by non-malignant respiratory diseases was not significantly altered for the 2 cohorts (SMRs: 88 and 109 for the full and entry cohort, respectively). Similarly, for chronic obstructive pulmonary disease; stratified analyses, revealed no clear associations between excess of lung cancer, non-malignant respiratory diseases or chronic obstructive pulmonary disease and length of employment, time since the first employment, or time since cessation of employment for any of the cohorts. Also "all-cause mortality", "all cancer mortality" and "all heart disease mortality" was decreased (SMRs: 78, 79 and 78, respectively) in the full cohort. This was also statistically significant, albeit less pronounced in the entry cohort (SMRs: 86, 87 and 84, respectively). The authors acknowledge that their data set shows a strong healthy worker effect. Also, the smoking frequency over time, in the general and CB working population is a possible strong bias. The deficit in lung cancer deaths observed in general and in particular amongst certain groups may suggest that workers were less likely to have been smokers (IARC, 2009).

Table 2. Epidemiological studies of plants in the USA

Reference	Location and exposure	Cohort	Endpoints/Results
(Ingalls, 1950; Ingalls and Risquez-Iribarren, 1961)	1 CB manufacturer with multiple units in southwest USA. There is no direct information on CB exposure in mg/m^3	476 workers per year recruited in the period of 1940 to 1949 or 1940-1956 and working for more than 12 months at the facility. Smoking habits were unknown	The observed death rate was 0.21 per 1 000 per work year in both tested periods (expected was 0.49). The result was that CB workers face no excess risks for cancer
(Dell et al., 2006)	18 CB plants in the USA. There is no direct information on CB exposure in mg/m^3	5011 male and female CB workers (worked at the plant for more than 12 months) hired since the 30s and followed until end of 2003. *Age-, race-, sex-, and calendar year-adjusted SMRs were calculated*	No increased risk for mortality of lung, bladder, non-malignant respiratory diseases. Statistically reduced risk for all-cause (SMR 74 and all-cancer mortality (SMR 83). No trends in risks were observed with duration of employment or time since first hire for any cause of death incl.

		using state-specific mortality rates Smoking status and history unknown	lung cancer.
(Dell et al., 2015)	18 CB plants in the USA. Exposure level was between 0-43 mg/m^3 (1980s) with a decreasing trend to 0-12 mg/m^3 (2000s). The vast majority of all measurements (1975-2007) were in the range 0-5 mg/m^3.	Full cohort: 6634 male and female CB workers. A smaller entry cohort of 3890 male hourly workers with an assumed larger CB exposure. All with more than 12 months hire at one of 18 US CB facilities. Follow-up through 2011. A job-exposure matrix was based on measurement data from 7 previous industry campaigns. Personal exposure was estimated via coupling to personal work history. State, race- and sex-specific mortality rates by age and calendar interval Smoking history unknown	Lung cancer mortality was stat. significantly decreased in both cohorts (SMR 77 & 87). Similarly, "all-cause", "all cancer" and "all heart disease" mortality was stat. significantly decreased in the full cohort (SMR 78, 79 and 78) and the entry cohort (SMR 86, 87 and 84). No increased risk for mortality caused by non-malignant respiratory disease and chronic obstructive pulmonary disease. The authors conclude: No support for increased lung cancer mortality for CB workers. No associations with total employment, time since the first employment, or time since cessation of employment for any of the cohorts was observed. HR: 1.0 for 20-50 mg/m^3*year; HR: 1.3 for 50-100 mg/m^3*year; and HR: 1.4 for 100 or more mg/m^3*year compared with referent (<20 mg/m^3*year)

Germany

There are a range of studies on a cohort of CB workers from one production plant in Germany followed in 1976 to 1998. The cohort consisted of 1528 male workers that produced furnace black, lamp black, and gas black (Büchte et al., 2006; Morfeld et al., 2016, 2006a, 2006b, Morfeld and McCunney, 2009, 2007; Wellmann et al., 2006). And one study was likely in the same cohort (same number of deaths due to lung cancer, 50) in 1960 to 1998 (Vital status and causes of death were assessed for 1976 to 1998) (Wellmann et al., 2006).

Concerning the study by Wellman and co-workers, the mortality of the cohort of 1535 male CB manufacturing plant workers was investigated. The workers were employed at the plant for at least 1 year during the period of 1960 to 1998 (vital status and causes of death assessed for the period of 1976 to 1998). Risk was calculated based on national referent rates, and state referent rates. The SMR for all-cause-mortality (332 deaths) was 120 (95% confidence interval: 108 to 134), that of mortality from lung cancer (50 deaths) was 218 (95% confidence interval: 161 to 287) using national rates as a reference.

Comparisons to regional rates gave SMRs of 120 (95% confidence interval: 107 to 133) and 183 (95% confidence interval: 136 to 241), respectively. There was no dose-response relationship between lung cancer mortality and such indicators of occupational exposure as years of employment and exposure to CB. The authors of the study concluded that: *"The mortality from lung cancer among German carbon black workers was increased. The high lung cancer standardised mortality ratio cannot fully be explained by selection, smoking, or other occupational risk factors, but the results also provide little evidence for an effect of carbon black exposure."* (Wellmann et al., 2006).

Buchte and co-workers performed a case–control study of lung cancer nested within the cohort of 1528 subjects and the years were 1976–1998. Fifty lung cancer deaths were analysed and showed no association to CB exposure (Büchte et al., 2006). Another study was conducted on a sub-cohort comprising only subjects with information on their smoking status. Moreover, an inception sub-cohort consisting of all study subjects from the cohort who started working at the CB plant on or after January 1, 1960, was identified to reduce the healthy worker selection biases. Combining the criteria, 4 study groups were defined for analyses: The full cohort (cohort 1); The full cohort with smoking information (cohort 2); The inception cohort (cohort 3); And the inception cohort with smoking information (cohort 4). No positive association was found between the 50 lung cancer deaths and CB exposure indices. However, these authors found that certain models provided an indication that there was an increasing risk across duration of work in the lamp black producing department. The authors of the study conclude that their results do not suggest that CB exposure is a lung carcinogen. The lamp black results may point at historical exposures to polycyclic aromatic hydrocarbons." (Morfeld et al., 2006b). A sensitivity analysis of the lung cancer SMRs was also applied to the data on 1522 of the German CB workers observed in the period of 1976 to 1998. Risks were calculated based on national and regional mortality rates. Based on 47 lung cancer deaths, the SMRs were 1.62, 1.72, and 2.08 (local, state, and national rates, respectively). Adjustment for previous exposures and smoking provided additional correction factors of 0.64 or 0.74. The authors of the study concluded: "*Lung cancer standardised mortality ratios (95% confidence intervals) for the full cohort ranged from 1.20 (0.88 –1.59) to 2.08 (1.53–2.77) in this sensitivity analysis. Thus, overall standardised mortality ratios are only weak measures of causal associations and should be complemented by internal modelling of exposure effects whenever possible* (Morfeld et al., 2006a).

In a follow-up to a British study of CB production workers (Sorahan and Harrington, 2007) in which the risk of lung cancer was reported to decline after cessation of employment. The German cohort was re-evaluated by focusing on the first 15 years after cessation of employment in terms of lung cancer SMR. This was analysed in the German cohort of 1528 male workers and in an inception cohort consisting of 1271 males. In contrast to the British study a rising trend in lung cancer SMR was observed (Morfeld and McCunney, 2007). In a study of the same German cohort, a new analysis was undertaken. The reason for this is that an analysis of a UK cohort had been published (Sorahan and Harrington, 2007). In this publication the most recent 15 years of exposure were assessed (''lugging'') to support the hypothesis that CB acts as a late stage lung carcinogen. Thus, this was also tested in the German cohort of 1528 CB workers. Negative coefficients were returned by all tested models. The authors of the study

conclude that: "*Despite extensive searching, no exposure scenario suggested an adverse effect of ''lugged'' carbon black exposure on lung cancer mortality. Our analysis does not support the hypothesis of carbon black being a late stage carcinogen.*" (Morfeld and McCunney, 2009).

SMR and Cox proportional hazards results from cohort studies of US, UK and German CB production workers were combined. Mortality from all causes, heart disease, ischemic heart disease and acute myocardial infarction were analysed. Full cohort meta-SMRs (random effects) were 1.01 (95% confidence interval: 0.79–1.29) for heart disease; 1.02 (95% confidence interval: 0.80–1.30) for ischemic heart disease, and 1.08 (95% confidence interval: 0.74–1.59) for acute myocardial infarction mortality. The authors of the study conclude that "*Our results do not demonstrate that airborne CB exposure increases all-cause or cardiac disease mortality*" (Morfeld et al., 2016).

Table 3. Epidemiological studies of plants in Germany (and Germany, USA and UK combined)

Reference	Location and exposure	Cohort	Endpoints/Results
(Wellmann et al., 2006)	A CB manufacturing plant in Germany. There is no direct information on CB exposure in mg/m^3, however, an expert committee evaluated the exposure and established a job-exposure matrix and intensity of exposure was assigned to each job title. The highest score (20 units) was assigned to jobs where carbon black was shovelled into bags. This was performed up till the early 1960s. A score of zero was assigned to the jobs with no contact to CB	1535 male CB workers, who had worked for at least 1 year and were employed between 1960 and 1998 at a single CB production plant. Age- (five-year groups), sex-, and calendar year-adjusted SMRs were calculated using national (West) Germany or regional -specific mortality rates North-Rhine Westphalia	The authors of the study conclude that: "*the results also provide little evidence for an effect of carbon black exposure"* on all-cause-mortality and lung cancer mortality
(Büchte et al., 2006)	A CB production plant in Germany. There is no direct information on CB exposure in mg/m^3. Applied an adjusted job-exposure matrix	1528 CB workers, 1976 – 1998, producing furnace black, lamp black, and gas black	No effect on lung cancer
(Morfeld et al., 2006b)	A CB production plant in Germany. A few CB measurements were performed at the plant. However, the authors did not consider them valid for the purpose of the article. Thus, there is no direct	1528 CB workers, 1976 – 1998, producing furnace black, lamp black, and gas black	No positive association was found between the 50 lung cancer deaths and CB exposure indices. However, these authors found that certain models provided an indication that there was an increasing risk across

	information on CB exposure in mg/m^3. Also, certain changes were done in the job-exposure matrix compared to Wellman et al., 2006		duration of work in the lamp black producing department
(Morfeld et al., 2006a)	A CB production plant in Germany. There is no direct information on CB exposure in mg/m^3	1528 CB workers, 1976 – 1998, producing furnace black, lamp black, and gas black	Based on 47 lung cancer deaths, the SMRs were 1.62, 1.72, and 2.08 (local, state, and national rates, respectively). Adjustment for previous exposures and smoking provided additional correction factors of 0.64 or 0.74
(Morfeld and McCunney, 2007)	A CB production plant in Germany. There is no direct information on CB exposure in mg/m^3	1528 CB workers, 1976 – 1998, producing furnace black, lamp black, and gas black	The cohort was re-evaluated by focusing on the first 15 years after cessation of work. A rising trend in lung cancer SMR was observed
(Morfeld and McCunney, 2009)	A CB production plant in Germany. There is no direct information on CB exposure in mg/m^3	1528 CB workers, 1976 – 1998, producing furnace black, lamp black, and gas black	The most recent 15 years of exposure was assessed by so-called ''lugging''. No exposure scenario suggested an adverse effect of ''lugged'' CB exposure on lung cancer mortality.
(Morfeld et al., 2016)	A CB production plant in Germany. 18 CB plants in the USA (also described above) 5 CB plants in the UK (also described above). Exposures for all 3 cohorts were converted to 100 mg/m^3-years (UK and US) and unit/years (Germany). The job-exposure matrix for all 3 cohorts were used	SMR and Cox proportional hazards results from cohort studies of US, UK and German CB production workers were combined	Full cohort meta- SMRs (random effects) were 1.01 (95% confidence interval (CI) 0.79–1.29) for heart disease; 1.02 (95% CI 0.80–1.30) for ischemic heart disease, and 1.08 (95% CI 0.74–1.59) for acute myocardial infarction mortality

Other studies

Employees (n=1755) at 22 North American plants were systematically administered questionnaire and spirometry tests. In conclusion, the study shows a relationship between exposure to CB and small reductions in the 1-second forced expiratory volume (-2 mL/mg-year/m^3 total dust and -0.7 mL/mg-year/m^3 for the inhalable fraction) and increased prevalence of chronic bronchitis for large exposures. Although the effect may be limited, the authors conclude that workplace exposures to CB should be controlled to the lowest practical levels (Harber et al., 2003).

Lung and bladder cancer was investigated in a group of 2286 longshoreman occupationally exposed to CB dust at the dock of Genova, Italy. Workers exposed to high

concentrations of CB (n=14) had a significantly increased frequency of bladder cancer (standardised incidence ratios 204, 112–343). Gender and age-specific incidence rates for the City of Genova were used to compute standardised incidence ratios. The authors conclude that the increase in bladder cancer in longshoremen is probably related to high CB exposure (Puntoni et al., 2001). In a follow-up study, cancer incidence was analysed amongst 2101 longshoremen from the same dock. The workers CB dust exposure was listed as low, moderate, and high. Incidence rates were calculated as mentioned above. A positive CB exposure–response relationship was detected for bladder cancer (SIR 204, 95% CI 112–343; high CB exposure) (Puntoni et al., 2007).

Inhalation of CB NM (30 - 50 nm) and altered lung function and inflammation were analysed in 81 CB-exposed male workers and 104 non-exposed male workers. The exposure concentration was 14.9 mg/m^3 where 50.8% were less than 0.5 μm, and 99.6% were less than 2.5 μm in aerodynamic diameter. A reduction of lung function parameters including FEV1%, FEV/FVC, MMF%, and PEF% in CB workers was observed, and the IL-1β, IL-6, IL-8, MIP-1beta, and TNF- alpha had 2.86-, 6.85-, 1.49-, 3.35-, and 4.87-folds increase in serum of CB workers, respectively. The authors conclude that the data strongly suggests that CB NM could be responsible for a reduction in lung function and increased inflammation in the workers (Zhang et al., 2014).

As high levels of CB exposure had previously been associated with an increased prevalence of chest radiograph abnormalities, the authors examined to what extent current levels of exposure in the CB industry are associated with such effects. Longitudinal analyses of workers in the European CB industry who provided three full-size chest radiographs sequentially between 1987 and 1995. Data from 675 workers were included. An association between cumulative CB exposure and new cases of chest radiograph abnormalities and progression in small opacities was observed (majority came from one factory). A large fraction of workers with abnormalities reversed to normal; however, after adjusting for other confounders, this was not associated with levels of exposure to CB dust. In conclusion, the results show that exposure to CB is associated with increased risk of chest radiographic abnormalities, which may be reversible after reduction or cessation of exposure (van Tongeren et al., 2002).

