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REVIEW ARTICLE

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Evaluating the evidence on genotoxicity and reproductive toxicity of carbon black: a critical review

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ABSTRACT

Carbon black is produced industrially by the partial combustion or thermal decomposition of gaseous or liquid hydrocarbons under controlled conditions. It is considered a poorly soluble, low toxicity (PSLT) particle. Recently, results from a number of published studies have suggested that carbon black may be directly genotoxic, and that it may also cause reproductive toxicity. Here, we review the evidence from these studies to determine whether carbon black is likely to act as a primary genotoxicant or reproductive toxicant in humans. For the genotoxicity endpoint, the available evidence clearly shows that carbon black does not directly interact with DNA. However, the study results are consistent with the mechanism that, at high enough concentrations, carbon black causes inflammation and oxidative stress in the lung leading to mutations, which is a secondary genotoxic mechanism. For the reproductive toxicity endpoint for carbon black, to date, there are various lung instillation studies and one short-term inhalation study that evaluated a selected number of reproduction endpoints (e.g. gestational and litter parameters) as well as other general endpoints (e.g. gene expression, neurofunction, DNA damage); usually at one time point or using a single dose. It is possible that some of the adverse effects observed in these studies may be the result of non-specific inflammatory effects caused by high exposure doses. An oral gavage study reported no adverse reproductive or developmental effects at the highest dose tested. The overall weight of evidence indicates that carbon black should not be considered a direct genotoxicant or reproductive toxicant.

Abbreviations: AM: alveolar macrophages; BAL(F): broncho-alveolar lavage (fluid); BAL: broncho-alveolar lavage; CHO: Chinese Hamster Ovary; cytoB: cytochalasin B; CBPI: cytokinesis-block proliferation index; dev: development; ELF: epithelial lining fluid; Endo III: endonuclease III; ESTR: Expanded simple tandem repeat; FLG: few layer grapheme; Fpg: formamidopyrimidine DNA glycosylase; gd: gestational day; GL: guideline; HPRT: hypoxanthine phosphoribosyltransferase; i.t.: intratracheal; IARC: International Agency for Research on Cancer; LDH: lactate dehydrogenase; LOEL: Lowest Observed Effect Level; Mg/kg bw: milligrams per kilogram body weight; Nm: nanometers; NOEL: No Observed Effect Level; OECD: Organization for Economic Co-operation and Development; PAH: polycyclic aromatic hydrocarbons; PBS: Phosphate buffered saline; PMN: polymorphonuclear neutrophils; PSLT: poorly soluble, low toxicity; ROS: reactive oxygen species; SA: surface area; SCCS: Scientific Committee on Consumer Safety; SCE: sister chromatid exchanges; TK: toxicokinetic

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Introduction

Carbon black [CAS. No. 1333-86-4] is elemental carbon in the form of particles that are produced industrially by the partial combustion or thermal decomposition of gaseous or liquid hydrocarbons under controlled conditions. Commercially available grades of carbon black differ in particle size, surface area, average aggregate mass, morphology, or structure. Potential health effects of carbon black have been investigated extensively in laboratory animal experiments and in epidemiological studies of carbon black production workers. The main health concerns associated with carbon black and other poorly soluble, low-toxicity (PSLT) particles are lung effects resulting from inhalation exposure. The International Agency for Research on Cancer (IARC 2010) proposed an overall mode of action for carbon black toxicity in rat lungs. Particle deposition above certain concentrations in the rat lungs may lead to a phenomenon known as "lung overload", which in turn leads to sustained inflammation, production of reactive oxygen species (ROS), depletion of antioxidants and/or impairment of other defense mechanisms, cell proliferation, and gene mutations. These changes in the rat lung can lead to the induction of alveogenic tumors. Similar tumors have not been observed in mouse or hamster lungs.

Recently, a number of authors have indicated that carbon black may have toxic effects that fall outside the well-understood mechanism of action for PSLT particles; namely, that carbon black may be directly genotoxic, and that it may cause reproductive toxicity (these studies are summarized in Tables 1–3). The purpose of this review is to critically examine the evidence on genotoxicity and reproductive toxicity for carbon black.

Literature search strategy

As toxicologists involved in understanding carbon black toxicology and undertaking a REACH registration of carbon black, we have been compiling data on carbon black health effects over a period of at least 20 years. Journal articles related to carbon black toxicology were identified from comprehensive literature searches primarily in the United States National Library of Medicine PubMed and TOXNET databases, supplemented by the evaluation of the reference lists of reviews and key publications. For this paper, any studies published by April 2017 related to genotoxicity, mutagenicity, reproductive toxicity, or developmental toxicity of carbon black were identified and reviewed. This paper is a targeted comprehensive review of these endpoints, therefore published *in vitro* and *in vivo* studies related to these endpoints were included for review regardless of quality or limitations of study design.

Carbon black composition, uses, and exposure potential

The physical appearance of carbon black is that of a black, finely divided powder, consisting of aggregates (in the size range between 100 and 1000 nm) of aciniform morphology (i.e. aggregates that have been strongly fused together in random configuration that resemble grape-like clusters) (Gray and Muranko 2006). Primary particles, which are defined according to ISO as "the original source particles of agglomerates or aggregates or mixtures of the two" are not discernible anymore after completion of the manufacturing process. The aciniform aggregates constitute the smallest inseparable entities in manufactured carbon black and are hence the fundamental structural units of carbon black. However, even the carbon black aggregates are not readily available outside the closed reaction chamber of the manufacturing process as, within the reaction chamber, the aggregates rapidly form larger agglomerates held together by van der Waals forces. Carbon black agglomerates often are compressed into even larger-sized pellets as a final step in the manufacturing process.

Carbon black is sometimes used in toxicology studies to represent environmental carbonaceous particles in air pollution studies (Reisetter et al. 2011). However, it is important not to confuse carbon black with "soot" or "black carbon" which are names applied to carbonaceous emissions from fires and incomplete combustion of carbon-containing fuels and products (e.g. waste oil, fuel oil, gasoline fuel, diesel fuel, coal, coal-tar pitch, oil shale, wood, paper, rubber, plastics and resins, household refuse, etc.). Such emissions are not only comprised of some elemental carbon but also of significant quantities of organics and other compounds (Watson and Valberg 2001). For example, combustion soot is a highly heterogeneous substance that generally includes a major organic carbon fraction (often >50% of total mass) and significantly higher ash and extractable organic matter contents than carbon black. The chemical and physical properties of combustion soot are highly variable depending on its source (Long et al. 2013). In contrast, commercially produced carbon black is composed of mainly carbon with only traces of other substances (McCunney et al. 2012).

Carbon black has been commercially produced worldwide for more than 100 years. Worldwide production in 2012 was about 24 billion pounds [11 million metric tons] (ICBA 2016). About 89% of total manufactured carbon black consumption is in the rubber industry. Of that, 70% goes toward automotive and truck tires, and related tire products; and, approximately 10% is in other automotive rubber products, such as belts, hoses, and related accessories. The final 9% is consumed in rubber products unrelated to the automotive industry. The remaining 11% of total production is used in non-rubber applications such as paints, inks, coatings, plastics, electrostatic discharge compounds, ultraviolet light absorption applications, and as a chemical reagent (Wang et al. 2004).

Occupational exposure to carbon black may arise during the manufacture of carbon black and during its use in the formulation of rubber, printing ink, paints as well as other uses. The results of large-scale multiphase industry-wide exposure assessment surveys in Europe and the USA have shown that by the mid- to late-1990s the geometric mean levels of inhalable dust in manufacturing facilities were below 2 mg/m^3 [discussed in IARC (IARC 2010)]. In nearly all cases of use, carbon black is incorporated into a rubber or polymer matrix, in which carbon black is tightly bound within other materials. A study of carbon black in food contact plastics showed that carbon black particles do not migrate out of

Table 1. *In vitro* tests with carbon black related to mutagenicity/genotoxicity endpoints.