Power of the epidemiological studies

The ability to detect the effect of exposure to occupational carcinogens is also determined by the population-specific lung cancer incidence. In Denmark, the life time risk of developing lung cancer (0-74 years) is now 4.9% for men and 4.5% for women, respectively, according to The National Board of Health. In the US, life time lung cancer risk is similar, 7% for men and 6% for women (American Cancer Society 2018). The lung cancer incidence has historically been much higher and is largely determined by the smoking. The relative lung risk caused by occupational exposure to a carcinogen, which causes lung cancer the different risk levels, 1%, 0.1% and 0.01% are given in Table 4. As can be seen, exposures that cause 1% excess lung cancer will give relative risks of 1.2. According to power calculation, detection of 1% excess cancer incidence with 5% lung cancer incidence in the reference group would require group sizes of 8 000 participants (with 80% chance of detecting the effect at 5% significance level). On the other hand, occupational exposures that cause 0.1% excess lung cancers (1 of 1 000, which is the

acceptance level in the US), corresponds to a relative risk (RR) of 1.04, which requires group sizes of 750 000 persons if the background cancer incidence is 5%.

Table 4. Relative risk of lung cancer for carcinogens that cause 1%, 0.1% or 0.01% excess lung cancer risk in a population with the current Danish lung cancer incidence

	Men	Women
Life time risk (0-74 years) 2011-2015 in Denmark[1]	4.9 %	4.5 %
Excess lung cancer risk level		
1: 100	RR= (4.9+1)/ 4.9= 1.20	RR= (4.5+1)/4.5= 1.22
2: 1 000	RR= (49+2)/49= 1.041	RR= (45+2)/45=1.044
1: 1 000	RR= (49+1)/49= 1.02	RR= (45+1)/45=1.02
1: 10 000	RR= (490+1)/490= 1.002	RR= (450+1)/450= 1.002
1: 100 000	RR= (4 900+1)/4 900= 1.000 2	RR= (4 500+1)/4 500= 1.000 2

RR: Relative risk

Thus, in spite of a small subsection of the cohorts being exposed to very high CB levels; the epidemiological studies on CB and lung cancer risk have limited statistical power to detect carcinogenic effects of CB exposure, unless the excess lung cancer risk associated with CB exposure is very high.

Conclusion

The overall epidemiological evidence is not conclusive. The two European production cohorts show evidence of excess cancer incidence. A very high prevalence for all causes mortality (SMR 146) was observed in the British cohort. This was mainly driven by a large increase at two facilities (SMR 230). In the German cohort similar trend on both all-cause mortality and mortality from lung cancer was observed, but this was unrelated to years of exposure/employment. Workers employed and exposed for many years should have a higher occupational risk compared to workers recently employed. In contrast the study of American CB employees demonstrated no excess occurrence of cancer mortality when compared to the general population. A significantly decreased mortality was observed in spite of some very high estimated exposure doses based on a job exposure matrix. Concerning cardiac disease mortality, a study combining exposures from both Germany, USA and UK showed no increased mortality associated with CB exposure (Morfeld et al., 2016). When conducting epidemiological studies, it is a challenge to select a control group with minimal bias; especially, when knowledge about the participants is limited. Possible bias in all studies may come from the healthy worker effect, possible misclassifications of exposures, previous occupational exposures and the unknown smoking history. Smoking restrictions were implemented at American facilities in the mid-90s, and this may have an impact on the lung cancer cases over time. In the UK cohort there was no excess risk of other diseases known to be associated with smoking. Also, high exposure exposures may have forced a healthy worker effect. None of the epidemiological studies provided information regarding the particle size distribution of the CB exposure and thus, did not allow to identify a possible CB NM exposure. CB particles may likely have been larger in the earlier years leading to less deposition and effect. The American insurance-based health plan generated through the employer may give bias towards willingness or ability to seek medical aid and therefore, the general population cannot be used as reference group for CB-exposed workers. The working

group finds the difference between the European studies and especially the British and the US cohort striking.

Thus, in conclusion, we cannot exclude a carcinogenic potential of CB NM based on the present human epidemiological studies. Furthermore, the available epidemiological data cannot be used for risk assessment.

TOXICOKINETICS

As CB is the black dye of the world and hence one of the most used chemicals (IARC, 2010), exposures may occur in many places via inhalation, dermal exposure or via primary or secondary ingestion. Highest exposures and risks are expected to occur through breathing of air in occupational settings. Dermal exposure occurs both in occupational and consumer settings. However, dermal exposure levels are not expected to be critical, and uptake through healthy undamaged skin are expected to be low or non-existing. End-users of rubber, ink or paint products are not exposed to CB per se, as it is bound within a product matrix (IARC 2010). Focus in this section will thus be on the most critical exposure pathway; inhalation. Pulmonary deposition and systemic biodistribution is of importance as it describes key sites for possible secondary effects caused by translocation.

In general; the smaller the diameter of inhaled particles the deeper the pulmonary penetration and deposition will be. Especially nanoparticles will deposit in the alveolar ducts and alveolar sacs (ICRP, 1994; Jacobsen et al., 2009). Materials deposited from the respiratory bronchioles to the larynx will be cleared by the ciliated epithelium (the mucociliary escalator). The respiratory bronchioles are only partially ciliated, and the alveolar sacs and ducts are not. Here the main mechanism for particle clearance is macrophage phagocytosis.

The success of particle clearance relies on attracting the alveolar macrophages to the site of particle deposition and particle uptake (phagocytosis). It has been shown that nanoparticles are less efficiently phagocytized by macrophages than larger particles of identical composition (Geiser et al., 2008; Kreyling et al., 2002; Oberdorster et al., 2005; Oberdörster et al., 1992; Semmler et al., 2004). Twenty-four hours after inhalation of various sized particles increased retention of particles within the lung is seen with decreasing size of particles.

The poor phagocytosis of NMs could be due to a reduced response when phagocytes and NM encounter. However, it could also be that the nanoparticles are too small to initiate a cell generated substantial chemotactic signal or it could be caused by increased adherence or uptake by epithelial cells. Actually, it has been suggested that the size range of phagocytosis may be optimal in the lowest μm range (Tabata and Ikada, 1988). Increased or long-term lung retention of nanoparticles may increase inflammation and proximal as well as distal translocation to secondary target organs via the circulation.

Kinetics of CB particles are in general challenging to study as carbon is the second most abundant element in the body. Thus only a few studies have performed quantitative assessment of pulmonary retention of inhaled CB. In general, small variations over a method of digesting dried tissues before measuring the optical density of the re-suspended insoluble particles are used. The spectrophotometric results are compared to a standard curve (Rudd and Strom, 1981).

Strom and co-workers exposed male rats for 20 h/d, 7 d/week for 1, 3, or 6 weeks to filtered air or 7 mg/m^3 CB NM (Elftex-12, 37 nm, Cabot Corp., Boston, Mass.). After 1-, 3-,

and 6-wk of exposures, the lung burdens were 1.1, 3.5, and 5.9 mg, respectively. One year after the 1-, 3-, or 6-wk exposure, 8%, 46%, and 61% of the initial lung burden remained in the lungs, respectively. Lymphatic translocation was determined 1-year post exposure and showed lymph node burdens of 1%, 21%, and 27% of the initial lung deposited material for the three exposure doses, respectively. A combined retention of lung/lymph nodes was 9%, 67%, and 89% for the 1-, 3-, and 6-wk exposed animals showing a clear decrease in lung clearance with increasing dose (Strom et al., 1989).

Rats were exposed 19 h/day, 5 days/week for 24 months to 12 mg/m^3 of CB NM (7.5 mg/m^3 for the first 4 months) (Printex 90, Degussa-Hüls, Germany). Following the exposure 43.9 mg of CB NM material was retained in the lungs with determined half times of 550 days and 6.7 mg in the lung associated lymph nodes (Creutzenberg et al., 1990). The high half-times indicate a severely impaired or collapsed clearance function.

Another study focused on comparing three rodent species (female rats, mice and hamsters) for particle retention kinetics. Four exposure concentrations were used; 0, 1, 7, and 50 mg/m^3 high surface area CB (Printex 90, Degussa-Hüls, Germany; 300 m^2/g) and 50 mg/m^3 low surface area CB NM (Sterling V, Cabot Corp., Boston, Mass.; 37 m^2/g)(Elder et al., 2005). Exposure was 6 h/day, 5 days/week for 13 weeks. Increased dose resulted in decreased clearance and low surface area CB NM was cleared faster than high surface area CB NM. For low and middle dose, the retention half times were longest for mice (133 and 343 days) followed by rats (64 and 115 days) and hamsters (42 and 53 days). Thus, these doses overwhelmed primarily the mouse lungs and to a lesser extent the rat lungs leading to a very low and slow clearance. At the highest tested dose, the rat lungs did show substantially longer retention half times compared to mice (322 days) and hamsters (309 days). Normal retention half times has been suggested around 70 days for rats and 55 days for mice (Kreyling, 1990; Oberdörster, 1995). Hamsters have the most efficient clearance mechanisms also leading the least severe responses of the three species.

One study has attempted to determine short term (24h) full body translocation of carbon particle aggregates (25 nm) containing ~1% iridium (^{192}Ir). Inhalation of pure ^{192}Ir aggregates 20 nm or 80 nm was also performed (Kreyling et al., 2009). In general, most material deposited in upper airways was rapidly cleared to the gastro intestinal tract to the faeces. Here, as also shown before (Kreyling et al., 2002), absorption from the gastro intestinal tract was negligible (Geraets et al., 2014). Twenty-four hours following the inhalation 78 % of the retained dose was still in the lung; of this the 8 % was accessible by lavage. Translocated carbon / iridium material agglomerates were detected; 0.2 % liver; 0.08 % heart; 0.06 % kidney; 0.05 % spleen; 0.05 % blood; 3% carcass (smooth tissue and bone). Translocation was higher following inhalation of 20 nm ^{192}Ir aggregates and was similar or slightly less following 80 nm ^{192}Ir aggregates (Kreyling et al., 2009).

A few short-term studies (generally 24h but one study up to 3 days) in humans using carbon particles with attached ^{99m}technetium have also been performed (Brown et al., 2002; Mills et al., 2006; Möller et al., 2008; P. Wiebert et al., 2006; Pernilla Wiebert et al., 2006). All studies found that whether material size was 4-20 nm, 35 nm or 100 nm by far

the most material remained in the lung and immediate surroundings and systemic translocation was low and mainly consisted of free and not particle bound ^{99m}technetium.

Translocation of pulmonary deposited CB NM (Printex 90, Degussa-Hüls, Germany) to the liver has also been documented using bright field and enhanced dark field hyperspectral microscopy. In this study female mice were exposed by single intratracheal instillation of 162 μg of CB NM suspension. CB NM was detected in the liver but detection in other organs was not attempted (Modrzynska et al., 2018).

In summary; although little work has been performed on the kinetics of pulmonary deposited CB NM, the data clearly shows low and slow pulmonary clearance. Three or 6 weeks of inhalation at 7 mg/m^3 resulted in a pulmonary and lymphatic retained dose of 68% and 89% 1 year after the inhalation ended (Strom et al., 1989). These results are from a rat study but as shown by Elder and co-workers, mice actually have even slower clearance rates compared to rats at this and lower inhalation concentrations (Elder et al., 2005). It is the opinion by the present working group that there are no reasons to believe that retention and systemic translocation and excretion of CB NM would be much different for CB NM than for other low solubility and low toxic potency NMs. It has been shown for several metals and metal oxides that smaller particles translocate from the lung to the system to a greater extent than larger counterparts. This means higher material accumulation in an increased number of organs for the smallest NM (Kreyling et al., 2018, 2014, 2009; Sadauskas et al., 2009)(Balasubramanian et al., 2013; Ferin et al., 1992; Kreyling et al., 2009, 2002; Oberdörster et al., 1994). The same conclusion has been drawn in regards to translocation across placental barriers during rat pregnancy (Semmler-Behnke et al., 2014). I.e. translocated particles may be found in several organs. Knowledge from the general NM literature (radioactive gold, iridium or carbon nanotubes) shows clear evidence that NM cross membranes and reach secondary target organs where they accumulate. Although higher levels have been observed following instillation with 1.4 and 2.8 nm gold (Schmid et al., 2017), evidence in general show that only a smaller fraction of inhaled particles (<1 %) will translocate beyond the lung and immediately associated tissue (lymph nodes and pleura) (Jacobsen et al., 2017; Kreyling et al., 2002; Schmid et al., 2017). However, it is important to note that even if <1% escape the lung and translocate into secondary organs such as liver, spleen, kidneys, reproductive system and brain it may represent a very high number of NMs.

ANIMAL STUDIES

Rodent versus human response

Inhalation studies in mice and rats are used to assess potential human hazard where human exposure studies and epidemiological studies are not available or inconclusive. There is very limited data available on effects following controlled inhalation of CB NMs in humans. Rats are the preferred animal model in particle toxicology and are more sensitive than mice to particle-induced lung cancer and fibrosis (Kratchman 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 inhalation exposure. However, the deposited pulmonary dose can be difficult to monitor precisely following inhalation of certain materials e.g. CB NM. For these, inhalation models can be used taking into account differences in sizes of the aerosolised particle agglomerates and deposition frequency making it possible to estimate the delivered dose. In addition, exposure by inhalation requires a substantial amount of material and specialised inhalation facilities, and it poses an occupational health risk to the technicians handling the NMs.

Pulmonary deposition by intratracheal instillation is used in screening studies (Bourdon et al. 2012;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. Intratracheal installation has previously been shown to give widespread distribution of particles throughout the lung (Mikkelsen et al., 2011; Poulsen et al., 2016). This exposure method ensures that the same precise dose is delivered to the lung for all NM exposures, demands less material and is more user-friendly.

Several studies have compared the toxicological response following inhalation and instillation of NMs. Two studies have compared the global transcriptional profiles to investigate the pulmonary biological response after inhalation compared to instilled or aspirated NMs. Inhalation and intratracheal instillation of a surface modified titanium dioxide (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.*, (Kinaret et al. 2017) compared the global transcriptomic profiles of lung tissue from mice exposed to a straight and long multiwalled carbon nanotubes (MWCNT) by inhalation or aspiration. The authors concluded that the perturbed pathways were very overlapping, suggesting that the transcriptomic 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. 2012;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 was delivered. The authors concluded that intratracheal instillation is useful for quantitative ranking of NP hazards, but not for quantitative hazard assessment (Baisch et al. 2014).

Selection of studies and endpoints

In the present report inhalation studies will be prioritised. For the description of toxicological endpoints and mechanism of toxicity, studies using intratracheal instillation for pulmonary deposition, will be included to support the findings from the inhalation studies. Hazard assessments, however, are solely conducted based on subchronic and chronic inhalation studies. Endpoints were evaluated based on reported adverse effects of CB NM exposure in reports and in the scientific literature. Cancer and cardiovascular disease have been identified as two of the main mortality causing diseases in the world (World Health Organization 2018;Cancer Risks UK 2018). Both diseases are potentially initiated by inflammation, as described in the section *Mechanism of toxicity*. The critical endpoints were chosen based on literature wide review.

Pulmonary inflammation

Concerning inflammation there are a long range of CB NM inhalation studies and intratracheal instillation addressing this endpoint. The studies considered most relevant for OEL derivation are described below; and chronic and subchronic inhalation studies are also presented in Table 5.

In a chronic inhalation study by Mauderly and co-workers, male and female rats were exposed to CB NM by inhalation. The dosage regimen was a mass concentration of 2.5 or of 6.5 mg/m^3 for 16 h/day, 5 days/week for 12 or 24 months. The CB NM was Elftex-12 furnace black from Cabot Corp. (MA, USA) (Mauderly et al., 1994). This CB NM has been reported elsewhere to have a diameter of 37 nm (SCCS, 2015). Neutrophils were measured in bronchoalveolar lavage fluid (BAL) in lungs after 12 months of exposure. The mass concentration of 2.5 mg/m^3 was determined to be the lowest observed adverse effect concentration (LOAEC) in terms of increased neutrophil numbers in BAL.

Other endpoints investigated in the study were survival, additional BAL fluid endpoints, organ weight, non-neoplastic lesions as well as neoplastic lesions. Concerning survival, the median survival in days was for females: Control 696 days, low CB NM 707, high CB NM 675 (statistically significantly decreased); and for males Control 639 days, Low CB NM: 605 (statistically significantly decreased) and High CB NM 599 (statistically significantly decreased). Concerning other BAL fluid endpoints, lactate dehydrogenase and beta glucoronidase were increased at both dose levels. Concerning lung weight, the weight was increased for all CB NM dosed groups. By the authors of the report it was suggested that this reflected the inflammatory, proliferative and fibrotic lesions resulting from the exposure. Notably the relative lung weights (lung weight/body weight) were not increased. Concerning non-neoplastic lesions there are a range of described effects at time points ranging from 3 months of exposure until 6 weeks after the end of the experiments (Table 5). Notably, although clearly increased as evaluated by looking at the absolute numbers given in the appendix of the graph, no statistical analysis was

included. Because of this and because of the presence of neoplastic lesions in another pivotal study (as described below) we have not focused on non-neoplastic changes here.

Concerning sub-chronic inhalation studies and neutrophil inflammation there are a range of relevant studies. Of these we consider two to be the most important (Driscoll et al., 1996; Elder et al., 2005). Elder and co-workers, investigated CB NM Printex 90 (14 nm) in three species, mice, rats and hamster. The mass concentrations were 1, 7 or 50 mg/m^3. The duration was 6h/day 5 days /week for 13 weeks. The animals were evaluated at 5 weeks, and 13 weeks (end of exposure), and after recovery periods of 3 or 11 months post exposure. The NOAEC for neutrophil numbers in BAL was at the end of exposure 1 mg/m^3 for rats, mice and hamsters. Other endpoints investigated were body weight, lung weight, as well as histopathology. Only minimal effects were seen on body weight. Only high dose in hamsters showed decreased body weight at the end of exposure. The lung weight was increased at high dose in all three animal species, and also at the mid dose in mice at the end of exposure. Regarding histopathology, the density of alveolar type II cells was increased at high dose at the end of exposure but only for rats and hamsters. The percentage of cells in the S –phase was increased at high dose at the end of exposure but only in rats (Elder et al., 2005).

Driscoll *et al.,* investigated CB NM Monarch 880 (16 nm) in rats. The inhalation mass concentration was 1.1, 7.1 or 52.8 mg/m^3 and the duration was 6h/day, 5 days/week for 13 weeks. The NOAEC for increased neutrophil numbers in BAL was 1.1 mg/m^3. Other investigated endpoints included hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene mutation frequencies in alveolar epithelial cells, which were increased at 7 and 53 mg/m^3 at the end of exposure (Driscoll et al., 1996) suggesting that the used CB NM is mutagenic by inhalation.

Two other inhalation studies in rats also measured pulmonary inflammation by increased neutrophil numbers in BAL. These were done with 12 weeks of exposure and resulted in LOAECs of 3.5 and 10 mg/m^3 (Henderson et al., 1992; Wolff et al., 1990). In addition, there is a range of intratracheal instillation studies in mice and rats that also reported pulmonary inflammation by increased neutrophil numbers in BAL. These were done with CB NMs in the size range of 10 to 100 nm. These exposures resulted in lowest observed adverse effect levels (LOAELs) in the range of 0.02 to 16 mg/kg body weight (bw) and no observed adverse effect levels (NOAELs) in the range of 0.2 to 3.3 mg/kg bw (Bend et al., 2007; Bengtson et al., 2017; Bourdon et al., 2012; Chang et al., 2005; Chen et al., 2015; Cho et al., 2010; Danielsen et al., 2010; Götz et al., 2011; Hadrup et al., 2017; Husain et al., 2015; Jacobsen et al., 2015, 2009, Kyjovska et al., 2015a, 2015b; Li et al., 1999; Lu et al., 2009; Renwick, 2004; Roberts et al., 2015; Saber et al., 2012b, 2012a; Saber et al., 2016; Sager and Castranova, 2009; Schinwald et al., 2012; Shvedova et al., 2005; Shwe et al., 2005; Teeguarden et al., 2011; Yang et al., 1999).

In summary; inhalation of CB NM induced long lasting inflammation in rats, mice and hamsters. Taking the data on increased neutrophil numbers in BAL from both the chronic and the two subchronic studies into account (Driscoll et al., 1996; Elder et al., 2005; Mauderly et al., 1994) we suggest that a NOAEC is placed at 1 mg/m^3. This is based on a LOAEC of 2.5 in a chronic study and NOAECs of 1 and 1.1 mg/m^3 in two

subchronic studies. We acknowledge that the finding of a LOAEC of 2.5 mg/m^3 could warrant a somewhat lower NOAEC level, but that also depends on a chosen assessment factor for using a LOAEC instead of a NOAEC. If this factor for example is set to 3 (ECHA, 2012) the LOAEC in the chronic study would give a NOAEC also of approximately 0.8 mg/m^3.

Table 5. Overview of non-neoplastic endpoints in chronic subchronic CB inhalation studies used for evaluation of pulmonary inflammation hazard levels in the current report

Reference	CB NM	Animal species / Exposure	Endpoints/Results
(Mauderly et al., 1994)	Elftex-12 furnace black, 37 nm	Rats: Inhalation for 16 h/day, 5 days/week for up to 2 years (12 months for inflammation measurements), to 0, 2.5, or 6.5 mg/m^3 Regarding recovery time after exposure the following was stated in Mauderly *et al.* (Mauderly et al., 1994): "*The exposures were terminated at 24 months, and the remaining rats were transferred to an animal housing room where they were maintained in shoebox cages with hardwood-chip bedding (Murphy Forest Products, Montville, NJ) until mortality reached approximately 90%. All remaining rats in blocks A and B were killed and necropsies were performed between March 21 and 25, and between April 10 and 12, 1991, or 41 to 45 and 40 to 42 days after the end of the 24-month exposures, respectively.*"	BAL neutrophils after 12 months of exposure. Effects were observed at both doses. Also, lactate dehydrogenase and beta glucuronidase were increased in both doses. Survival (median) in days: Females: Control: 696 days, CB NM 2.5 mg/m^3: 707 days, CB NM 6.5 mg/m^3: 675 days (statistically significantly decreased). Males: Control 639 days, CB NM 2.5 mg/m^3: 605 days (statistically significantly decreased), CB NM 6.5 mg/m^3: 599 days (statistically significantly decreased). Lung weight: Increased for all CB NM dosed groups. No effect on relative lung weight. Non-neoplastic lesions there are a range of described effects (as compared to control) at time points ranging from 3 months of exposure until 6 weeks after the end of the experiments. Lesions described to be related to CB exposure consisted of alveolar macrophage hyperplasia and alveolar epithelial hyperplasia. Notably, although clearly increased as evaluated by looking at the absolute numbers given in the appendix of the graph, no statistical analysis was included
(Elder et al., 2005)	CB NM Printex 90 (14 nm)	Mice, rats and hamster: Inhalation of 1, 7 or 50 mg/m^3. The duration was 6h/day 5 days /week for 13 weeks. The animals were evaluated at 5 weeks, and 13 weeks (end of exposure), and after recovery periods of 3 or 11 months post exposure	BAL neutrophils 1-day post 13 week exposure: Rats: Increased numbers at 7 and 50, but not at 1 mg/m^3 Mice and hamster: The effects were similar to those observed in rats. Body weight: Decreased for hamsters at high dose (50 mg/m^3). No effects were observed at other doses and no effects were observed in mice and rats. Lung weight: Increased for all species at high dose (50 mg/m^3), and for mice at the mid dose 7

			mg/m^3). Histopathology, showed increased density of alveolar type II cells at high dose (50 mg/m^3) for rats and hamsters. Cells in the S –phase was increased for rats at high dose (50 mg/m^3)
(Driscoll et al., 1996)	CB NM Monarch 880 (16 nm)	Rats. Inhalation of 1.1, 7.1 or 52.8 mg/m^3. The duration was 6h/day, 5 days/week for 13 weeks	BAL neutrophils increased at 7.1 and 52.8 but not 1.1 mg/m^3 Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene mutation frequencies in alveolar epithelial cells: Increased at 7.1 and 52.8 but not 1.1 mg/m^3

Genotoxicity and cancer

Genotoxicity and cancer are well-studied endpoints and possible adverse effects of exposure to CB NM. Genotoxicity are often studied shortly after exposure, whereas cancer is a more complex pathological endpoint that requires longer time to develop. In the below section, the working group has chosen to differentiate between genotoxicity in shorter-term studies and cancer in long-term studies.

Cancer

IARC has classified CB as *possibly carcinogenic to humans* (group 2B). This classification was based on inadequate evidence for carcinogenicity in humans, but sufficient evidence of carcinogenicity in experimental animals (IARC, 2010).

There are some CB NM inhalation studies (Heinrich et al., 1995; Mauderly et al., 1994), and intratracheal instillation studies (Davis 1975, Rat; Dasenbrock 1996, Rat; Pott 1994, Rat;) having genotoxicity and cancer as main endpoints. The two pivotal chronic inhalation carcinogenicity studies are by Heinrich and co-workers using CB NM Printex 90 (14 nm) (Heinrich et al., 1995) and by Mauderly and co-workers using Elftex-12 furnace black (37 nm)(Mauderly et al., 1994). These will be addressed in detail below.

The Heinrich study (CB NM Printex 90)

Female rats were exposed to CB NM Printex 90 by inhalation (18 h/day, 5 days/week for up to 24 months). Printex 90 has a diameter of 14 nm, surface area 337 m^2/g and is specified as >99% pure CB (SCCS, 2015). The mass concentration was 7.2 mg/m^3 for the first 4 months and then 12.2 mg/m^3 for the next 20 months. This amounts to an average exposure of 11.6 mg/m^3 for 104 weeks. The rats were investigated for lung tumour incidence. The tested dose resulted in a significantly increased (tumour) incidence. Details of this study are presented in Table 6. Other endpoints such as mortality, body weight, organ weight and BAL endpoints were also analysed. It was shown that the lifetime of the rats was significantly shortened by CB NM exposure as compared to controls. The body weight was significantly reduced from day 300 for CB NM exposed rats as compared to controls. The lung weight was increased by CB NM exposure. Concerning BAL endpoints, differential cell count, and the concentration of lactate dehydrogenase, beta-glucuronidase, OH-proline and total protein in BAL showed CB NM exposure related effects. The BAL cellularity was not detailed further than in the article.

Female mice were exposed to CB NM Printex 90 by inhalation. The duration was 18 h/day, 5 days/week for up to 13.5 months. It is noted by the working group of the current report that 13.5 months is a relatively short study of carcinogenicity where guidelines suggest 24 months. The mass concentration was 7.2 mg/m^3 for the first 4 months and then 12.2 mg/m^3 for the next 9.5 months. This amounts to an average exposure of 10.7 mg/m^3. The mice showed no increase in lung tumour incidence. Details of this study are presented in Table 6. Other endpoints included mortality, body weight and lung weight. Increased mortality was described in the mice as being 20% for CB NM exposed animals as compared to 10% in the control group. However, it was not stated whether this was

statistically significant. The body weight was decreased and the lung weight was increased for the CB NM exposed mice as compared to controls (Heinrich et al., 1995).

The Mauderly study (CB NM Elftex-12 furnace black)

Female and male rats inhaled CB NM Elftex-12 furnace black (Cabot Corp. Boston, MA, USA) at mass concentrations of 2.5 or 6.5 mg/m^3 for 16 h/day, 5 days/week for 12 or 24 months. Elftex-12 has a diameter of 37 nm, a Brunauer–Emmett–Teller (BET) surface area 43 m^2/g and extractable organic material about 0.04–0.29%. The size was not reported in the study (Mauderly et al., 1994), but has been described elsewhere in the literature (e.g. (SCCS, 2015)). Mauderly and co-workers reported a Mass Median Aerodynamic Diameter of 101 nm as measured by small fraction parallel flow diffusion battery and a Mass Median Aerodynamic Diameter of 2000 nm as measured by large fraction cascade impactor (Mauderly et al., 1994). Neoplasms in lungs of female rats were found with "statistical significance". The lowest dose with a significant increased tumour incidence was 2.5 mg/m^3. There was no carcinogenic effect in male rats. Details of this study are presented in Table 6. Inflammatory effects, also observed by Mauderly and co-workers are described above in the section "Inflammation" (Mauderly et al., 1994). The same results of Mauderly et al., which is a report, is also reported in the article (Nikula et al., 1995).

Summary

In summary, Heinrich *et al.,* found significantly increased carcinogenicity at 11.6 mg CB NM/m^3 in female rats whereas Mauderly *et al.,* found significantly increased carcinogenicity at 2.5 mg CB NM/m^3 in female rats. This was the lowest tested dose in both studies. Heinrich and co-workers additionally conducted a negative cancer study in mice exposed for 10.7 mg/m^3, however, this exposure was only for 13.5 months. It is also noted that Mauderly *et al.* did not find an effect in male rats. CB has been classified as possibly human carcinogen by IARC (group 2B) based on sufficient evidence of carcinogenicity in experimental animals (IARC, 2010).