Test system	Method	Testing conditions	Test material/SSA	Result	Remark	References
Mutagenicity						
Bacterial cell systems						
Ames test with CB particles	OECD 471	As per GL	Printex 90, N339, Unipure Black, 100–300 m^2/g	Negative		(Kirwin et al. 1981; Degussa 1998; Hobson 2011)
Ames test with CB extracts	OECD 471	As per GL, Soxhlet extraction	Printex 70, Printex 90, N339	Negative		(Kirwin et al. 1981; Degussa 1997, 1998)
	Similar to OECD 471	Soxhlet extraction (benzene, 16 h (Agurell and Löfroth 1983, 1993) or toluene, 48 h (Rosenkranz et al. 1980))	N330, Black Pearls L	Positive; some negative	Mutagenic activities varied widely between different samples; positive findings in pre-1979 material were due to nitropyrene impurities	(Rosenkranz et al. 1980; Agurell and Löfroth 1983, 1993)
Mammalian cell systems						
FE1-MML MutaMouse™ epithelial cell line	Transgenic cell line, Comet (Fpg)	75 μg/mL, cumulative CB dose 6 mg in 8 × 72 h incubation	Printex 90, 295 m^2/g	Small (1.2- to 1.4-fold) increases in transversion mutations at the cII and LacZ loci of a transgenic cell line (consistent with ROS damage); DNA strand breaks and oxidized DNA	The mutant frequency for the cII gene of the negative control cells increased 1.27-fold	(Jacobsen et al. 2007, 2011)
L5178Y Mouse Lymphoma	OECD 476, HPRT test	GLP, 3 h, up to 120 μg/mL	Carbon Black E3000281, containing Unipure Black LC 902, 200–260 m^2/g	Negative	The SCCS (2015) is of the opinion that 3 h exposure may have been too short for cellular uptake	(Lloyd 2011)
L5178Y Mouse Lymphoma	OECD 476	4 h (extended because of the difficulty of separating test material from cells), 10–40 mg/mL (-S-9); 5–15 mg/mL (+S-9)	N339, 100 m^2/g	Negative		(Kirwin et al. 1981)
Clastogenicity – Aneuploidy/micronucleus assays *in vitro*						
A549 human lung cancer cell line	Micronuclei	0.02–200 mg/L (0.003–34 μg/cm^2); 6 h; no cytoB	Printex 90	Increase in micronuclei (max. 3.3%; controls: 0.7%) with plateau at 2 mg/L; cell growth reduced by 60% at the highest dose tested	Solvent control cultures (0.9% saline with 0.05% v/v Tween 80, serum) also showed an increase in micronucleus frequency after 48 h. No further data were reported on the solvent controls nor on cytotoxicity	(Totsuka et al. 2009)
RAW 264.7 mouse macrophage cell line	Micronuclei	0.01–100 mg/L, 48 h; 4 mg/L cytoB (28 h delayed co-treatment), 10% serum	Printex 90	Negative	Cytotoxicity: trypan blue exclusion, CBPI	(Migliore et al. 2010)
RAW 264.7 mouse macrophage cell line	Micronuclei	1, 3, and 10 μg/cm^2, 48 h; cytoB added after 44 h of incubation; after further 28 h of incubation, the cells were harvested	Degussa Huber NG90, diameter 200–250 nm	Positive (22, 36.5, and 50 micronuclei/1000 cells at 1, 3, and 10 μg/cm^2, controls: 14/1000 cells), 3 and 10 μg/cm^2 were cytotoxic (MTT assay); increased apoptosis		(Poma et al. 2006)
RAW 264.7 mouse macrophage cell line	Micronuclei, chromosome aberrations	1, 3, and 10 mg/L, 48 h; cytokinesis-block method for MNT, but no details reported; chromosome	Printex 90	1 mg/L: MNT negative 3 and 10 mg/L: ↑MN, less than 2-fold); acentric chromosome fragments at	Cytotoxicity: 50 and 100 mg/L (trypan blue exclusion, 72 h, MTS); CBPI; chromosomal effects: relevance	(Di Giorgio et al. 2011)

(*continued*)

Table 1. Continued

Test system	Method	Testing conditions	Test material/SSA	Result	Remark	References
		aberrations at 24, 48, and 72 h post-exposure; ROS production at 50 mg/L after 5 and 24 h		all concentrations; accumulation in phagolysosomes, causing acute necrosis; ↑intracellular ROS production, similar at 5 and 24 h	uncertain (insufficient control data in RAW 264.7 cells)	
Chinese hamster ovary (CHO)	OECD 479, sister chromatid exchange (SCE)	+/− metabolic activation 0.00032–1 mg/mL	N339, 100 m^2/g	Negative	OECD 479 was deleted in 2014	(Kirwin et al. 1981)
Chinese hamster ovary (CHO)	OECD 487 (2010), micronuclei	GLP, 10, 20, and 30 μg/mL for pulse treatments in the absence and presence of S9-mix; 10, 15, and 20 μg/ml for the continuous treatment in the absence of S9-mix; vehicle: DMSO	Carbon Black E3000281, containing Unipure Black LC 902, 200–260 m^2/g	Negative	CytB not used during the 3 h exposure, but added immediately afterwards (delayed co-treatment), SCCS questions the validity of the study (SCCS 2015)	(Lloyd 2012)
Undifferentiated hamster epithelial cell line M3E3/C3	Micronuclei	0.1, 1, and 2 μg/mL, 30 h	Carbon black, not specified	Inconclusive (<2-fold increase over controls)		(Riebe-Imre et al. 1994)
PAH-DNA adducts						
A549 human lung epithelial cells	^{32}P post-labeling	Particles: 100 μg/cm^2, DMSO extracts: 30–300 μg/cm^2 for 24 h	Printex 90 (PAH: 0.039 ppm, 300 m^2/g), Sterling V (PAH: 8.8 ppm, 30–40 m^2/g), N330 (PAH: 2.4 ppm, 70–90 m^2/g), Lampblack 101 (PAH: 0.057 ppm, 20 m^2/g)	Negative (Printex 90, N330, Lampblack 101); some adducts were found with Sterling V extract. The identity of those could not be identified and they were not dose-dependent with regard to the spot intensity	The *in vitro* conditions showing the effect with Sterling V will not be encountered *in vivo* and renders this mechanism in particle-induced lung cancer at *in vivo* exposures highly unlikely	(Borm et al. 2005)
DNA damage & repair/comet assays						
RAW 264.7 mouse macrophage cell line	Comet	1, 3, 10, and 50 mg/L, 24 h	Printex 90	1, 3, and 10 mg/L: negative 50 mg/L:↑DNA damage		(Di Giorgio et al. 2011)
RAW 264.7 mouse macrophage cell line	Comet +/− Fpg and Endo III	1, 10, and 100 mg/L, 24 h incubation for cytotox test, not reported for Comet assay;	Standard carbon black (particle size 500 nm) and N330 (20–50 nm)	Negative for standard carbon black; positive for N330 at 1 mg/L (in buffer, +EndoIII), 10 mg/L (+EndoIII), and negative at 100 mg/L; both products ↑ NFkappaB at 10 and 100 mg/L and (not dose-dependently) of TNF-alpha and COX-2		(Rim et al. 2011)
Primary mouse embryo fibroblasts	Comet	5 concentrations up to 100 mg/L, 24 h	CB from Nano-Innovation Co. China, C > 99.4%, 12 nm	Cytotoxicity and oxidative damage; induced fewer DNA single strand breaks than the other materials tested	Comparative study with other materials, authors state importance of further *in vivo* tests	(Yang et al. 2009)
Human A549 and monocytic T-cells (THP-1)	Comet	16, 160, and 1600 ng/mL for 48 h	Vulcan M, furnace black, 100 nm from Cabot	Positive at 1.6 μg/mL; dichloromethane extracts negative; no cytotoxicity (AlamarBlue assay)		(Don Porto Carero et al. 2001)
A549 cells	Comet (Fpg)	1, 20, and 40 μg/cm^2 for 4 h	Carbon powder, Sigma Aldrich, <30 nm	Negative	SCCS (2015) notes shortcomings in methodology	(Karlsson et al. 2008)
Human embryonic lung Hel 299 fibroblasts and Chinese hamster V79 cells	Comet	4 concentrations up to 138 μg/cm^2, 3 h	Carbon black, Cabot, 37 nm	Negative		(Zhong et al. 1997)

(*continued*)

Table 1. Continued

Test system	Method	Testing conditions	Test material/SSA	Result	Remark	References
FE1-MML MutaMouse™ epithelial cell line	Comet (Fpg)	75 μg/mL, cumulative CB dose 6 mg in 8 × 72 h incubation	Printex 90, 295 m^2/g	↑Oxidized purines		(Jacobsen et al. 2007)
A549 cells	Comet	25 mg/m^2 (100 μg/mL), 0.5–24 h, suspensions in culture medium sonicated for 20 min	Huber 990, 260 nm, Printex 90, 14 nm	Positive at ≥3 h exposure Both particle types: NFkappaB activation, only Printex 90: DNA single strand breaks, activation of p53 and DNA repair	Preliminary work, only one concentration tested. No double strand breaks (however those were detected in parallel experiment with urban dust)	(Mroz et al. 2007, 2008)
Human umbilical vein endothelial cells (HUVEC)	Comet (Fpg)	100 mg/L for 3 h	Printex 90	Oxidative DNA damage		(Frikke-Schmidt et al. 2011)
HepG2 cells	Comet (Fpg, hOGG1)	25 mg/L for 3 h	Printex 90	Oxidative DNA damage		(Vesterdal et al. 2014)
Caco-2 human intestinal carcinoma cell line	Comet (Fpg)	20 μg/cm^2 for 4 h	Printex 90	Negative		(Gerloff et al. 2009)
Cell transformation assays						
C3H/10T1/2 CL8 mouse embryo cells	EU method B.21	4 concentrations, 2–16 mg/mL	N339, 100 m^2/g	Negative		(Kirwin et al. 1981)
Hamster M3E3/C3 epithelial cell line	Similar to EU method B.21	100, 300, and 500 μg/mL	CB, not specified	Inconclusive	Non-validated cell line	(Riebe-Imre et al. 1994)
Cell-free systems						
Supercoiled plasmid	–		Fine CB and Printex 90, 253.9 m^2/g	Single strand breaks in directly exposed DNA after treatment with Printex90		(Stone et al. 1998)
Oxidative stress						
A549 cells	MTT, oxidative stress	Up to 0.78 μg/mm^2	Fine CB and Printex 90, 253.9 m^2/g	Printex 90 induced oxidative stress	These results suggest that ultrafine CB induces a greater oxidative stress than fine CB	(Stone et al. 1998)
Human primary bronchial epithelial cells	Western blot analysis	6.13–30.7 μg/cm^2	CB, 250 nm, 7.8 m^2/g; ultrafine CB, 11 nm, 457 m^2/g	30.7 μg/cm^2: Ultrafine CB stimulated proliferation of human airway epithelium via epidermal growth factor (EGF) receptor-mediated signaling pathway and oxidative stress; NOEC (DNA synthesis): 12.3 μg/cm^2	Stimulation was similar to that caused by 10% fetal bovine serum	(Tamaoki et al. 2004)
THP-1 monocytes, THP-1 derived macrophages (THP-1a), HUVECs	WST-1, trypan blue, ROS	2.5, 12.5, 25, and 100 mg/L	Printex 90	↑cytotoxicity at ≥12.5 mg/L (THP1, -1a) and all cell types at 100 mg/L; ↑LDH at 100 mg/L only in THP-1; no effect on trypan blue exclusion; ↑intracellular ROS, plateau at 12.5 mg/L		(Cao et al. 2014)

CB: carbon black; CBPI: cytokinesis-block proliferation index; DMSO: dimethylsulfoxide; Endo III: endonuclease III; Fpg: formamidopyrimidine DNA glycosylase; GL: guideline; GLP: Good Laboratory Practice; HPRT: hypoxanthine guanine phosphoribosyltransferase; i.t.: intratracheal; MN: micronuclei; MNT: micronucleus test; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SCCS: Scientific Committee on Consumer Products, opinion on carbon black (nano-form), December 2015; SSA: specific surface area.

Table 2. *In vivo* tests with carbon black related to mutagenicity/genotoxicity endpoints.

Test system	Method	Testing conditions	Test material/PPS/SSA	Result	Remark	References
Mutagenicity						
Germ cell test						
Female germline mutations	ESTR mutations in oocytes	Mouse, i.t., 4 × 67 μg/animal during gestation	Printex 90	Negative		(Boisen et al. 2013)
Somatic cells						
RLE-6TN rat alveolar epithelial cells (type II cells) + BAL cells (*ex vivo*)	HPRT test	BAL cells *ex vivo* from rats 15 months after single intratracheal instillation of saline or 10 or 100 mg/kg bw CB	Monarch 900, 15 nm, 230 m^2/g	10 mg/kg: NOEL 100 mg/kg: positive (factor 7.6) at 15 months after exposure	Supports threshold and secondary genotoxicity by ROS species	(Driscoll et al. 1997)
Alveolar epithelial cells (type II cells)	HPRT test	Rat, 90 days inhal., 6 h/day, 5 days/week; 1.1, 7.1, and 52.8 mg/m^3 and recovery	Monarch 880, 16 nm. 220 m^2/g, MMAD 0.88 μm	1.1 mg/m^3: NOAEL 7.1 mg/m^3: positive (factor 3.4); negative after recovery; 52.8 mg/m^3: positive (factor 4.3, 3.2, and 2.7 after exposure and 3- and 8-month recovery, respectively)	Mutations only at inflammatory doses, with epithelial hyperplasia	(Driscoll et al. 1996, 1997)
BAL	HPRT test	Rat, mouse, hamster (all female); 90-day inhalation, 1, 7, and 50 mg/m^3 + recovery	Printex 90	1 mg/m^3: NOEL 7.50 mg/m^3: ↑hprt in BAL cells, rat ≫ mouse; not in hamster		(Carter et al. 2006)
Lung, kidney	gpt (lung, kidney) and Spi (only lung) mutations	Male gpt transgenic mouse, 200 μg 1× or 4×, examined 8 or 12 weeks after exposure for mutations, 5/group	Printex 90	No gpt mutations were induced; (frequency of gpt and Spi-mutations/deletions in lungs slightly, but not significantly higher than in controls)	(Microscale kaolin was positive in both assays); rarely used assay with high standard deviation	(Totsuka et al. 2009)
Retina pigment epithelium	DNA deletions	Mouse, oral, 500 mg/kg bw	Not specified	No effects on DNA deletions in the retina pigment epithelium of offspring		(Reliene et al. 2005)
Lung carcinoma	K-ras, p53 mutations; hypermethylation of p16 gene	F344 rat, 24 months (16 h/day, 5 days/week), 0. 2.5, and 6.5 mg/m^3)	Diesel exhaust or CB (Elftex-12, 43 m^2/g)	No mutations in p53 were observed; a low frequency (3/50) and variable pattern of activating mutations were identified in K-ras independent of exposure	Small sample size; no significant differences between the yields of mutants recovered from diesel exhaust, carbon black, or sham-exposed rats	(Nikula et al. 1995; Swafford et al. 1995; Belinsky et al. 1997)
Clastogenicity						
Peripheral human blood lymhocytes	Cytogenetic assay	28 workers (2–8 years exposed, tire industry), 15 controls; 100 metaphases analyzed	Not specified; co-exposure to substances in tire industry	Positive association (5.07% versus 2.27 in unexposed controls)	Only abstract available	(Babu et al. 1989)
DNA Adducts						
Lung	^{32}P post-labeling	F344 rat (female), 13 week, 6 h/day, 5 days/week; Printex 90: 0, 1, 7, and 50 mg/m^3 Stering V: 50 mg/m^3 all in filtered air; 3/group	Printex 90 (PAH: 0.039 ppm, 300 m^2/g), Sterling V (PAH: 8.8 ppm, 30–40m^2/g)	No adduct formation		(Borm et al. 2005)
Lung, liver	^{32}P post-labeling, HPLC	F344 rat, 0.64 mg/kg, 1× p.o. (gavage) or i.t., 7–10/group	Printex 90	No adduct formation in lungs; ↑etheno-adducts in liver after gavage; no change after i.t.	Large variation in the levels in liver within the control and exposure groups	(Danielsen et al. 2010)
Lung	^{32}P post-labeling	Wistar rat (f), 11.3 mg/m^3, 18 h/day, 5 days/week, 20 months, whole-body	Printex 90; MMAD, 0.65 μm; 270 m^2/g;	No adduct formation		(Gallagher et al. 1994)