Table 6. Overview of chronic rat inhalation studies

Reference	CB NM	Animal species / Exposure	Lung tumour incidence
(Heinrich et al., 1995)	Printex 90, 14 nm	*Rats (only females were investigated):* 18 h/day, 5 days/week for up to 2 years to 0, or 11.6 mg/m^3 (average exposure over the 2-year period) followed by 6 months without CB NM exposure. *Mice (only females were investigated):* 18 h/day, 5 days/week for up to 13.5 months (the animals were kept at clean air for an additional 9.5 months) to 0, or 10.7 mg/m^3 (average exposure over the 13.5 month period) followed by 6 months without CB NM exposure	*Rats:* 11.6 mg/m^3: Increased (p<0.001 by Fischer's exact test done by the authors of the current report) Exposed: 39/100: Total number with tumours 20/100: Keratinizing cystic squamous-cell tumours 4/100: Squamous cell carcinoma 13/100: Adenoma 13/100: Adenocarcinoma Controls: 1/217: Total number with tumours 1/217: Adenocarcinomas *Mice:* 10.7 mg/m^3: There was no carcinogenic effect
(Mauderly et al., 1994)	Elftex-12 furnace black, 37 nm	Rats (female and male): Inhalation for 16 h/day, 5 days/week for up to 2 years, to 0, 2.5, or 6.5 mg/m^3 Regarding recovery time after exposure the following was stated in Mauderly *et al.* (Mauderly et al., 1994): "*The exposures were terminated at 24 months, and the remaining rats were transferred to an animal housing room where they were maintained in shoebox cages with hardwood-chip bedding (Murphy Forest Products, Montville, NJ) until mortality reached approximately 90%. All remaining rats in blocks A and B were killed and necropsies were performed between March 21 and 25, and between April 10 and 12, 1991, or 41 to 45 and 40 to 42 days after the end of the 24-month exposures, respectively.*"	***2.5 mg/m^3:*** Increased in females (p<0.01 by Fischer's exact test done by the authors of the current report). *Female rats*: 8/107: Malignant or benign lung neoplasms 7/107: Malignant lung neoplasms 2/107: Adenoma 6/107: Adenocarcinoma 1/107: Mixed mesenchymal and epithelial type tumour *Male rats:* 2/106: Malignant or benign lung neoplasms 1/106: Malignant lung neoplasms 1/106: Adenoma 1/106: Adenocarcinoma

			6.5 mg/m³: Increased in females (p<0.001 by Fischer's exact test done by the authors of the current report). *Female rats:* 28/105: Malignant or benign lung neoplasms 21/105: Malignant lung neoplasms 13/105: Adenoma 20/105: Adenocarcinoma 1/105: Squamous cell carcinoma 1/105: Adenosquamous carcinoma *Male rats:* 4/106: Malignant or benign lung neoplasms 4/106: Malignant lung neoplasms 1/106: Adenocarcinoma 2/106: Squamous cell carcinoma 1/106: Adenosquamous carcinoma **Controls**: *Female rats:* 0/105: Malignant or benign lung neoplasms *Male rats:* 3/109: Malignant or benign lung neoplasms 2/109: Malignant lung neoplasms 1/109: Adenoma 1/109: Adenocarcinoma 1/109: Squamous cell carcinoma

Genotoxicity

The genotoxic potential of CB NM exposure has been tested in several *in vivo* studies. Therefore, we have chosen not to include *in vitro* or *ex vivo* exposures in this section. Several of these are, however, included in the section "Mechanism of toxicity" where we also discuss evidence for a non-threshold mechanism for genotoxicity and carcinogenicity. The endpoints within this section include deoxyribonucleic acid (DNA) strand breaks, DNA adducts and *Hprt* mutation. It is a general consensus that exposures causing permanent changes to the DNA, e.g. mutations are also carcinogenic. The same strong association has not yet been documented for DNA strand breaks measured by the comet assay. Comet assay is a popular assay in the area of nanotoxicology (Karlsson, 2010), but based on the transient nature of the detected damage to DNA and the lack of a clear link to carcinogenesis it will bear less weight as also discussed in a recent review (Møller and Jacobsen, 2017). However, genotoxicity detected by the comet assay is always a course for concern and should merit further studies clarifying the results.

Mutations have been detected in rat pulmonary alveolar epithelial cells following inhalation exposure 6h/day, 5 days/week for 13 weeks to 7.1 and 52.8 mg/m^3 CB NM (Monarch 880, 16 nm, 220 m^2/g, Cabot Corp., MA, USA). The lung burdens were estimated to 1826 and 7861 mg, respectively. *Hprt* mutation frequency was significantly increased immediately after the 13 weeks of inhalation (7.1 and 52.8 mg/m^3) but also after a 3- and 8-month recovery period (52.8 mg/m^3). For the high exposure dose the increase in *hprt* mutation frequencies were 4.3-, 3.2-, and 2.7-fold greater than the air control group, immediately after exposure and 3- and 8-months after exposure, respectively. No adverse effects were detected following inhalation of 1.1 mg/m^3 (deposited dose 354 mg) (Driscoll et al., 1996). Instillation of a much smaller dose 0.2 mg Printex 90/animal did not lead to any increase in mutant frequencies in the lungs of the *gpt* delta transgenic mouse model (Totsuka et al. 2009).

Rats inhaled CB NM Printex 90 (14 nm; 300 m^2/g) or CB NM Sterling V (70 nm; 37 m2/g) 6h/day 5 days/week for 13 weeks. The mass concentration of CB NM Printex 90 was 1, 7 or 50 mg/m^3 whereas it was 50 mg/m^3 for CB NM Sterling V. Exposure to CB NM Printex 90 resulted in the formation of 8-Oxo-2'-deoxyguanosine (8-oxo-dGua) in lungs at the highest dose immediately following the exposure period, and at both the high and middle dose following a 44-week recovery period. No effect was observed at 1 mg/m^3. There was no effect of CB NM Sterling V, despite a retained particle surface area comparable to the middle dose of Printex 90 (Gallagher 2003, Rat). Using very low exposure doses (0.16 mg/m^3 for rats and 0.14 mg/m^3 for mice) for 4 h (rats) or 4 and 3x4 h (mice) Wessels and co-workers did not detect oxidative DNA damage by the formamidopyrimidine DNA glycosylase (FPG) modified comet assay in lung tissue of pulmonary epithelial cells (Wessels et al., 2011).

Danielsen and co-workers exposed male rats orally to 0.64 mg/kg CB NM Printex 90. A significant increase in 8-oxo-dGua was observed in liver, but not lung. No increase in bulky PAH-DNA adducts were observed 24 h post exposure in lung or liver tissue (Danielsen et al., 2010). Analysis for DNA adducts formed by CB NM exposure have been examined in a few studies. Borm and co-workers tested Printex 90 (1, 7 and 50 mg/m^3) and Sterling V (50 mg/m^3) in a 13-week rat inhalation study (particles as

mentioned in the above section). No PAH-DNA related adducts were observed in the lung tissue compared to sham exposed rats (Borm et al., 2005). A lack of bioavailability of surface PAHs was suggested due to tight adhesion. One study has reported a significant increased level of DNA adducts in isolated rat type II alveolar cells when compared to the filtered-air controls was reported (Bond et al. 1990). The rats were exposed to 6.2 mg/m^3 (16 h/day, 5 days/week) for 12 weeks. The same material (CB NM Elftex-12, Cabot Corp. Boston, MA, USA) tested negative for induction of DNA adducts in another rat inhalation study (7 h/day, 5 days/week for 12 weeks at 10.0 mg/m^3) (SCCS, 2015).

There is a range of inhalation and intratracheal instillation studies in which CB NM induced DNA strand breaks using comet assay were examined. Mice inhaled CB NM Printex 90 (aerosol agglomerate size was 65 nm) at a mass concentration of 20 mg/m^3 and the total duration was 6 h distributed over 4 days. DNA strand breaks were detected in BAL cells. Tissues were not analyzed (Saber et al., 2005). Pregnant mice inhaled CB NM Printex 90 at a mass concentration of 42 mg/m^3. The duration was 1 h on each of gestation days 8 to 18; 11 h in total. This resulted in elevated DNA strand breaks damage in the liver as measured by comet assay at 3 days of exposure. DNA damage was not increased in BAL cells. Other tissues were not examined (Jackson et al., 2012a).

Concerning intratracheal instillation studies in mice and rats there are a range of studies that report effects in the comet assay following CB NM Printex 90 exposure at doses in the range of 0.02 for to 8.1 mg/kg bw (Bengtson et al., 2017; Bourdon et al., 2012; Hadrup et al., 2017; Husain et al., 2015; Kyjovska et al., 2015a, 2015b; Saber et al., 2012a). However, there are also studies in which no effect was observed using the same Printex 90 and Lampblack 101 in the dose range of 0.6 to 8.1 mg/kg bw (Danielsen et al., 2010; Jackson et al., 2012a; Saber et al., 2012b; Saber et al., 2012c; Saber et al., 2016, 2005).

In summary, the present working group concludes that there is clear evidence for genotoxicity of CB NM. Above-mentioned *in vivo* studies show that CB NM can induce mutations, oxidative damage to DNA as well as DNA strand breaks in rats and mice. It is clear that inflammation is closely linked to genotoxicity via secondary cell driven production of reactive oxygen species (ROS). Primary and secondary particle effects can be challenging to separate within *in vivo* studies; however, the present working group do find support for primary production of ROS could have some importance in the genotoxicity of CB NM. This primary genotoxic effect does not include DNA bulky adducts. Although a limited number of materials have been tested regarding adduct formation, it is noteworthy that Sterling V, a dirty CB material containing high level of PAHs, is amongst those. Therefore, this working group does not find sufficient evidence for a direct acting mechanism via bulky PAH-DNA adduct formation.

Cardiovascular effects

The term cardiovascular effects cover all pathological changes in the entire circulatory 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/ischemic heart disease and stroke.

Only few studies have investigated the cardiovascular effects of pulmonary CB NM exposure and some of these have investigated the acute phase response; a promising early biomarker for cardiovascular disease. Below is a brief overview of the scientific literature touching on the subject of CB NM-induced cardiovascular effects.

Cardiac physiology

Concerning inhalation studies, mice were exposed to CB NM by inhalation of 0.55 mg/m^3 for 3 consecutive days (2 h/day). The CB NM aerosol was characterised by a count median diameter of 0.7 μm and a mass median aerodynamic diameter of 1.0 μm. The size and commercial name of the CB NM were not reported. CB NM exposure demonstrated considerable (although some inconsistent) changes in the associations between breathing and cardiovascular responses describing heart function (Hamade et al., 2010). The relevance of the finding in this study in connection to the derivation of an OEL is currently unclear and further investigation would be needed to clarify this. Rats were exposed by inhalation to CB NM (86 nm). The particle exposure concentration was 1.3×10^5, 6.2×10^5, or 4.2×10^6 particles/cm^3. Using a density of 1.7 g/mL this corresponds to ~0.07, 0.3 and 2.4 mg/m^3, respectively. The duration was 4 h/day, 5 days/week for 4 weeks. There was no effect on plasma coagulation, platelet aggregation, or on vasomotor function (Kim et al., 2011).

Mice were exposed to ultrafine CB NM (Hiblack 41Y, 19 nm) by intra-tracheal instillation (every 2 days for a total of 3 times) at 0.05, 0.15 and 0.6 mg/kg bw. At the two highest doses heart rate variability indices were decreased. At the highest dose slight pulmonary inflammation and myocardial injury were observed (Jia et al., 2012). Rats were exposed to CB NM (N330, 28-36 nm; N990, 250-350 nm) by intratracheal instillation at 1, 3, or 10 mg/kg bw. No cardiac symptoms were detected by electrocardiographic endpoints describing different measures of heart function and heart rate variability (Kim et al., 2012).

Accelerated plaque progression and vascular dysfunction

The lipid profile of mice significantly differs from that of humans. Mice do not develop atherosclerosis, because 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 apolipoprotein E knockout (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 for investigating cardiovascular effects.

Regarding studies in knockout mice, ApoE$^{-/-}$ mice were exposed to CB NM Printex 90 (14 nm) by intratracheal instillation at 8.5 or 26 μg/mouse (~0.4 or ~1.3 mg/kg bw). CB NM

exposure was not associated with promoted plaque progression in aorta or brachiocephalic artery; and there was no effect on *Saa3* messenger ribonucleic acid (mRNA) levels in lung. In contrast, plasma from CB NM exposed mice caused vasoconstriction in aorta rings isolated from naïve mice (Christophersen et al., 2016). ApoE$^{-/-}$ mice were exposed to CB NM (Printex 90, 14 nm) by intratracheal instillation. Two consecutive CB NM doses each of 0.5 mg/kg bw caused decreased acetylcholine-induced vasorelaxation in aorta segments. This was not observed following single doses in the range of 0.05 to 2.7 mg/kg bw. CB NM exposure did not affect the progression of atherosclerotic plaques in aged ApoE$^{-/-}$ mice. Moreover, CB NM did not affect vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression and did not affect the 3-nitrotyrosine levels in the vascular tissue of either young or aged ApoE$^{-/-}$ mice (Vesterdal et al., 2010). Low density lipid receptor (LDLr)$^{-/-}$ Mice were exposed to CB NM by intratracheal instillation 1 mg/week for 10 weeks. This was done in the presence or absence of 0.51% cholesterol diet. The CB NM had an agglomerate size of 121 nm in diameter; no manufacturer name was given, and the total dose given over 10 weeks was 500 mg/kg bw. CB exposure resulted in accelerated development of atherosclerosis in mice receiving a high-cholesterol diet as compared to control mice on cholesterol diet (Niwa et al., 2007).

Acute phase response

The acute phase response is an early defence system induced in e.g. humans in response to e.g. infection, infarction, inflammation and trauma. 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 are intimately linked to risk for cardiovascular disease in both epidemiological (human) and animal studies (Johnson et al., 2004; Lowe, 2001; Mezaki et al., 2003; Ridker et al., 2000). In mice, the four 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). In a recent review, evidence for a close correlation between the deposited surface area of nanoparticles, the generated pulmonary inflammation (neutrophil influx) and the resulting level of acute phase response was presented (Saber et al., 2014). These results implied that even smaller elevations of the inflammatory state have an influence on the risk for cardiovascular disease.

Several studies have examined changes in *Saa* expression. Mice were exposed to CB NM Printex 90 by inhalation. The mass concentration was 20 mg/m^3 and the duration was 1.5h/day for four consecutive days. There was no evidence of the induction of an acute phase response in the livers of the mice. However, when considering this result the short exposure time should be taken into account (Saber et al., 2009). Mice were exposed to CB NM by intratracheal instillation of 0.67, 2, 6, or 162 μg Printex 90 CB NM (~0.04, 0.1, 0.3 and 8.1 mg/kg bw). There was only increased *Saa3* mRNA levels in lung at 8.1 mg/kg bw (Kyjovska et al., 2015a). Mice were exposed to CB NM, Printex 90 by intratracheal instillation at a dose of 162 μg/mouse (8.1 mg/kg bw). The *Saa3* mRNA level was increased in lung at all three investigated time points 1, 3 and 28 days (Kyjovska et al.,

2015b). Mice were exposed to CB NM at 18, 54 or 162 μg/mouse by intratracheal instillation and humanely killed at 1, 3 or 28 days of recovery (~0.9, 2.7 and 8.1 mg/kg bw). CB NM exposure resulted in increased expression of *Saa3* mRNA in lung tissue on day 1 (all doses), 3 (all doses) and 28 (middle and high dose), but not in liver (Bourdon et al., 2012).