(*continued*)

Table 2. Continued

Test system	Method	Testing conditions	Test material/PPS/SSA	Result	Remark	References
Lung	^{32}P post-labeling	F344 rat (m), 10 mg/m^3; 7 h/day, 5 days/week, 12 weeks; whole-body; 6/group	Elftex-12 furnace black; 2 μm MMAD (large mode); 0.1 μm MMAD (small mode); 43 m^2/g	No adduct formation	Significant localized inflammation in the rat lung was induced	(Wolff et al. 1990)
Lung alveolar epithelial type II cells	^{32}P post-labeling	F344 rats, 6.2 mg/m^3 (respirable fraction);16 h/day, 5 days/week, 12 weeks; whole-body	Elftex-12 furnace black; 2 μm MMAD (large mode); 0.1 μm MMDD (small mode); 43 m^2/g	25 and 5 adducts/10e9 in exposed and filtered-air controls, respectively		(Bond et al. 1990)
DNA damage and repair						
Studies in rats						
Lung, alveolar lining cells	PAR, γ-H2AX, 8-OH-dG, OGG1, 1 month after last instillation	Rat, i.t., 6 mg/rat 1×/month, 3 months (total dose 18 mg/rat); CB was dispersed in phys. saline with Tween 80 (conc. not reposted) before instillation	Printex 90	↑γ-H2AX- and 8-OH-dG-positive nuclei in lung tissue (2.1- and 1.8-fold, respectively); number of OGG1-positive nuclei (↓); frequency of OGG1-positive cytoplasm ↑(4-fold)	γ-H2AX is a pre-toxic lesion (Nikolova et al. 2014); alveolar lining cells displayed a granular pattern of OGG1 in the cytoplasm, probably reflecting mitochondrial expression of the enzyme	(Ziemann et al. 2011; Rittinghausen et al. 2013)
Lung	8-Oxo-dG	Rat (f), 13 weeks, 6 h/day, 5 days/week inhalation, 5/g (1, 7, and 50 mg/m^3 Printex90, 50 mg/m^3 Sterling V); 5/group, + recovery	Printex 90, 300 m^2/g, Sterling V, 30–40 m^2/g	1 mg/m^3: NOEL 7 mg/m^3: ↑oxidative damage after 44 weeks recovery; max. factor 1.5; 50 mg/m^3: ↑lung burden, particularly Sterling V, ↑oxidative damage (only Printex 90) at 0 and 44 weeks after exposure, lung load 1–7 mg/lung, PMNs↑		(Gallagher et al. 2003)
Liver, lung	8-Oxo-dG, εdG, εdA, OGG1, mRNA expression, 24 h after exposure	F344 rat, 0.64 mg/kg, 1× p.o (gavage). or i.t., 7–10/group	Printex 90	mRNA levels of genes related to oxidative stress unchanged after i.t., but ↑after p.o.; 8-oxo-dG increased by 23% after p.o.; no change in OGG1	Endotoxin content of test preparation: 25 mg/L	(Danielsen et al. 2010)
Comet assays – inhalation						
BAL	Comet assay (± hOGG1), 1 day and 14 days post-exposure	Wistar rat (m), 6 mg/m^3 for 14 days (6 h/day, 5 days/week) + 14 d recovery, $n = 5$/group	Printex 90	No strand breaks, no oxidative DNA damage; positive controls ($KBrO_3$, i.p.) were functional)		(Lindner et al. 2017)
BAL, liver	Comet, 5 and 24 days after exposure	Mouse, inhalation (whole body), 42 mg/m^3, 1 h/day, gd 8–18), 5–6/group	Printex 90	No effect on gestation and lactation; strand breaks in BAL not increased (5 and 24 days after exposure), but higher levels in liver (max 1.6-fold increase) of dams and offspring	Pregnancy usually affects liver function; no historical control data provided – questionable association with CB exposure; in inhalation study oral exposure of damns probable (whole-body chamber)	(Jackson et al. 2012a)
BAL	Comet, 1 h after last exposure	TNF+/+ and TNF−/− mice, 4 × 20 mg/m^3 for 1.5 h, inhalation, 4/group; nose-only	Printex 90, 295 m^2/g; diesel exhaust particles (DEP), filtered air	% neutrophils in BAL not different from air controls (but significant increase in DEP treated animals); ↑IL-6	TNF induces neutrophil apoptosis as a mechanism for removal of neutrophils (Murray	(Saber et al. 2005)

(*continued*)

Table 2. Continued

Test system	Method	Testing conditions	Test material/PPS/SSA	Result	Remark	References
				mRNA in lung; ↑DNA strand breaks in the BAL cells of DEP- and CB-exposed $TNF^{-/-}$ (by a factor of approximately 2 in carbon black exposed animals)	et al. 1997); this might explain the reduced DNA damage in TNF +/+ mice as compared to TNF−/− mice	
Comet assays – intratracheal instillation						
BAL cells, lung, liver	Comet, 1, 3, or 28 days after exposure, +/− fpg	C57BL/6 female mice, i.t. 18, 54, and 162 μg/mouse; suspensions in physiological saline with 10% added acellular BAL (or BAL +10% saline; both reported in publication); endotoxin 0.142 EU/mg Printex 90	Printex 90	BAL 18 μg: ↑DNA strand breaks at ayd1 and day 28; 54 μg: ↑DNA strand breaks at day 28; 162 μg: ↑ DNA strand breaks at day 1, day 3, and day 28; Lung 18 μg: ↑DNA strand breaks at day 1; 54 μg: ↑DNA strand breaks at day 3 and day 28; 162 μg: ↑ DNA strand breaks at day 1, day 3, and day 28; Liver 18, 54, and 162 μg: ↑DNA strand breaks at day 1 and day 28; not at day 3. At day 1, not dose-related Inflammation 18, 54, and 162 μg: significant and dose-dependent increase in BAL neutrophil count still present at day 28	Results indicate clearance and repair activity; maximum neutrophil influx at day 1, maximum recruitment of other cells at day 3; vehicle contained particles >20 nm; ↑FPG sensitive sites in lung at day 1 at all dose levels, and at day 3 only at 162 μg	(Bourdon et al. 2012b, 2012c)
BAL	Comet, 3 and 24 h after exposure	Mouse, wild-type (C57) and ApoE-/-, i.t., (18, 54 μg/mouse); inhal, 60 mg/m^3 (30, 90 min); 7 animals/group	Printex 90	54 μg: single strand DNA damage in BAL cells at 3 h after i.t. treatment; inh caused only marginal effects (increases in cytokine mRNA, distribution between neutrophils and macrophages)	Compared to normal mice, the ApoE-/- model is more sensitive to inflammatory effects; results of comet assay after inhalation not reported; no comet results either for the 24 h measurement after i.t. instillation; 60 mg/m^3 was selected as similar dose to highest it dose regarding inflammation	(Jacobsen et al. 2009)
BAL	Comet, 24 h after exposure	C57BL/6 mouse (f), i.t., 54 μg/mouse; in physiol. Saline containing 10% BAL from unexposed mice	Printex 90, Lampblack 101	Negative (examined 24 h post-instillation); macrophage depletion and increase in neutrophils with Printex 90 only	DNA damage may have been repaired by 24 h	(Saber et al. 2012)

(*continued*)

Table 2. Continued

Test system	Method	Testing conditions	Test material/PPS/SSA	Result	Remark	References
BAL, lung	Comet, 3 h; 1, 2, 3, 4, 5, 14, and 42 days post-exposure; included also toxicogenomic analysis	C57BL/6 mouse (f), i.t. 162 μg/mouse, suspension in nano-pore water	Printex 90	↑neutrophil at all time points; ↑DNA strand breaks in BAL cells 3 h and 3 days post-exposure, and in lung tissues 2–5 days post-exposure; ca. 2600 genes were differentially expressed (± 1.5 fold; $p \leq .05$) across all time-points in the lungs; gene expression changes associated with inflammatory response followed a biphasic pattern, with initial changes at 3 h declining to base-levels by 3 days, increasing again at 14 days, and then persisting to 42 days post-exposure	Max. ↑neutrophils between day 1 and day 5, but still increased at day 42; max ↑macrophages between day 2 and day 5; altered transcript levels were associated with immune-inflammatory response and acute phase response pathways; genes involved in DNA repair, apoptosis, cell cycle regulation, and muscle contraction were also differentially expressed.	(Husain et al. 2015)
BAL cells, lung, liver	Comet, 1, 3, or 28 days after exposure	C57BL/6JBomTac mice, i.t., 0.67, 2, 6, 162 μg/mouse under 4% isoflurane anesthesia	Printex 90	0.67 μg: ↑neutrophils (day 1 and 3), eosinophils (3 days), lymphocytes (3 days); ↑DNA strand breaks (BAL, liver) 2 μg: ↑neutrophils (day 1), eosinophils (3 days) ↑DNA strand breaks (BAL, lung); 6 μg: ↑neutrophils (day 1); ↑DNA strand breaks (day 3, day 28; BAL, lung, liver) 162 μg: ↑neutrophils (day 1, day 3, and day 28), macrophages (day 1, day 28), ↓lymphocytes (day 1), ↑lymphocytes (day 28), total BAL cells (day 1, day 3, and day 28); ↑DNA strand breaks (only day 1, lung)	The study authors "interpret the increased DNA strand breaks occurring following these low exposure doses of nanoparticulate carbon black as DNA damage caused by primary genotoxicity in the absence of substantial inflammation, cell damage, and acute phase response".	(Kyjovska et al. 2015)
BAL, liver	Comet, 5 and 24 days after exposure	Mouse, i.t. (4× up to 67 μg/animal), gd 8–18), 5–6/group	Printex 90	No effects in BAL In offspring 1.5-fold single strand breaks in liver	Pregnancy usually affects liver function; no historical control data provided – questionable association with CB exposure;	(Jackson et al. 2012a)
Lung	Comet assay, 3 h and 24 h after exposure	C57BL/6J mouse (m), 0.05 and 0.2 mg 1× i.t., examined 3 and 24 h after exposure; 5/group	Printex 90	0.05 mg: no effect 0.2 mg: positive (factor 1.1 to 2, 3 h after administration, no change in level of DNA damage at 24 h)		(Totsuka et al. 2009)
Other						
Lung, BAL macrophages	Profibrotic growth factors and procollagen gene expression in lungs, 1 and 21 days post-exposure	Rat, single oropharyngeal aspiration, 2mg/kg bw	Raven 5000 Ultra II, 8 nm, 350–583 m^2/g	No induction of mRNA encoding for platelet-derived growth factor (PDGF)-A, -B, or –C, which are known to be associated with fibrosis	No fibrotic reaction in lung as evidenced by histopathology, no overt inflammatory response, >95% macrophages in BAL	(Mangum et al. 2006)

(*continued*)

Table 2. Continued

Test system	Method	Testing conditions	Test material/PPS/SSA	Result	Remark	References
Lung	Toxicogenomic analysis	Mouse, i.t., 18, 54, and 162 μg/animal	Printex 90	162 μg: persistent pulmonary inflammation; gene expression changes similar to those typical for pulmonary injury and fibrosis; activation of the atherogenic HMG-CoA reductase pathway	Authors calculated BMDs for quantitative risk assessment based on adaptive responses (and not, as usually in risk assessment, based on adverse outcomes); all data based on i.t. mouse data; there is no concordance with human exposure routes and relevant gene expressions;	(Bourdon et al. 2012a, 2013)
Lung, liver, offspring	Toxicogenomic analysis	Mouse i.t., 0, 11, 54, or 268 μg/animal subdivided in four instillations on gestational days 7, 10, 15, and 18	Printex 90	11 μg: no effects; ≥54 μg: CB retention 268 μg: neutrophil-marked inflammation; altered expression of several cytokines and chemokines, both at the transcriptional and tissue protein levels; liver: female offspring more sensitive; cellular signaling, inflammation, cell cycle, and lipid metabolism pathways affected.	Probably secondary toxic effects in target and non-target tissues due to maternal toxicity	(Jackson et al. 2012b)

8-OH-dG: 8-hydroxyguanosine; BAL: broncho-alveolar lavage; ESTR: expanded simple tandem repeat; DE: diesel exhaust; γ-H2AX: phosphorylated H2AX; hOGG1: human 8-hydroxyguanine DNA-glycosylase; HPRT: hypoxanthine guanine phosphoribosyl transferase; i.t.: intratracheal; NOAEL: No observed adverse effect level; NOEL: No observed effect level; OGG1: 8-oxoguanine DNA glycosylase; PAR: poly(ADP-ribose); ROS: reactive oxygen species.

Table 3. Reproductive toxicity tests with carbon black.

Test system	Test material	Material preparation	Exposure dose and timing	Findings	Remarks	References
Sexual maturation, fertility, and reproduction						
Mouse (C57BL/6/BomTac); intratracheal instillation	Printex 90	Carbon black was suspended in filtered water and subsequently sonicated for 8 min	Total doses of 11, 54, and 268 μg/animal; administered on gd 7, 10, 15, and 18	No changes in gestational, litter parameters, or sexual development in offspring. Inflammation seen in 268 μg/animal (females): female offspring showed altered habituation pattern during open field test	Effect found only in females	(Jackson et al. 2011)
Mouse (C57BL/6J) Intratracheal instillation	Printex 90	Carbon black was suspended in filtered water	67 μg/animal administered on gd 7, 10, 15, and 18: total dose of 268 μg/animal	↓sperm production in F2 when F1 fathers were exposed *in utero*	Few animals studied; effect seen at high, non-physiologically administered dose. Statistically significant, but numerically modest reduction of sperm production: without historical controls, biological relevance not assessable	(Kyjovska et al. 2013)
Intratracheal instillation in male ICR mice	14 nm (Printex 90), 56 nm (Printex 25), and 95 nm (Flammruss 101)	Carbon black was suspended in a normal saline solution containing 0.05% Tween 80 for instillation (no information provided on sonication)	100 μg/mouse 10 times at weekly intervals, resulting in a total dose of 1000 μg.	Increased testosterone with 14 nm CB and 56 nm CB, changes in seminiferous tubules and decreased daily sperm production with all CB.	The high dose of CB may result in inflammation and oxidative stress, which could be responsible for the reproductive effects. However, other organs were not observed for adverse effects.	(Yoshida et al. 2009)
Intratracheal instillation in ICR mice	14 nm CB – source not provided	Carbon black was suspended in normal saline solution with 0.05% (v/v) Tween 80 and sonicated for 5 min.	200 μg/mouse on gd 7 and 14, resulting in a total dose of 400 μg.	Male offspring showed histological changes in the testes, as well as decreases in daily sperm production. There was no marked effect on body weight, testicle weight, epididymis weight, or serum testosterone concentration.	Only one high dose was tested	(Yoshida et al. 2010)
Developmental effects						
Rat, oral gavage, OECD 414 (Prenatal developmental toxicity study)	Unipure BlackLC 902	Carbon black was suspended in 0.5% aqueous sodium carboxymethylcellulose and given at 10 ml/kg bw.	Daily oral gavage at 0, 100, 300, or 1000 mg/kg/day during the sensitive period of organogenesis [gd 5 through 19].	No adverse maternal effects on embryo-fetal development. NOAEL was 1000 mg/kg/day for both maternal and developmental effects		(Ramesh 2012); as cited in SCCS 2015
Mouse (C57BL/6/BomTac) inhalation	Printex 90		42 mg/m^3, 1 h/day, between gd 8–18; whole body exposure	No increase in gestational or developmental toxicity. Liver: Increased DNA strand breaks in liver of dams and in liver of in utero exposed offspring – ↑max. 1.5-fold. Lungs: persistent inflammation; DNA strand breakage in BAL comparable to controls		(Jackson et al. 2012a)