Other cardiac endpoints

Mice were intratracheally instilled with 162 μg CB NM Printex 90 (~8 mg/kg bw) and humanely killed at 1, 3 or 28 days post exposure. CB NM exposure was associated with increased plasma levels of markers of endothelial inflammation (soluble-E-selectin, soluble-ICAM-1, soluble-VCAM-1) and total PAI-1. CB NM exposure did not alter cardiac gene expression as measured by gene array. It was concluded that CB NM exerted adverse cardiovascular effects, in absence of changes in cardiac tissue gene expression (Bourdon et al., 2013a). Mice were exposed to CB NM by oropharyngeal aspiration at doses of 10 or 40 μg (~0.5 or ~2 mg/kg bw). The size of the CB NM ranged from 71 to 96 nm as measured by dynamic light scattering (DLS). Twenty-four hours after the instillation, the animals had their hearts perfused and excised. A 20 min period of experimental cardiac ischemia was applied to the perfused hearts. There was no effect of CB NM exposure on cardiac functional recovery, infarct size, and coronary flow rate in the isolated perfused hearts (Tong et al., 2009). Rats were exposed to CB (N330, 28-36 nm; N990, 250-350 nm) by intratracheal instillation at 1, 3, or 10 mg/kg bw. N330 caused accelerated platelet-dependent blood clotting at the highest dose. Both particles caused prolonged activated partial thromboplastin time but only at the mid doses. No effect was observed on prothrombin time (a measure of coagulability, the time it takes plasma to clot after addition of tissue factor) (Kim et al., 2012).

In summary; one of the above studies on cardiovascular toxicity study finds effects at 0.55 mg CB NM/m^3 (2 h/day for 3 days). Considerable changes in heart function response were observed. However, as the reported changes were not consistent, this study group does not consider these effects to be suitable for OEL derivation. The other inhalation study was performed using a mass concentration of 20 mg/m^3, a much higher level compared to studies on inflammation described above.

Concerning studies with intratracheal instillation, the above described studies reported that doses having effect were in the range of 0.15 mg/kg bw to 500 mg CB NM/kg bw. A NOAEL based on these intratracheal studies would be set to 0.05 mg/kg (bw). This NOAEL could be hypothesised to translate to a mass concentration of ~0.7 mg/m^3 with a deposition fraction of 50% and using this NOAEL as a NOAEL per day. However, inhalation studies are deemed of higher importance than intratracheal instillation studies. The NOAEC in inflammation studies (described above) was 1 mg/m^3, a value not far from a potential LOAEC of 0.55 mg/m^3 from the above described inhalation study. Taken together, the present working group find that the data on cardiovascular toxicity caused by CB NM are not strong enough to lower the suggested NOAEC of 1 mg/m^3 for pulmonary inflammation.

Reproductive toxicity

Time mated female mice were exposed to CB NM Printex 90 either 1) by inhalation of 42 mg/m^3 for 1 h/day on gestation days 8-18; or 2) by intratracheal instillation to CB NM Printex 90 on gestation days 7, 10, 15 and 18 at cumulative doses of 11, 54 and 268 μg CB NM/mouse (equal to 0.55, 2.7 and 13.4 mg/kg bw). CB NM exposed mothers exhibited persistent lung inflammation at 24-27 days after exposure for both administration routes, albeit only at the highest intratracheal instillation dose. CB NM exposure did not affect gestation or lactation. DNA strand breaks were increased in the liver of mothers and their offspring following inhalation exposure only (Jackson et al., 2012a). Offspring from the highest dose level of intratracheal instillation were subsequently tested in the open field test. Female offspring showed an altered habituation pattern (Jackson et al., 2011). Furthermore, DNA microarray was performed on tissues from male and female offspring from the highest intratracheal instillation level. Liver was recovered on postnatal day 2 and from dams 26-27 days after exposure. Maternal instillation exposure to CB NM changed expression of several genes, significantly more in female compared to male offspring, indicated that female offspring were more sensitive than male offspring in regard to changes in liver mRNA levels. Affected pathways in female offspring included: cellular signalling, inflammation, cell cycle regulation and lipid metabolism. In males there were subtle changes in metabolism-related genes (Jackson et al., 2012b). Finally, female and male F1-offspring were raised to maturity and subsequently mated with unexposed animals. Testicles were collected from mature F1- and F2-males. F2-offspring, whose fathers were prenatally exposed to CB NM Printex 90, showed lowered sperm production (Kyjovska et al., 2013). The expanded simple tandem repeat germline mutation rates were not different in CB NM-exposed F2 female offspring as compared to controls (Boisen et al., 2013).

Pregnant mice were exposed to CB NM by intranasal instillation on gestational day 5 and 9 at a cumulative dose of 190 μg/kg bw. Splenocyte phenotypes as well as cytokine mRNA levels were determined at day 1, 3, 5, and 14 days postpartum. In the CB NM group there was a decrease in numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells in the spleen from 1, 3 and 5-day-old offspring. In addition, CB NM exposure was associated with an increased Interleukin-15 mRNA level in the spleen of new-born male offspring; and increased Ccr7 and Ccl19 mRNA levels of female offspring spleen. The authors conclude that exposure of pregnant mice to CB NM partially suppressed the development of the offspring immune system (Shimizu et al., 2014). In another study by the same research group, pregnant mice were exposed to CB NM using the same dosing regimen. CB NM increased total thymocyte count and certain specific immunophenotypes ($CD4^-CD8^-$ and $CD4^+CD8^+$ cells). In addition, an increase in total lymphocytes, and $CD4^-CD8^-$cells was observed. The authors conclude that that maternal respiratory exposure to CB NM during middle and late gestation may have allergic or inflammatory effects in male offspring (El-Sayed et al., 2015). Several studies have used this exposure regimen and investigated brains of the male offspring 6 to 12 weeks after birth. CB NM exposure was generally associated with enlarged granules in perivascular macrophages and a decrease in the number of periodic acid Schiff positively stained perivascular macrophages. Furthermore, the expression level of glial fibrillary acidic protein was increased in astrocytes, indicative of long-term activation of astrocytes and reactive astrogliosis. In an

exposure-effect study, using cumulative doses of 5.8, 30 and 146 μg/kg body weight (bw), 5.8 μg/kg (bw) could be identified as the NOAEL. The authors conclude that maternal CB NM exposure is associated with a decrease in *normal* perivascular macrophages, and the changes in expression of glial fibrillary acidic protein in astrocytes are indicative of long-term activation of astrocytes and reactive astrogliosis in a wide area of the CNS of the offspring. Overall, CB NM maternal exposure may increase the risk for neurological changes in the offspring (Onoda et al., 2017b, 2017a, 2017c, 2014). These latter outcomes have also been investigated following inhalation exposure. Time mated mice were exposed for 45 min/day to 0, 4.6 or 37 mg/m^3 aerosolized Printex 90 on gestation days 4–18, i.e. for a total of 15 days. No lung inflammation was observed in the exposed females, when measured 11 or 28 days post-exposure. In the offspring, glial fibrillary acidic protein expression levels were dose-dependently increased in astrocytes in the cerebral cortex and hippocampus at six weeks of age, as also described above for the instillation studies. Lysosomal granules were also enlarged in the brain perivascular macrophages. Assessment of behaviour in the open field test at 90 days of age showed dose dependent alterations, with prenatally exposed female offspring moving a longer distance and males spending longer time in the central zone of the maze. Finally, the number of parvalbumin-positive interneurons and the overall expression level of parvalbumin were assessed at the highest dose level. Both were decreased in the motor and prefrontal cortices at weaning and 120 days of age in prenatally exposed compared to control offspring. The authors conclude that the effects are similar to those observed after instillation exposure and furthermore that some of the observed effects resemble those observed in mouse models of neurodevelopmental disorders (Umezawa et al., 2018).

In summary, the present working group finds that the experimental studies indicate possible adverse reproductive effects such as genotoxicity in liver and suppressed development immune system in the offspring; to the least such an effect cannot be excluded. It should, however, be underlined that the available data is far too limited and does not yet include any pulmonary exposure guideline studies. Overall, within the above studies on reproductive toxicity, effects were observed at exposure to 4.6 CB NM/m^3 for 45 min /day. A NOAEL as low as 5.8 μg/kg (bw) was observed following intranasal instillation exposure, and with a hypothetical deposition fraction of 50% this could be translated to a mass concentration of 0.07 mg/m^3. However, inhalation studies are deemed of higher importance than intratracheal instillation studies. When using intratracheal instillation a bolus dose is given. This results in a very high dose rate and the importance of dose rate for inflammation and translocation to sites for reproductive toxicity is unknown. The lowest observed LOAEC/LOAEL is following intratracheal instillation. This taken together with the LOAECs and NOAECs observed for neutrophil accumulation in BAL in the inhalation studies, described above, leads us to suggest that reproductive toxicity is not the critical effect in the current report. This, however, may change if experiments with reproductive endpoints with lower inhalation mass concentrations of CB NM are undertaken in the future.

Other toxicological endpoints

Following the overview of the literature, the present working group suggests that it is highly likely that the critical endpoint of pulmonary exposure to CB NM is to be identified among inhalation studies and the toxicity endpoints described above (inflammation, genotoxicity and cancer, cardiovascular toxicity, reproductive toxicity). However, to ascertain that no toxicological endpoints had been overlooked, a literature search was done in the PUBMED database on the following: "Carbon black AND sensitisation", "Carbon black AND neurotoxicity AND inhalation", "Carbon black AND liver AND toxicity AND inhalation" and "Carbon black AND kidney AND toxicity AND inhalation". No relevant articles were identified following any of these searches.

MECHANISMS OF TOXICITY

Pulmonary inflammation, genotoxicity and cancer

Toxicity of CB NM depends on the pulmonary deposition dose which is expected to follow traditional fractional deposition patterns as described in the literature (Jacobsen et al., 2009; Oberdörster et al., 2005). Pulmonary exposure to CB NM has consistently been shown to cause dose-dependent pulmonary inflammation, with close correlation to deposited surface area and inverse correlation to particle size (Bourdon et al., 2013b; Elder et al., 2005; Saber et al., 2012b; Stoeger et al., 2007, 2006). One study has demonstrated that this correlation is also valid using 6 finely tuned carbon materials all within a narrow nano size range (primary particle size 10-50 nm and specific surface area 30-800 m^2/g)(Stoeger et al., 2007, 2006).

Based on this, the present working group concludes that inhalation of CB NM induces dose dependent pulmonary inflammation and that neutrophil influx is predicted by the total surface area of deposited particles. In addition, the present working group considers inflammation as a threshold effect.

The International Agency for Research on Carcinogenicity (IARC) has classified CB NM as possibly carcinogenic to humans (group 2B) based on sufficient evidence of carcinogenicity in experimental animals and *inadequate evidence* in humans for the carcinogenicity of CB NM. In the evaluation, IARC does not distinguish between CB and CB NM (IARC 2010). One pathway towards cancer suggested by IARC followed the following line of events: Inhalation and deposition leading to inflammation with cell injury and proliferation. This could directly cause mutations and carcinogenesis. A second described pathway depended on increased levels of ROS leading to mutations and carcinogenesis. The importance of inflammation in carcinogenesis of CB NM is supported by the observation that 13 weeks of inhalation of CB NM only resulted in mutations in the lung epithelium at doses that caused inflammation (7.1 and 52.8 mg/m^3). A lower dose (1.1 mg/m^3) did not result in inflammation or mutations (Driscoll et al., 1996); this supports the idea of a threshold effect.

Several studies have shown that CB NM particles generate very high levels of ROS in a concentration-dependent manner in both acellular and cellular tests (Folkmann et al., 2009; Foucaud et al., 2007; Høgsberg et al., 2013; Jacobsen et al., 2008b; Koike and Kobayashi, 2006; Saber et al., 2012b; Wilson et al., 2002). This opens for the possibility for a primary and directly particle-driven imbalance in the oxidative stress defence. This will be elaborated further below.

Secondary genotoxicity due to particle-induced inflammation and activated phagocytes is another important and well-documented mechanism of action for the development of lung cancer. It has previously been documented that exposure to CB NM may result in damage to DNA as e.g. measured by the comet assay. Increased levels of DNA strand breaks caused by CB NM exposure have been observed both *in vitro* and *in vivo* in BAL cells and lung and liver tissue (Bourdon et al., 2012; Jackson et al., 2012a; Jacobsen et al., 2009, 2007; Saber et al., 2005). The oxidatively damaged DNA lesions detected in CB NM

Printex 90-exposed cultures encompass 8-oxo-dGua as well as ring-opened formamido-pyrimidine lesions. These were detected in the formamidopyrimidine DNA glycosylase (FPG) modified comet assay (Jacobsen et al., 2007). Increasing the exposure duration has shown that long-term non-cytotoxic exposures to CB NM Printex 90 are associated with a modest, but statistically significant increase in the *cII* and *lacZ* mutation frequency in FE1-Muta™Mouse cells (Jacobsen et al., 2007). The level of mutations was similar to that observed following exposure to the standard reference material (SRM) 1650; a standard reference diesel exhaust particle from a heavy-duty truck available from the National institute for standards and technology (Jacobsen et al., 2008a).

The CB NM induced mutation spectrum was found to be in line with a fingerprint of oxidative damage to DNA. E.g. the most frequent mutation was G:C→T:A transversions that is likely caused by formation of 8-oxo-dGua mispairing with dA during replication. It was suggested that the observed spectrum of CB NM Printex 90-induced mutations was a direct consequence of oxidative damage to DNA, which in turn is a consequence of the high ROS production (Jacobsen et al., 2008a).

Rats were exposed to saline or saline suspensions of 10 and 100 mg/kg bw of CB NM by intratracheal instillation. Fifteen months after exposure, neutrophilic inflammation was detected in all CB NM exposed rats. Additionally, *Hprt* mutation frequency was increased in alveolar type II cells and epithelial hyperplasia was observed in the high exposure group (Driscoll et al., 1997).

The cell mediated secondary genotoxicity was examined by exposing RLE-6TN cells to macrophage or neutrophil enriched BAL cells from rats treated with 100 mg/kg bw of CB NM. Both exposures (macrophages or neutrophils) increased *Hprt* mutation frequency; however, the mutagenic activity appeared greatest for neutrophils. Addition of catalase to the BAL cell exposures:RLE-6TN co-cultures inhibited the increase in *Hprt* mutation frequency (Driscoll et al., 1997) suggesting a mechanism via oxidants and hydrogen peroxide. Overall, the study suggests that exposures generating significant inflammation are associated with increased level of mutations in epithelial cells.

However, inflammation alone does not always result in genotoxicity. Mice exposed for one of 3 NM (TiO_2, CeO_2 or CB NM; Printex 90) showed a strong and very similar inflammatory response. All 3 materials translocated and were detected in the livers. None of the materials caused increased genotoxicity at day 1. However, only the CB NM, which was also the only NM to produce ROS, caused an increased level of DNA strand breaks and this was observed after 1 month and 180 days (Modrzynska et al., 2018).

Particulates generated by combustion processes are often complex mixtures of organic compounds and smaller levels of metals adhered to a carbon core. The same is true for CB NM products, although the carbon content is high with normally only smaller traces of other substances (Bingham and Cohrssen, 2012). It has been suggested that since CB NM normally contains small amounts of PAHs (compared to soot) and desorption occurs slowly and to a very low degree, the adverse health effects are associated with the insoluble particle core (Borm et al., 2005; Jacobsen and Clausen, 2015).