(*continued*)

Table 3. Continued

Test system	Test material	Material preparation	Exposure dose and timing	Findings	Remarks	References
Mouse (C57BL/6/BomTac); intratracheal instillation	Printex 90	Carbon black was suspended in filtered water and subsequently sonicated for 8 min	Total doses of 11, 54, and 268 μg/animal; administered on gd 7, 10, 15, and 18	No increase in gestational or developmental toxicity. All doses: No effects on DNA strand breaks in BAL and liver of dams and in liver of offspring CB retention in lungs of animals dosed with 54 and 268 μg/animal 27 days post-exposure Persistent lung inflammation only in high dose animals (268 μg/animal) High dose: altered expression of cytokine and chemokines in dams. Altered expression of hepatic genes in offspring: female offspring more sensitive.	Hepatic gene profiling in offspring: statistical threshold applied to identify significant change was less stringent $p = .1$ versus conventional $p = .05$ or higher	(Jackson et al. 2012a, 2012b)
Intranasal instillation in ICR mice	Printex 90	Suspended in distilled water, sonicated for 30 min and then filtered through a 450-nm filter	95 μg/kg/day on gd 5 and 9; resulting in approx. 6 μg/mouse	Brains collected from offspring at 6 and 12 weeks after birth, showed enlargement of granules of perivascular macrophages, decrease in number of perivascular macrophages, change in astrocyte phenotypes	The authors state that these changes indicate increased risk of dysfunction and disorder in the offspring brain, although no further studies were conducted to support this claim.	(Onoda et al. 2014)
Intradermal injection in pregnant rhesus macaques	Printex 90	Test material was suspended in 0.1% Tween 80- PBS at dose of 10 mg/ml, and sonicated for 10 min just before administration	Intradermal injection of test material suspension 4–6 times at intervals of 10–12 days. Total dose is not clear as the amount injected is not listed	Brains collected from new-born infants showed higher levels of hemoglobin.	The authors state that higher levels of hemoglobin are reportedly neurotoxic; however, the level of increase necessary to cause neurotoxicity is not clear.	(Mitsunaga et al. 2016)
Intranasal instillation in ICR mice	Printex 90	Carbon black was suspended at 1 mg/ml in saline with 0.05% (v/v) Tween 80 and sonicated for more than 30 min just before administration.	50 μg/mouse on gd 5 and 9; resulting in a total dose of 100 μg/mouse	Increased expression of collagen type VIII in the kidney of 12-week-old offspring mice but not in 3-week-old offspring mice. There was no difference in levels of serum creatinine or blood urea nitrogen, which are indicators of renal function.	Relevance of these findings is not clear as changes were noted in one endpoint of kidney function, and the change was seen at only one time-point	(Umezawa et al. 2011)
Mouse, (C57BL/6JP^{un}/P^{un}), Oral	Unclear	Test material was suspended in PBS	500 mg/kg bw; from gd 10.5–15.5 post-coitum	CB exposure did not increase the number of eye-spots.		(Reliene et al. 2005)
Intranasal instillation in ICR mice	Printex 90	Suspended in distilled water, sonicated for 30 min and then filtered through a 450-nm filter	95 μg/kg/day on gd 9 and 15; resulting in approx. 6 μg/mouse[a]	Increased total thymocyte and lymphocytes in thymus and spleen of offspring.	Relevance of findings is unclear; different outcomes between two similar studies (see below).	(El-Sayed et al. 2015)

(*continued*)

Table 3. Continued

Test system	Test material	Material preparation	Exposure dose and timing	Findings	Remarks	References
Intranasal instillation in ICR mice	Printex 90	Suspended in distilled water, sonicated for 30 min and then filtered through a 450-nm filter	95 µg/kg/day on gd 5 and 9; resulting in approx. 6 µg/mouse	Partial suppression of immune system development in offspring. Decrease in splenic T cells in offspring on post-natal days 1 through 5. Splenic T cells recovered on post-natal day 14.	Relevance of findings is unclear; different outcomes between two similar studies (see above).	(Shimizu et al. 2014)
Mouse (Balb C), intranasal	CB received from EPA or Harvard; no further description	Test material was suspended in PBS	Single dose application of 50 µg/animal on Day 14 of gestation	↑susceptibility to allergy in offspring of CB treated mothers. Authors conclude CB causes enhanced immune response in pregnancy and increased allergic susceptibility in offspring	Test material identity not apparent. Methodology to determine airway responsiveness controversial	(Fedulov et al. 2008)

Gd: gestation day; PBS: phosphate buffered saline.
[a]Assuming 12-week female ICR mouse weight of 33 g (https://animals.ekmd.huji.ac.il/He/home/animalOrder/Documents/ICR.pdf), the dose of 95 µg/kg translates to 3 µg. This amount was given twice.

plastic materials once incorporated (Bott et al. 2014). Indeed, IARC recognized and noted the unique nature of carbon black and the distinction between pure and bound-in-matrix carbon black: "End-users of these products (rubber, ink or paint) are not exposed to carbon black per se, since it is bound within the product matrix" (IARC 2010).

Mechanism of toxicity of carbon black

Long-term inhalation exposure of rats to carbon black leads to the development of lung tumors (Mauderly et al. 1994; Heinrich et al. 1995; Nikula et al. 1995). The mechanism of tumor induction in rats by carbon black is considered to be representative of and common to PSLT particles, such as titanium dioxide (Mauderly 1997; Nikula 2000). Studies with carbon black and other PSLT particles indicate that the lung tumors in rats are a generic response to lung overload and subsequent chronic inflammation. At high particle concentrations, the ability of the rat lung to remove particles becomes impaired. A persistent particle burden in rat lungs leads to pulmonary inflammation, which causes DNA damage, epithelial cell proliferation, and fibrotic changes (Mauderly and McCunney 1996; Nikula 2000).

The adequacy of the rat as a reliable model for predicting human lung cancer risk of PSLT particles is a matter of controversy in the scientific community and continues to be questioned (ILSI 2000; ECETOC 2013; Morfeld et al. 2015; Warheit et al. 2016). Compared to other species, it appears that rats exhibit an exaggerated response to high concentrations of inhaled, insoluble particles. In contrast to rats, mice do not develop lung tumors after long-term exposure to carbon black or other PSLT (Heinrich et al. 1995). Lung overload effects after exposure to carbon black have also not been observed in hamsters (Elder et al. 2005). Moreover, epidemiological evidence from carbon black production workers chronically exposed to carbon black has not shown consistent patterns of either an elevated risk of lung cancer or a dose–response trend [discussed in IARC (2010) and Dell et al. (2015)]. Also, no association of carbon black exposure with inflammation markers (number of white blood cells, neutrophils, lymphocytes, T cells, CD4+, CD8+, B cells, NK cells, and monocytes) in blood of exposed workers was found in a recent epidemiological study (0.66 mg/m^3 elemental carbon, measured in personal samples from 8 packing workers); a borderline, possibly allergy-induced, increase in eosinophil count, was only significant in workers that have never smoked (Dai et al. 2016). At an excessive workplace concentration of 14.90 mg/m^3 (measured by personal air samplers), however, increases in IL-1β, IL-6, IL-8, MIP-1beta, and TNF-alpha were found (Zhang et al. 2014).