Non-threshold carcinogenic effect

In a recent evaluation of the genotoxicity of CB (Chaudhuri et al., 2018) the authors argue that the apparent lack of DNA-PAH adducts following CB exposure strongly supports the view of no direct interaction between DNA and CB. The general statement of no PAH adducts is correct and is based on the lack of DNA-adduct formation in the lungs of rats in several studies (Borm et al., 2005; Gallagher et al., 1994; Wolff et al., 1990). Bond and co-workers did observe DNA adduct formation in rat alveolar type II cells, in a similar inhalation experiment and using the same material as Wolff and co-workers (Bond et al., 1990). Also, DNA-PAH adducts were analysed in A549 lung epithelial cells following exposure to CB NM (Printex 90, Lampblack 101, N330 or Sterling V). Only Sterling V, the CB NM with the highest PAH content, showed adduct spots using the ^{32}P post-labelling detection method (Borm et al., 2005). However, the bioavailability of CB surface PAH has been questioned several times. In general, removing such PAH requires very harsh chemical treatments. E.g. Borm and co-workers did not observe leakage of PAHs from CB particles when shaken in a range of saline/dipalmitoyl-phosphatidyl-choline concentrations (24 h at 37°C in the dark) (Borm et al., 2005). Combined, the above results could indicate no direct DNA interaction, but they could also indicate a low bioavailability of PAH for the majority of CB NM. However, the present working group see several evidences in favour of a non-threshold mechanism, thus supporting the latter statement.

As detailed described above, CB NM has been shown to induce ROS in acellular and cellular assays. The ROS induction is proportional to the specific surface area of the carbon black particles (Saber et al., 2012b). ROS would be expected to cause oxidative DNA damage (Møller et al., 2015) and DNA strand breaks, but not bulky DNA adducts. It has been shown several times that CB NM can enter cell cytosol. E.g. transmission electron microscopy of 16HBE cells exposed to CB NM (13 and 21 nm) for 24h showed aggregates of NMs present either inside endosomes. About 80% of analysed cells contained NM. Uptake was additionally supported by flow cytometry which showed a dose dependent uptake of CB NM (Hussain et al., 2009). However, a central question is whether CB NM enters the cell nuclei to exert a potential primary and direct genotoxic effect. In this regard, it has been demonstrated *in vitro* that oxidised CB (127 nm in hydrodynamic diameter) labelled with a fluorescent marker entered the nucleus of cells in two cell lines; murine macrophage cells (RAW 264.7) and epidermal cervical carcinoma calls (CaSki) (Amornwachirabodee et al., 2018).

CB NMs are great ROS producers. Hydrogen peroxide and hydroxyl radicals accounted for < 20% of the ROS produced by Printex 90. Other CB-based black tattoo inks showed similar results (Høgsberg et al., 2013). In this regard it is interesting that hydrogen peroxide is a relatively stable ROS which are frequently used as positive control cell exposures for the comet assay. Thus, a proportion of ROS produced outside of CB exposed cells may move to the nucleus causing genotoxicity.

Two chronic cancer studies exist for inhalation of CB NM. In case of a non-threshold mechanism the dose response curve for tumour induction should be linear and extrapolate to the background tumour frequency. In Figure 1 the dose response curve of

the combined data from female rats in these two long-term chronic cancer studies are presented (Heinrich et al., 1995; Mauderly et al., 1994). Both studies found increased cancer incidence at their lowest tested CB NM concentration. The control groups (0 mg/m^3) of the two studies overlap with a tumour frequency of 0.0% and 0.5%. We have chosen to present the data as constrained; fixed through x=0 mg/m^3 and y= 0.25% (the mean of the controls in the 2 studies) and without constrictions (free floating). The computed regression lines have a very high coefficient of determination (R^2); around 0.98 supporting the linear effect.

The regression lines are expressed as:

Constrained:	y= 3.494x + 0.25
Not constrained:	y= 3.462x + 0.5357

This means that the "free floating" linear regression suggests a tumour incidence about 0.5%. If we eliminate the background data (0 mg/m^3) the regression line for the remaining three points would be expressed as: y= 3.414x + 0.956 (R^2=0.96).

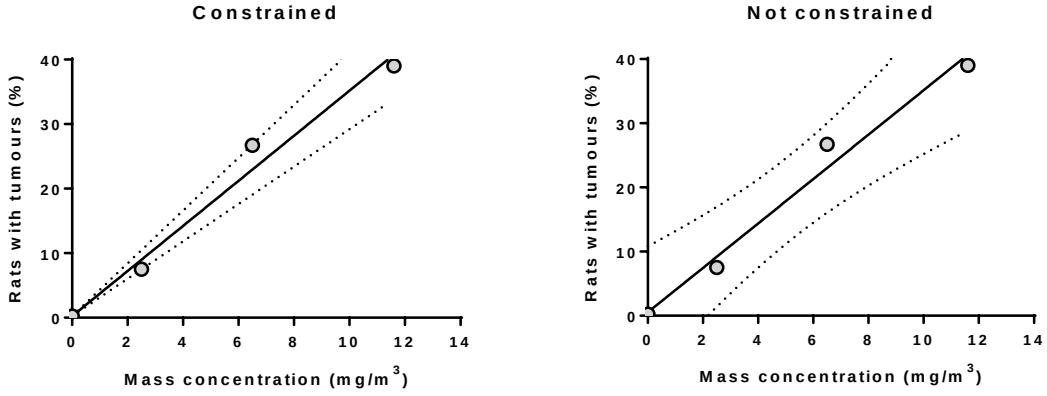

Figure 1. Frequency of female rats with tumours as a function of CB NM mass concentrations in the chronic inhalation studies by (Heinrich et al., 1995; Mauderly et al., 1994). Notably the 2 values at 0 mg/m^3 overlap (0 and 0.5%). Left: The graph has been constrained and forced through (0; 0.25). Right: Data represented without constrictions. Dotted lines represent 95% confidence interval for the regression lines.

As mentioned previously it is challenging to separate primary and secondary genotoxicity and to show if CB NM induces genotoxicity in the absence of inflammation. Below we will show that a strong NM induced inflammation does not always lead to genotoxicity and that CB genotoxicity has been observed without concomitant inflammation.

As described above, mice were exposed to TiO_2 NM, CeO_2 NM and CB NM (Printex 90) by a single intratracheal instillation of 162 μg/mouse. All three materials caused a very similar and long lasting pulmonary inflammation. A strong inflammatory response was observed following 1 day, much less but still very similar inflammation for all 3 materials following 1 month and a return to baseline was observed after 180 days; the

last of the 3 tested time-points. DNA strand breaks were detected using comet assay in liver, lung and BAL cells. None of the materials caused increased genotoxicity at day 1. However, only the CB NM caused an increased level of DNA strand breaks and this was observed after 1 month and 180 days. None of the materials gave any significant increase in BAL cells or lung tissue. All 3 materials translocated and were detected in the livers using enhanced dark-field hyperspectral microscopy; only the CB NM produced ROS measured with the $DCFH_2$ assay. Therefore, the authors conclude that their findings indicate that hepatic genotoxicity are caused by a direct genotoxic effect of translocated CB NPs rather than being caused by inflammatory responses (Modrzynska et al., 2018).

Mice were exposed to CB NM (Printex 90) to a single intratracheal instillation of 0.67, 2, 6, and 162 μg/mouse. Animals were examined following 1, 3, and 28 days post exposure. The 3 low doses of CB NM induced a slight or no neutrophil influx one day after exposure. DNA strand breaks in BAL cells, lung, and liver tissue were assessed and an increase in genotoxicity were observed in BAL cells on day 1 (0.67 and 2 μg), on day 28 in lung tissue (2 μg) but not in liver tissue at any time point. The authors interpret the genotoxicity as increased DNA damage and repair activity occurring in the absence of substantial inflammation and therefore as being caused by primary particle genotoxicity (Kyjovska et al., 2015a).

The cell line Muta™Mouse - FE1 was previously developed via spontaneous immortalization of lung tissue from a male Muta™Mouse. A characterization of development and the cell line that retain pulmonary epithelial characteristics have previously been published (White et al., 2003). Additional, results have shown the origin of the FE1 lung cell line as presenting a phenotype of both type I and type II alveolar and have a similar Global transcriptional characterization and response as primary lung epithelial cells were derived from mature male Muta™Mouse (Berndt-Weis et al., 2009). The cell line was recently exposed for 3 levels of Mitsui-7 CNT (12.5, 25 and 100 μg/ml corresponding to 3.9, 7.8 and 31.19 $\mu g/cm^2$ of Petri dish, respectively) for 24 h. A global transcriptomic analysis showed that the cell line did not express inflammatory genes to the extent of pulmonary tissue from exposed mice (Poulsen et al., 2013). This could be interpreted as the Muta™Mouse - FE1 is a cell line expressing low or no inflammation upon NM exposure and then indicate that the genotoxicity previously observed using the same cell line (Jacobsen et al., 2007; Jackson et al., 2015) was based on primary genotoxicity caused by ROS production. In these cases, FE1 cells were exposed to CB NM (Printex 90) for 75 μg/ml, 3 h and for 200 μg/ml, 24 h, respectively. In the latter genotoxicity was only observed as tail length (TL) and not % tail DNA. No genotoxicity was observed following CB NM (Printex 90) exposure up to 200 μg/ml, 24 h (Bengtson et al., 2016).

In summary; for the mechanism of toxicity, the present working group notes that there is limited available data on the biological effects of different physical - chemical properties but concludes that most of the available data support that increased surface area (and decreased size) is a critical driver of particle-induced inflammation, genotoxicity and carcinogenicity in the lungs.

Although a direct interaction between CB NM and DNA leading to genotoxic effect cannot be ruled out, it seems that the major pathway for genotoxicity involves a primary ROS production and a secondary cell mediated indirect genotoxicity.

The present working group does not find convincing evidence for a threshold carcinogenic effect. On the contrary we find evidence pointing towards pathways of genotoxicity involving both a primary ROS production and a secondary and indirect cell mediated ROS production. A non-threshold effect is also supported with regards to carcinogenicity. To the least, based on the above presented data, a non-threshold mechanism of carcinogenicity cannot be excluded, and in such cases, we choose a precautionary approach and recommend a linear extrapolation in the hazard assessment of carcinogenicity. This precautionary approach is based on ECHA REACH R8 (ECHA, 2012) in which it is stated that: *"It is to be noted that the decision on a threshold and a non-threshold mode of action may not always be easy to make, especially when, although a biological threshold may be postulated, the data do not allow identification of it. If not clear, the assumption of a non-threshold mode of action would be the prudent choice. For mutagens/carcinogens, it should be stressed that the Carcinogens and Mutagens Directive (2004/37/EC) requires that occupational exposures are avoided/minimised as far as technically feasible. As REACH does not overrule the Carcinogens and Mutagens Directive, the approach to controlling workplace exposure should therefore comply with this minimisation requirement."*

Consequently, the present working group decided to perform the hazard assessment based on both a threshold effect for inflammation and a non-threshold mechanism of action for carcinogenesis.

Cardiovascular effects

NM exposure can lead to cardiovascular effects either: 1. Directly, by translocation of NMs from the lung to the vascular system. 2. Indirectly, as a consequence of pulmonary inflammation and acute phase response. 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 NMs 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 (Cybulsky et al., 2001; Hansson and Libby, 2006). Inside the intima layer, the monocytes differentiate into macrophages and internalise fatty deposits (mainly oxidised 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 stabilises 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 NMs may also promote accelerated atherosclerosis indirectly through an induced pulmonary acute phase response. Introduction of NMs to the lung promotes neutrophil influx and release of pro-inflammatory 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., 2015), 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 oxidised LDL in the macrophages (Lee et al., 1985). In addition, SAA-HDL has a lower capacity to promote cellular cholesterol efflux from macrophages than native HDL (Artl et al., 2000). Pulmonary neutrophil influx has been shown to correlate with pulmonary Saa3 mRNA levels, SAA3 levels in blood and with deposited surface area of instilled particles (Saber et al., 2014), which links deposited particle surface area with biomarkers of risk of developing cardiovascular disease.

In conclusion, the present working group is of the opinion that pulmonary exposure to particles including CB NMs can lead to accelerated plaque progression directly, through translocation, or indirectly, through an induced acute phase response. No single physicochemical property has been identified as the driver of cardiovascular effects, but CB NM surface area is likely important due to the close association with pulmonary inflammation. As for inflammation, we consider cardiovascular effects as a threshold effect. This is based on identified dose-response relationships between particle exposure dose and induced acute phase response (Poulsen et al., 2015; Saber et al., 2013), and the close interplay between inflammation, acute phase response and plaque progression.

Dose-response relationships

Inflammation

Strong dose-response relationships have been observed following inhalation (Driscoll et al., 1996; Elder et al., 2005; Mauderly et al., 1994) when dose is expressed as mass. Inhalation and intratracheal instillation studies have shown that when rats and mice were exposed to CB NM the larger the BET surface area cause stronger pulmonary inflammation. Pulmonary exposure to CB NM has consistently been shown to cause

dose-dependent pulmonary inflammation, with close correlation to deposited surface area and inverse correlation to particle size (Bourdon et al., 2013b; Elder et al., 2005; Saber et al., 2012b; Stoeger et al., 2007, 2006). One study has demonstrated that this correlation is also valid using 6 finely tuned carbon materials all within a narrow nano size range (primary particle size 10-50 nm and specific surface area 30-800 m^2/g)(Stoeger et al., 2007, 2006). The dose response relationship has been observed for a number of low-toxicity, low-solubility particles and it is generally accepted that the inflammatory response of these materials including CB is proportional to the surface area of the deposited particles rather than the mass as reviewed by (Oberdörster et al., 2005).

Cardiovascular toxicity

Dose-response relationship was observed for pulmonary *Saa* mRNA expression levels in mice intratracheally instilled with CB NM (Kyjovska et al., 2015b)(Bourdon et al., 2012). A weak dose response relationship was observed in hear rate variability indices following intratracheal instillation of CB NM in mice (Jia et al., 2012). For the other cardiovascular studies there was only effect at highest dose, only one dose investigated of no effect at any of the investigated doses.

Cancer

The Heinrich study with CB NM Printex 90 inhalation in rats has only one dose and therefore the results cannot form basis for an evaluation of dose response or not (Heinrich et al., 1995). The Mauderly *et al.,* study was done with two mass concentrations 2.5 or 6.5 mg/m^3 (Mauderly et al., 1994). In females there was dose response as the highest dose also exhibited increased number of rats with lung neoplasms and this number was substantially higher than the increase observed at 2.5 mg/m^3 (Table 6). Overview of chronic rat inhalation studies). Notably the Heinrich study was conducted with CB NM Printex 90 which has a diameter of 14 nm and a BET surface area of 300 m^2/g. This study gave rise to lower air concentrations resulting in different excess lung cancer incidences as compared to a calculation based on Mauderly and co-workers and CB NM Elftex-12 Furnace Black having a diameter of 37 nm and BET surface area of 43 m^2/g (as described below) (Mauderly et al., 1994). This suggests that the smaller Printex 90 having a larger BET surface area induces cancer at a lower mass concentration as compared to Elftex-12 Furnace Black. Suggesting that when taking these two studies together, a dose response effect based on BET surface area is observed.

Particle characteristics / dose metrics

CB NMs may vary regarding size (and therefore also surface area), and in regard to the levels of impurities. Of the described impurities PAHs are deemed to be of highest concern. *In vitro* PAHs from CB NM have shown to become available for forming PAH-DNA adducts. However, in the same publication it was stated that the *in vitro* conditions showing this effect will not be encountered *in vivo*, and thus this mechanism is observed to be highly unlikely *in vivo* (Borm et al., 2005). Therefore, the surface area of CB NM is likely the best dose predictor for both the inflammatory response and for lung tumours.

The present working group notes that there is limited available data on the biological effects of different physico-chemical properties, but the current working group

concludes that the majority of available data support that the surface area (and therefore also the size) of CB NM is a critical driver of particle-induced inflammation and the acute phase response in the lungs.

PREVIOUS HAZARD AND RISK ASSESSMENTS OF CB

During the last couple of years, IARC and the Scientific committee on consumer safety (SCCS) have published evaluations of CB and highlights of these are presented below. No evaluations of CB (NM) were found from: National Institute for Occupational Safety and Health (NIOSH), New Energy and Industrial Technology Development Organization (NEDO), Risk Assessment Committee (RAC), The Nordic Expert Group (NEG) and The EU Scaffold project.

International Agency for Research on Cancer

In 2006, the International Agency for Research on Carcinogenicity (IARC) classified CB as *possibly carcinogenic to humans* (group 2B). This classification was based on sufficient evidence for carcinogenicity in rodent experiments. *"Three studies of female rats that inhaled carbon black and three additional studies of female rats exposed intratracheally found significant increases in the incidence of malignant lung tumours, providing sufficient evidence that carbon black can cause cancer in animals. Solvent extracts of carbon black were used in one study of rats in which skin tumours were observed after dermal application and several studies of mice in which sarcomas were seen following subcutaneous injection, providing sufficient evidence that carbon black extracts can cause cancer in animals."* Additionally, IARC notes that there was inadequate evidence to assess whether CB inhalation causes cancer in humans (IARC, 2010).

In Denmark, substances classified as group 1, 2A and 2B by IARC are considered carcinogenic by the Danish Working Environment Authority.

Scientific Committee on Consumer Safety

SCCS established a dossier evaluating the safety of CB NM (SCCS, 2015). The latest recommendations regarding NMs were included. The main aim of the dossier was to answer if CB in its nanoform is safe to use as colorant in cosmetics products. Generally, the conclusion was that CB NM, with a size of 20 nm or larger, and a purity >97%, at a concentration up to 10%, is considered to not pose any risk of adverse effects in humans if applied to healthy, intact skin. The opinion paper specifies that the conclusion is for intended use as a colorant in cosmetic products and does not apply to applications that might lead to inhalation exposure.

As part of the opinion the SCCS evaluated the literature on toxicity and carcinogenicity of CB. They conclude that "*No carcinogenic effect was observed after oral or dermal exposure.*" However, they note that studies are old and incomplete and, therefore, no conclusion can be drawn. On the carcinogenicity following pulmonary exposure for CB the SCCS concludes that their opinion is "*that carbon black can induce malignant tumours in female rats after inhalation exposure or intratracheal instillations. The potency of carbon black particles with diameter of 14 nm was higher than the potency of carbon black particle with diameter of 95 nm. There is no empirical support for a dose threshold from the animal carcinogenicity studies*". The present working group has come to a similar conclusion.

SCCS additionally notes that they find "*that the animal cancer data are relevant to humans and that the use of nano carbon black in sprayable applications is not recommended*".

SCCS notes on inhalation toxicity that; "t*he responses after inhalation of carbon black at 1 and 7 mg/m^3 among rats, mice and hamsters were similar in magnitude. The NOAEL for inhalation of carbon black nanomaterials for rats, mice and hamster was 1 mg/m^3.*" This NOAEL (NOAEC) of 1 mg/m^3 is equal to the one the present working group is suggesting based on a review of the available literature on inhalation of CB.

Summary of the evaluations

The IARC and SCCS opinions are in accordance with the finding in the current report. SCCS suggests a NOAEL (NOAEC) of inhalation to be 1 mg/m^3 equal to the suggestions of the present working group. In addition, both opinions agree on the genotoxic and carcinogenic potential of CB. The present working group is also in line with the previous hazard assessments regards to the genotoxicity and carcinogenicity of CB.

SCIENTIFIC BASIS FOR AN OCCUPATIONAL EXPOSURE LIMIT

Different methods exist for calculating health-based OELs. The choice of method depends on the mode of action of the substance and can fundamentally be split up in two categories: Threshold effects or non-threshold effects. Threshold effect assumes 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. In this report, we will calculate proposed occupational exposure limits based both on threshold effects (inflammation) or non-threshold effects (carcinogenicity).

Endpoint: Inflammation

The derivation of a derived no effect level (DNEL) based on inflammation has been made under the assumption of a threshold driven mechanism of CB NM toxicity: CB NM induced oxidative stress/inflammation which may result in other effects such as e.g. cardiovascular disease. The lung epithelium is covered by a thin layer of lung lining fluid. This layer contains e.g. glutathione which gives it anti-oxidant capacity.

Our recommendation for an OEL for CB follows the traditional approach for setting health-based OELs:

1) Identification of critical effect
2) Identification of the NOAEC
3) Calculation of OEL using assessment factors adjusting for inter and intra species differences

In the current report we use the DNEL as recommended by ECHA as the method for calculating OEL for toxicological effects having a threshold [83].

Our calculation of a DNEL is based on pulmonary influx of neutrophils immediately after end of exposure. Inflammation is a key effect linked to several adverse outcomes. The calculation is based on a chronic inhalation study of rats (Elftex-12, 37 nm; 2.5 or 6.5 mg/m^3 for 16 h/day, 5 days/week for 12 or 24 months in rats) (Mauderly et al., 1994) and two sub-chronic inhalation studies performed by Elder and co-workers (Printex 90, 14 nm; 1, 7 or 50 mg/m^3, 6h/day 5 days/week for 13 weeks in mice, rats and hamster) and Driscoll and co-workers (Monarch 880, 16 nm; 1.1, 7.1 or 52.8 mg/m^3, 6h/day, 5 days/week for 13 weeks in rats). Mauderly and co-workers observed effect at the lowest tested mass concentration; resulting in a LOAEC of 2.5 mg/m^3 (Mauderly et al., 1994). No effect was observed at 1 and 1.1 mg/m^3 in the sub-chronic studies leading to a NOAEC of 1 mg/m^3.

Overall these studies support that a NOAEC level could be set to 1 mg/m^3.

The calculations of the DNEL follow the approach as set out in the REACH guidance [83]:

First, the NOAEC is modified to correct for an 8-hour working day with higher breathing rate of 10 m^3/day in workers compared to 6.7 m^3/day at rest. Mauderly *et al.*, used a 16 h exposure whereas the sub-chronic studies used a 6 h exposure period. Below we correct the NOAEC 6h at rest to NOAC 8h at higher breathing:

$NOAEC_{Corrected}$ = $NOAEC_{6h\ subchronic\ studies}$ * (6 hour/8 hour) * (6.7 m^3/10 m^3)
= 1 mg/m^3 * (6 hour/8 hour) * (6.7 m^3/10 m^3)
= 0.5025 mg/m^3

$NOAEC_{Corrected}$ = 0.5 mg/m^3

Secondly, the corrected NOAEC is adjusted by a number of assessment factors (most of these are default values suggested by ECHA (2008). The following default assessment factors are used:

Interspecies extrapolation:	2.5
Intraspecies interpolation (default factor for workers):	5
Extrapolation from sub-chronic to chronic:	2
The overall assessment factor AF_{Total} = 2.5 * 5 * 2 =	25

This results in a DNEL for chronic inhalation for pulmonary inflammation of:

DNEL = $NOAEC_{Corrected}/AF_{Total}$ = 0.5025 mg/m^3 / 25 = 0.0201 mg/m^3

<u>DNEL = 20 μg/m^3</u>

Alternatively, as no NOAEC was observed, the LOAEC of 2.5 mg/m^3 observed at 12 months of exposure in the chronic study by (Mauderly et al., 1994) could also be used for the calculation of a DNEL. In such a case an assessment factor in the range of 3 to 10 could be used to convert the LOAEC to a NOAEC as described by (ECHA, 2012). If we used the least conservative safety factor of 3 as recommended by ECHA in the majority of cases (ECHA, 2012), we would obtain a NOAEC of 0.83 mg/m^3. In this case the assessment factor for extrapolation from a subchronic study to a chronic (a factor of 2) is omitted. Adjusting for the longer inhalation period per day (16 h/8 h) and the higher breathing rate for workers (6.7 m^3/10 m^3) in the Mauderly study, we would obtain a corrected NOAEC of 0.83 mg/m^3. This is divided by a combined assessment factor of 12.5 (inter- and intra-species) and results in a DNEL of 90 μg/m^3. This value is higher than the 20 μg/m^3 value obtained based on a NOAEC value in the subchronic studies. However, it is noted that this calculation is based on the least conservative assessment factor for the use of a LOAEC instead of a NOAEC.

Endpoint: Cancer

Carcinogenicity is generally considered a non-threshold effect. The present working group recommends and have applied the same for CB NM-induced carcinogenicity, as the present working group finds evidence of non-threshold mechanism of CB-induced cancer. This approach is supported by and based on ECHA REACH R8 (ECHA, 2012). For comparison, however, towards the end of this section a calculation using a carcinogenic threshold approach is given.

Risk levels are calculated based on two investigations; Heinrich *et al.,* who used CB NM Printex 90 (14 nm) (Heinrich et al., 1995) and Mauderly *et al.*, who used CB NM Elftex-12 (37 nm) (Mauderly et al., 1994).

Heinrich et al., and CB NM Printex 90

The derivation of an OEL based on cancer has been made under the assumption of a non-threshold driven mechanism of CB NM toxicity.

The OEL is derived based on the chronic inhalation study of female mice and rats by Heinrich and co-workers (Heinrich et al., 1995). Lung tumour rate in mice exposed to CB NM was not statistically different from the lung tumour rate in mice exposed to filtered air. Therefore, as the most sensitive of the tested species, data from the rats are used for the hazard assessment.

The lowest effect level for lung cancer was observed in rats, where increased lung cancer incidence was found at 11.6 mg/m^3; the only tested dose in the study. The rats inhaled 7.2 mg/m^3 for the first 4 months and 12.2 mg/m^3 for 20 months. Thus, the average exposure was 11.6 mg/m^3 for 104 weeks. Lung cancer incidence in CB NM exposed rats was 39% (39/100), while the cancer incidence in control rats was 0.5% (1/217). Both malignant and non-malignant tumours were included in accordance with the REACH guideline stating that: *"malignant tumours as well as benign tumours that are suspected of possibly progressing to malignant tumours are taken into account in obtaining the dose-descriptor values"* (ECHA, 2012).

At 11.6 mg/m^3, the amount of pulmonary deposited CB NM after 2 years of inhalation was determined to be 43.9 mg/rat lung (Heinrich et al., 1995).

Table 7. Cancer incidence and lung burden in female rats after Heinrich et al.

	0 mg/m^3	11.6 mg/m^3
Total cancer incidences	1/217	39/100[#]
CB NM lung burden (mg/lung)		43.9

[#]Include benign keratinising cystic squamous-cell tumours

Method I

Observed excess cancer incidence at 11.6 mg/m^3:
(39/100- 1/217) / (1-1/217) = 0.387 = 39 %

The current working group has chosen to use the approach used by Kasai *et al.,* (Kasai et al., 2016) and Erdely *et al.,* (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. At 11.6 mg/m^3, the amount of pulmonary deposited CB NM after 2 years of inhalation was determined to be 43.9 mg/rat lung (Heinrich et al., 1995).

Human lung burden equals:

Rat lung burden × Human alveolar surface area / rat alveolar surface area

43.9 mg × 102 m^2 / 0.4 m^2

Human lung burden equals = 11 195 mg per human lung[4].

We assume using standard values that human ventilation is 20 L/min during light work (1.2 m^3/h), work-related exposure for 8 h per day, 5 days per week, 45 working weeks per year, over a working lifetime of 45 years. The deposition rate was not reported to in the Heinrich study. For the calculation, we have used a deposition of 8.6% based on an inhalation study with TiO_2 by (Hougaard et al., 2010). In that study, mice were exposed by inhalation 1h/day for 11 days to 42 mg/m^3 aerosolized powder of rutile TiO_2 with an average crystallite size of 21 nm. The pulmonary deposition fraction was estimated to be 8.6% based on the observed particle size distribution in the aerosol (Hougaard et al., 2010). A more conservative approach would be to use a higher deposition fraction as suggested by other studies. E.g. in a CB NM inhalation study of pregnant mice a deposition fraction of 34.8 % was used (Jackson et al., 2012a). If we used this higher deposition the suggested exposure limits would be reduced by approximately 4-fold.

A lung burden of 11195 mg in humans would require that workers are exposed, through a full work life for:

Air concentration = 11195 mg / (8h/day x 5 day/week x 45 weeks/year x 45 years x 1.2 m^3/h x 0.086) = 1.3 mg/m^3.

Thus, at an air concentration of 1.3 mg/m^3 during a 45-year work life, an excess lung cancer incidence of 39% is expected. If we assume a linear dose-response relationship, then 1% excess lung cancer would be expected at: (1.3 mg/m^3 /39) = 0.03 mg/m^3.

The CB NM air concentrations resulting in different excess lung cancer incidences for a deposition fraction of 8.6% are given in the table below.

[4] Human lung is defined as both lungs

Table 8. Calculated excess lung cancer incidence at different CB NM mass concentrations based on method I.

Excess lung cancer incidence	Deposition fraction: 8.6% CB NM concentration
1: 1 000	3 μg/m^3
1: 10 000	0.3 μg/m^3
1: 100 000	0.03 μg/m^3

If using a more conservative approach based on a deposition fraction of 34.8 % as described above the suggested exposure limits would be reduced by approximately 4-fold. For example, at 1: 1 000 this would be 0.8 μg/m^3.

Method II

ECHA (European Chemicals Agency (ECHA) 2012a;SCHER/SCCP/SCENIHR 2009), calculated based on the two year CB NM inhalation study in rats by (Heinrich et al., 1995) (Table 9). The calculations are based on results from female rats. No effects were observed in male rats:

Excess cancer risk:

Observed excess cancer incidence at 11.6 mg/m^3:
(39/100- 1/217) / (1-1/217) = 0.387 = 39 %

Correction of dose metric for humans during occupational exposure (8h/day):

$$11.6 \text{ mg/m}^3 \times (18 \text{ h/day}) / (8 \text{ h/day}) \times (6.7 \text{ m}^2/10 \text{ m}^2) = 17.5 \text{ mg/m}^3$$

Calculation of unit risk for cancer:

Risk level = exposure level x unit risk
0.39 = 17 500 μg/m^3 x unit risk
Unit risk = 2.2 x 10^{-5} per μg/m^3

At a dose of 1 μg/m^3, 2.2 x 10^{-5} excess cancers are expected.