Absorption, distribution, and excretion of carbon black

In order to understand the potential for carbon black to cause primary genotoxicity or reproductive toxicity, it is important to consider the toxicokinetic (TK) behavior of carbon black in the body. Inhalation exposure is the most relevant route for particles, as inhalation represents the main

route of exposure in the occupational setting. However, an inhalation absorption or TK study evaluating distribution to other parts of the body has not been identified for carbon black. In a study evaluating the kinetics of carbon black following oral administration, weanling (4 weeks old) and aged (18 months old) female Swiss mice received a single dose of 7 mg ^{7}Be-labelled carbon black particles (27 nm diameter) by gavage. Isotope distribution was measured at 4 h and 1, 2, 5, and 14 days after administration. Radioactivity in extra-intestinal viscera and blood was extremely low and practically all the label was excreted in the feces; most of it during the first day. At 4 h post-dosing, the total radioactivity in the body (excluding intestines) was approximately 0.01% of the dose in weanlings and 0.005% of the dose in adults. Less than 0.001% of the radioactive dose was also found in the urine at 1, 2, and 5 days post-dosing. The level of radioactivity in the intestines (tissues only) was 0.006% or 0.028% of the dose in weanlings and adults, respectively. About 30 and 20% of this radioactivity were associated with Peyer's patches in weanlings and in aged mice, respectively. This presence was confirmed histologically by the presence of carbon black particles within Peyer's patch macrophages (LeFevre and Joel 1986). These results indicate that carbon black is essentially non-absorbed following oral administration.

Graphenes are a form of insoluble carbonaceous material that have certain physical/chemical characteristics that are similar to carbon black. A pulmonary biodistribution study of few layer graphenes (FLG) is available, where FLGs intratracheally instilled into mice showed that greater than 93% of the instilled dose was retained in the lung or excreted through the feces (the dose in the lung was estimated to be 5 μg) (Mao et al. 2016). The FLG used in this study had hydrodynamic diameters between 100 and 1000 nm. The insolubility and chemical makeup of graphenes suggest that the absorption, distribution, and excretion characteristics of graphenes and carbon black are likely to be similar. In this study, ^{14}C-labeled FLG was utilized to quantify the *in vivo* distribution and excretion in mice up to 28 and 3 days after intratracheal instillation or oral gavage, respectively. Intratracheally instilled FLG was mainly retained in the lung with 47% remaining after 4 weeks. About 46.2% of the intratracheally instilled FLG was excreted through the feces 28 days after exposure. The results showed that intratracheally instilled FLG was mainly retained in the lung or excreted, and there was minimal distribution into other tissues.

Therefore, based on TK studies on carbon black and similar materials, and its known physico-chemical properties, industrially produced carbon black is unlikely to be absorbed to any meaningful extent or distributed in the body.

Evaluating the evidence for genotoxicity

An extensive database of *in vitro* and *in vivo* mutagenicity and genotoxicity studies exists for carbon black, comprising studies performed with standard protocols, non-standard protocols, or novel approaches. An overview of the available data can be found in Table 1 (*in vitro* data) and Table 2 (*in vivo* data). In general, *in vivo* tests are given more weight in determining whether a substance should be considered a mutagen. In this review, both *in vitro* and *in vivo* tests of mutagenicity are evaluated to determine the weight of evidence.

Summary of in vitro *data*

Mutagenicity – bacterial cell systems. Carbon black has been tested *in vitro* for gene mutations in bacteria (Ames tests) and mammalian cells [mouse lymphoma tests, hypoxanthine phosphoribosyltransferase (*HPRT*) tests], for aneugenic and clastogenic effects (several micronucleus tests), and for sister chromatid exchanges. In the majority of the published studies, however, only indicator tests were used, such as the alkaline comet assay. No evidence for mutagenicity was found in Ames tests performed according to the current Organization for Economic Co-operation and Development (OECD) testing guideline 471. The majority of tests performed with carbon black extracts (Soxhlet extraction with toluene for several hours) were negative. Some positive results were found with Soxhlet extracts of pre-1979 produced carbon blacks (N330, Black Pearls L) probably due to nitropyrene impurities, demonstrating the mutagenic potential of impurities in carbon black (Rosenkranz et al. 1980). However, more recent studies in biofluids have shown that polycyclic aromatic hydrocarbons (PAHs) from N330 are not bioavailable (Borm et al. 2005).

Mutagenicity – mammalian cell systems. Small increases in mutation frequency at cll and lacZ loci (1.4- and 1.2-fold, respectively) were reported in the FE1 MML MutaMouse^{TM} epithelial cell line after excessive exposure causing cytotoxicity (576 h, total 6 mg carbon black) (Jacobsen et al. 2007, 2011).

Mouse lymphoma cells were exposed for 3 h to carbon black particles both in the presence and absence of metabolic activation. No mutagenic activity was found (Lloyd 2011). As the EU Scientific Committee on Consumer Safety (SCCS 2015) speculate, the exposure might have been too short for cellular uptake of the particles (which has not been investigated). However, as separation of the insoluble carbon black particles from the cells after exposure is difficult, if not impossible, sufficient exposure of cells is likely [see also Kirwin et al. (1981)].

Clastogenicity. The chromosome-damaging potential of carbon black was explored *in vitro* using the micronucleus test which detects both potential aneugens and clastogens. In this assay, cytochalasin B (cytoB) is often used to block cytokinesis, resulting in binucleate cells and allowing for the identification and analysis of micronuclei in only those cells that have undergone a complete mitosis. CytoB also inhibits endocytosis, and thus might reduce or prevent cellular uptake of particles (Gonzalez et al. 2011).

No evidence for chromosomal damage was found in a comprehensive *in vitro* micronucleus study using mouse RAW 264.7 macrophages exposed to carbon black (Printex 90) concentrations of up to 100 mg/L for 48 h; a delayed co-treatment with 4 mg/L cytoB was used (Migliore et al. 2010). Another micronucleus test in RAW 264.7 macrophages was negative at 1 mg/L, but slightly increased (less than 2-fold) micronuclei frequencies were found at test concentrations of 3 and 10 mg/L;

there were acentric chromosome fragments present at all concentrations tested. However, due to insufficient historical control data, the relevance of the chromosomal effects was considered uncertain (Di Giorgio et al. 2011). A dose-dependent increase in the frequency of micronuclei was also reported in an earlier study with the RAW 264.7 cell line after 48 h of incubation at comparatively low doses (1, 3, and 10 μg/cm^2, corresponding to 2.2, 6.6, and 22 mg/L) (Poma et al. 2006). No clastogenic and no aneugenic activity was found in a test using Chinese Hamster Ovary (CHO) cells up to the highest tested concentration of 120 mg/L; however, the incubation time was only 3 h (Lloyd 2012).

An increase in the frequency of micronuclei was found in the human lung carcinoma cell line A549 when exposed to Printex 90 for 6 h; the response showed a plateau at 2 mg/L, with no further increase at higher concentrations. Cell growth was reduced by 60% at the highest dose tested (200 mg/L). In this study, no cyto B was used; however, solvent control cultures (0.9% saline with 0.05% v/v Tween 80, serum) also showed an increase in micronucleus frequency after 48 h. No further data were reported on the solvent controls nor on cytotoxicity (Totsuka et al. 2009). When treated for 48 h, with cytoB added 18 h before harvesting, a less than 2-fold increase in micronuclei and cytoskeleton disruption was found in an undifferentiated embryonic hamster epithelial cell line at "almost negligible cytotoxicity" (Riebe-Imre et al. 1994).