Calculation of dose levels corresponding to risk level of 10^{-5} (1: 100 000), 10^{-4} (1: 10 000) and 10^{-3} (1: 1 000).

10^{-5} risk level = exposure level x unit risk (2.2 x 10^{-5} per μg/m^3)
Exposure level (10^{-5}) = 0.45 μg/m^3

Thus, at 0.45 μg/m^3, 1:100 000 excess lung cancer cases can be expected.

Table 9. Calculated excess lung cancer incidence at different CB NM mass concentrations based on method II.

Excess lung cancer incidence	CB NM Air concentration
1: 1 000	45 μg/m^3
1: 10 000	4.5 μg/m^3
1: 100 000	0.45 μg/m^3

Mauderly et al., and CB NM Elftex-12

The derivation of an OEL based on cancer has been made under the assumption of a non-threshold driven mechanism of CB NM toxicity. The below OEL is derived based on the chronic inhalation study of rats by Mauderly *et al.,* (Mauderly et al., 1994). The calculations are based on results from female rats. No significant effects were observed in male rats.

The lowest effect level for lung cancer was observed in rats, where increased lung cancer incidence was found at 2.5 mg/m^3. Lung cancer incidence in CB NM exposed rats was 8% (8/107), while the cancer incidence in control rats was 0% (0/105). Both malignant and non-malignant tumours were included in accordance with the REACH guideline stating that: *"malignant tumours as well as benign tumours that are suspected of possibly progressing to malignant tumours are taken into account in obtaining the dose-descriptor values"* (ECHA, 2012).

At 2.5 mg/m^3, the amount of pulmonary deposited CB NM after 2 years of inhalation was determined to be 21.0 mg/rat lung (Mauderly et al., 1994).

Table 10. Cancer incidence and lung burden in female rats after Mauderly et al.

	0 mg/m^3	2.5 mg/m^3
Total cancer incidences	0/105	8/107[#]
CB NM lung burden (mg/lung)		21.0

[#]Include malignant and benign neoplasms

Method I

Observed excess cancer incidence at 2.5 mg/m^3:
(8/107- 0/105) / (1-0/105) = 0.075 = 8 %

The current working group has chosen to use the approach used by Kasai *et al.,* (Kasai et al., 2016) and Erdely *et al.,* (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. At 2.5 mg/m^3, the amount of pulmonary deposited CB NM after 2 years of inhalation was determined to be 21.0 mg/rat lung.

Human lung burden equals:

Rat lung burden × Human alveolar surface area / rat alveolar surface area

21.0 mg × 102 m^2 / 0.4 m^2

Human lung burden equals = 5 355 mg per human lung[5].

We assume using standard values that human ventilation is 20 L/min during light work (1.2 m^3/h), work-related exposure for 8 h per day, 5 days per week, 45 working weeks per year, over a working lifetime of 45 years. The deposition rate was not reported to in the Heinrich study. For the calculation, we have used a deposition of 8.6% based on an inhalation study with TiO_2 by (Hougaard et al., 2010). In that study, mice were exposed by inhalation 1h/day for 11 days to 42 mg/m^3 aerosolised powder of rutile TiO_2 with an average crystallite size of 21 nm. The pulmonary deposition fraction was estimated to be 8.6% based on the observed particle size distribution in the aerosol.

A lung burden of 5 355 mg in humans would require that workers are exposed through a full work life for:

Air concentration = 5 355 mg / (8h/day x 5 day/week x 45 weeks/year x 45 years x 1.2 m^3/h x 0.086) = 0.64 mg/m^3.

Thus, at an air concentration of 0.64 mg/m^3 during a 45-year work life, an excess lung cancer incidence of 8% is expected. If we assume a linear dose-response relationship, then 1% excess lung cancer is expected at: (0.64 mg/m^3 /8) = 0.08 mg/m^3.

The CB NM air concentrations resulting in different excess lung cancer incidences for a deposition fraction of 8.6% are given in the table below.

Table 11. Calculated excess lung cancer incidence at different CB NM mass concentrations based on method I.

Excess lung cancer incidence	Deposition fraction: 8.6% CB NM concentration
1: 1 000	8 μg/m^3
1: 10 000	0.8 μg/m^3
1: 100 000	0.08 μg/m^3

The above calculation are for 2.5 mg/m^3. Similar calculation for 6.5 mg/m^3 would result in 4 μg/m^3, 0.4 μg/m^3 and 0.04 μg/m^3, respectively for the three risk levels. These calculations are based on a rat lung burden of 38.5 mg as noted by Mauderly and co-workers.

If using a more conservative approach based on a deposition fraction of 34.8% as described above the suggested exposure limits would be reduced by approximately 4-fold. For example, at 1: 1 000 this would be 2 μg/m^3.

Method II

ECHA (European Chemicals Agency (ECHA) 2012a;SCHER/SCCP/SCENIHR 2009), calculated based on the two year CB NM inhalation study in rats by (Heinrich et al., 1995) (Table 12):

[5] Human lung is defined as both lungs

Excess cancer risk:

Observed excess cancer incidence at 2.5 mg/m^3:
(8/107- 0/105) / (1-0/105) = 0.075 = 8 %

Correction of dose metric for humans during occupational exposure (8h/day):

$$2.5 \text{ mg/m}^3 \times (16 \text{ h/day}) / (8 \text{ h/day}) \times (6.7 \text{ m}^2/10 \text{ m}^2) = 3.35 \text{ mg/m}^3$$

Calculation of unit risk for cancer:

Risk level = exposure level x unit risk
0.08 = 3350 μg/m^3 x unit risk
Unit risk =2.388 x 10^{-5} per μg/m^3

At a dose of 1 μg/m^3, 2.388 x 10^{-5} excess cancers are expected.

Calculation of dose levels corresponding to risk level of 10^{-5} (1: 100 000), 10^{-4} (1: 10 000) and 10^{-3} (1: 1 000).

10^{-5} risk level = exposure level x unit risk (2.388 x 10^{-5} per μg/m^3)
Exposure level (10^{-5}) = 0.42 μg/m^3

Thus at 0.42 μg/m^3, 1: 100 000 excess lung cancer cases can be expected.

Table 12. Calculated excess lung cancer incidence at different CB NM mass concentrations based on method II.

Excess lung cancer incidence	CB NM Air concentration
1: 1 000	42 μg/m^3
1: 10 000	4.2 μg/m^3
1: 100 000	0.42 μg/m^3

The above calculations are for 2.5 mg/m^3. Similar calculation for 6.5 mg/m^3 would result in 35 μg/m^3, 3.5 μg/m^3 and 0.35 μg/m^3, respectively for the three risk levels.

Summary

The CB NM air concentrations resulting in different excess lung cancer incidences for a deposition fraction of 8.6%, Heinrich and Mauderly data overview:

Table 13. Combined results based on Method I

Excess lung cancer incidence	**Based on Heinrich** CB NM concentration	**Based on Mauderly** CB NM concentration
1: 1 000	3 μg/m^3	8 μg/m^3
1: 10 000	0.3 μg/m^3	0.8 μg/m^3
1: 100 000	0.03 μg/m^3	0.08 μg/m^3

Table 14. Combined results based on Method II

Excess lung cancer incidence	**Based on Heinrich** CB NM concentration	**Based on Mauderly** CB NM concentration
1: 1 000	45 μg/m^3	42 μg/m^3
1: 10 000	4.5 μg/m^3	4.2 μg/m^3
1: 100 000	0.45 μg/m^3	0.42 μg/m^3

The present working group recommends a hazard assessment of carcinogenicity of CB NM based on a non-threshold mechanism as calculated above and thoroughly discussed earlier in the report (see section Mechanisms of toxicity).

Non-threshold based excess cancer risk (1: 1 000) at 3 μg/m^3 - 45 μg/m^3

However, for reference an alternative calculation based on a threshold approach is presented here. This calculation is based on the "potential NOAEC" of the Mauderly et al. and Heinrich et al. studies. However as both studies showed effects at all tested doses (2.5 and 6.5 mg/m^3 in Mauderly et al., 11.6 mg/m^3 in Heinrich et al.); this calculation is based on the lowest "LOAEC", 2.5 mg/m^3 and then the calculation is similar to the calculation on a DNEL of inflammation using the Mauderly et al. study above and result in a DNEL of 90 μg/m^3. This is calculated using the lowest assessment factor 3 (selected in the range of 3 to 10) and thus represents the least conservative assessment factor for converting a LOAEC to a NOAEC. It is stressed that the calculation is against the current presented evidence as well as against the ECHA guidelines (ECHA, 2012).

CONCLUSION

The present working group evaluated the relevant literature on CB NM concerning epidemiological and animal inhalation studies. None of the identified epidemiological studies provided information on the particle size range or purity. Also, the epidemiological data was inconclusive with results showing both large excess risks as well as reduced risks from working at CB factories. A strong healthy worker effect is expected in the latter case; but in general, the studies could not be corrected for smoking frequency and other exposures. However, within the cohort showing the largest excess risk for mortality caused by lung cancer, cigarette smoking is not expected to be a large confounder, as other cigarette smoke induced diseases were not increased. The results of epidemiological studies point in different directions, as to whether CB is carcinogenic or not. Based on the available human epidemiological studies, we cannot use the available epidemiological studies for risk assessment. Therefore, we decided to base the suggested health based OEL on data from experimental animal inhalation studies.

Pulmonary inflammation and carcinogenicity were observed in sub-chronic and chronic inhalation studies in rats. The present working group regards inflammation and carcinogenicity as the main adverse effects and the subsequent hazard assessments are conducted based on studies reporting these effects. CB NM induced cardiovascular and reproductive 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 hazard assessment. However, 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 dose response relationships for neutrophil influx as a marker of pulmonary inflammation (Driscoll et al., 1996; Elder et al., 2005; Mauderly et al., 1994). Neutrophil influx was predicted by deposited surface area. The working group considers inflammation as a threshold effect.

The present working group concludes that there is substantial evidence for genotoxicity of CB NM. CB NM can induce mutations, oxidative damage to DNA as well as DNA strand breaks in rats and mice. It is clear that inflammation is closely linked to genotoxicity via secondary cell driven production of ROS. Primary and secondary particle effects can be challenging to separate within *in vivo* studies; however, the present working group do find support for primary production of ROS could have some importance in the genotoxicity of CB NM. Additionally, some evidence for a linear and non-threshold relationship for CB NM-induced carcinogenesis are presented. Consequently, the present working group decided to perform the hazard assessment based on both a threshold (inflammation) and a non-threshold mechanism of action (cancer).

The working group considered that data from five rodent inhalation studies were the best basis for the hazard assessment. The following studies were selected to be used for calculation of DNEL and excess cancer risk, respectively: DNEL studies were: A 12-month chronic inhalation study in rats (0, 2.5, and 6.5 mg/m^3) (Mauderly et al., 1994), a

13-week sub-chronic inhalation study in mice, rats, and hamsters (0, 1, 7, and 50 mg/m^3) (Elder et al., 2005), and a 13-week sub-chronic inhalation study in rats (0, 1, 7, and 53 mg/m^3) (Driscoll et al., 1996). Cancer studies were: a 2-year chronic cancer inhalation study in rats (0 and 12 mg/m^3) (Heinrich et al., 1995) and a 2-year chronic cancer inhalation study in rats (0, 2.5 and, 6.5 mg/m^3) (Mauderly et al., 1994). Table 15 shows a DNEL for pulmonary inflammation derived based on the sub-chronic inhalation study of rats, and the lowest evaluated excess lung cancer risk at 1 in 1 000, 1 in 10 000 and 1 in 100 000 derived using two different approaches. As a precautionary principle, the lowest values are presented.

Table 15. Overview of threshold-based DNEL and non-threshold-based exposure levels leading to excess cancer risk using two different approaches.

		Suggestion of an OEL for CB NM		
Mechanism of action		Inflammation	Lung cancer (method I)	Lung cancer (method II)
Threshold based	DNEL	20 μg/m^3 #		
Non-threshold based	Excess cancer risk			
	1: 1 000		3 μg/m^3	45 μg/m^3
	1: 10 000		0.3 μg/m^3	4.5 μg/m^3
	1: 100 000		0.03 μg/m^3	0.45 μg/m^3

#Based on NOAEC values in 2 subchronic inhalation studies

Studies used for the hazard assessment used either CB NM Printex 90 (14 nm) or CB NM Elftex-12 Furnace Black (37 nm). CB NMs differ regarding size and surface area but also in the levels of impurities such as PAHs. The present working group notes that there is limited available data on the biological effects of different physico-chemical properties, but the current working group concludes that the majority of available data support that the surface area (and therefore also the size) of CB NM is a critical driver of particle-induced inflammation and the acute phase response in the lungs (Stoeger et al., 2006).

Two different approaches were used for calculating excess lung cancer risk based on the same two chronic inhalation studies. 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 hazard assessment, the resulting OEL suggestions were all very low. These levels are all more than 100-fold lower than the current Danish OEL for CB of 3.5 mg/m^3.

CB NMs are similar to the soot particles in diesel engine exhaust although with less adhered organics. The relative importance and general bioavailability of adhered organics are questioned and remains to be elucidated. Both CB NM and diesel particles consist mainly of an insoluble carbon core and both materials have shown similar results when tested for e.g. mutagenicity and carcinogenicity. Long-term non-cytotoxic *in vitro* exposures to CB NM Printex 90 were associated with a statistically significant increase in the *cII* and *lacZ* mutation frequency in FE1-Muta™Mouse cells (Jacobsen et al., 2007). The level of mutations was similar to that observed following exposure to SRM 1650 a reference diesel exhaust particle from a heavy-duty truck (Jacobsen et al., 2008a). This

similarity extends into a rat inhalation study which found no significant difference in carcinogenic potency between inhalation of CB NM and diesel exhaust (Nikula et al., 1995). Both materials have for long been registered as possibly or probably carcinogenic to humans by IARC (CB, Group 2B). However, IARC recently re-evaluated diesel exhaust to be a Group 1 carcinogen (previously Group 2A) (IARC, 2014). A recent epidemiological meta-analysis points towards carcinogenicity at very low diesel exposure levels (17 excess lung cancer deaths per 10 000 at life time occupational exposures of 1 μg diesel exhaust/m^3) (Vermeulen et al., 2014). Two hundred and 689 excess lung cancer deaths per 10 000 at a life time occupational exposure of 10 or 25 μg diesel exhaust/m^3, respectively. The recommended safe exposures are thus lower based on the human meta-analysis data compared to the rat study. We see many similarities between CB NM and soot particles and find a comparison of effects interesting.

The present working group recommends the hazard assessment approach estimating the excess lung cancer risk based on lung burden, since this approach takes the retained pulmonary dose into account. Thus, the expected excess lung cancer risk in relation to occupational exposure to CB NMs is 1: 1 000 at 3 μg/m^3, 1: 10 000 at 0.3 μg/m^3 and 1: 100 000 at 0.03 μg/m^3 CB NM.

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