Carbon black did not induce sister chromatid exchanges (SCE) in CHO cells (Kirwin et al. 1981). Cell transformations were not found in a validated mouse fibroblast model (Kirwin et al. 1981), while the results reported in a non-validated and undifferentiated embryonic hamster epithelial cell line (Riebe-Imre et al. 1994) are considered inconclusive.

Overall, the standard *in vitro* genotoxicity studies in mouse lymphoma and CHO cells were all negative. The results of micronucleus studies in macrophages and A 549 cells were however inconclusive.

PAH-DNA adducts. Industrially manufactured carbon black is produced by pyrolysis of hydrocarbons at high temperatures under controlled process conditions. This process results in the formation of unavoidable trace levels of organic impurities, such as PAHs. In a study to test the possible release of PAHs from commercial carbon black and their ability to form PAH adducts, PAH adducts were analyzed in lung epithelial cells (A549) after exposure to either original carbon black particles (Lampblack 101, Sterling V, N330, and Printex 90), toluene Soxhlet-extracted particles or to the toluene extracts transferred into DMSO. The cells were incubated with original and extracted carbon black particles in concentrations between 30 and 300 μg/cm^2. Adduct spots were found with Sterling V only. However, there was no dose–response relationship and the spot remained unidentified (Borm et al. 2005).

Indicator tests, including comet assays. Indicator tests detect DNA damage or specific mutations (Nikolova et al. 2014), which are the first in a line of events that may lead to permanent change. The comet assay is used to detect strand breaks induced in cellular DNA and involves electrophoresis at high pH. Strand breaks results in structures resembling comets, where the intensity of the comet tail relative to the head reflects the number of DNA breaks.

Due to the current lack of an agreed *in vitro* comet OECD testing guideline, different methods have been used to test carbon black. Therefore, it is difficult to derive any firm conclusions from the many reported tests on carbon black using the comet assay (Table 1). However, the data show that DNA damage was in most cases associated with cytotoxicity, and that there is evidence of oxidative DNA damage (as shown in the formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay; a modification that detects oxidized purines). Clearly cytotoxic doses induced oxidative damage in the FE1-MML MutaMouse^{TM} epithelial line (Jacobsen et al. 2007), single-strand breaks in human A549 cells (Mroz et al. 2007, 2008), and oxidative DNA damage in mouse embryo fibroblasts (Yang et al. 2009) and in HUVEC cells (Frikke-Schmidt et al. 2011). DNA damage at low or non-cytotoxic doses was noted in RAW 264.7 mouse macrophages after 24 h exposure (Di Giorgio et al. 2011; Rim et al. 2011), and in human A549 and monocytic T-cells (THP-1) after 48 h exposure (Don Porto Carero et al. 2001), while in another study no DNA damage was reported in A549 cells exposed to up to 40 μg/cm^2 for only 4 h (Karlsson et al. 2008). Oxidative damage was also found in HepG2 cells at a concentration of 25 mg Printex 90/L (Vesterdal et al. 2014). A dose of 20 μg Printex 90/cm^2 for 4 h did not induce DNA damage in human Caco-2 intestinal carcinoma cells (Gerloff et al. 2009), and up to 138 μg carbon black/cm^2 was negative in human embryonic lung cells and Chinese hamster V79 cells (Zhong et al. 1997).

Of note is that, after Printex 90 exposure, DNA single-strand breakage in human alveolar epithelial type II (A549) cells was significantly mitigated by the addition of epithelial lining fluid (ELF), suggesting that ELF plays a protective role against particle induced oxidative stress and DNA damage (Chuang et al. 2013).

Summary of in vivo *data*

In vivo studies with carbon black include tests for germ cell mutations, mutations in the *hprt* gene, mutations in the p-53 gene, DNA adducts, and indicator tests for DNA damage and repair. An overview of the available studies is presented in Table 2.

Germ cell mutations. The effects of Printex 90 on female germ cell mutagenesis were studied in pregnant mice intratracheally instilled four times with 67 μg per animal, given during the critical developmental stages of fetal oogenesis (gestation days 7, 10, 15, and 18) (Boisen et al. 2013). The dose induced persistent pulmonary inflammation in the animals. Female offspring were raised to maturity and mated with unexposed males. Expanded simple tandem repeat (ESTR) germline mutation rates in the resulting F2 generation were determined from full pedigrees (mother, father, and offspring) of F1 female mice. ESTR mutation rates in carbon black-exposed F2 female offspring were not statistically different from those of F2 female control offspring. The observed mutation rate in germ cells of carbon black-exposed F1 females was not significantly different from that of controls.

Although the study protocol has not been internationally validated, the sensitivity of the model and the high dose employed gives reasonable confidence that carbon black would not induce mutations in oocytes.

Somatic cell mutations. A significant and dose-related increase in the *hprt* mutation frequency in rat alveolar epithelial cells was detected immediately after 13 weeks of inhalation exposure to 7.1 and 52.8 mg/m^3 carbon black (Monarch 880, 220 m^2/g) as well as after 3- and 8-month recovery periods for the groups exposed to 52.8 mg/m^3. No effect was found in the epithelial cells of rats exposed to 1.1 mg/m^3 [which was the no observed adverse effect level (NOAEL)]. Exposure to 52.8 mg/m^3 carbon black resulted in *hprt* mutation frequencies which were 4.3-, 3.2-, and 2.7-fold greater than the air control group, immediately and after 3 and 8 months post-exposure, respectively. A significant increase in the frequency of *hprt* mutations was detected after 13 weeks of exposure to 7.1 mg/m^3 carbon black but not after 3 or 8 months of recovery. Lung tissue injury and inflammation, increased chemokine expression, epithelial hyperplasia, and pulmonary fibrosis were observed after exposure to 7.1 and 52.8 mg/m^3, with these effects being more pronounced at the higher exposure level (Driscoll et al. 1996).

In a subsequent study (Driscoll et al. 1997), the relationship between the severity of inflammation and mutations was confirmed by co-incubating lung lavage inflammatory cells from carbon black exposed rats with lung epithelial cells from unexposed rats. Bronchoalveolar lavage (BAL) cells were isolated from the lung of rats 15 months after intratracheal instillation of saline or saline suspensions of carbon black (Monarch 900, 230 m^2/g) at 10 and 100 mg/kg bw. When the percentage of neutrophils in the lavage fluid was $\geq$50%, the *hprt* mutation rate increased significantly, possibly related to the generation and release of reactive oxygen species (ROS) and/or depletion in antioxidants. The mutation spectrum was compatible with mutations being caused by ROS. Importantly, mutations in the *hprt* gene occurred only at the high-dose exposure concomitantly with inflammation and epithelial hyperplasia.

In a study comparing inflammatory responses and *ex vivo* *hprt* mutation frequencies in rats, mice, and hamsters after subchronic inhalation exposure to carbon black (1, 7, or 50 mg/m^3), rats demonstrated greater propensity for generating a pro-inflammatory response and *hprt* mutations at the higher doses of 7 and 50 mg/m^3. No effects on *hprt* mutation frequencies were found at a dose level of 1 mg/m^3, which was also a dose at which inflammatory effects were not observed. These results show that chronic inflammation at higher exposures may lead to a secondary indirect genotoxic response (Carter et al. 2006).

Some rat lung tumors from a carcinogenicity study with carbon black (Nikula et al. 1995) were analyzed for mutations in the K-ras or p53 genes. Only very low levels of either K-ras or p53 genes were mutated; there were no significant differences between the yields of mutants recovered from diesel exhaust, carbon black or sham-exposed rats (Swafford et al. 1995; Belinsky et al. 1997). The lung tumors analyzed for the K-ras gene mutation all came from a single carbon black-exposed rat. These small sample sizes limit the reliable interpretation of the reported results. It is further noted that in rat lungs – unlike the situation in human and mice lungs – the induction of p53 and/or K-ras mutations is generally very low (Rosenkranz 1996). In the same tumor samples, hypermethylation (inactivation) of the p16 gene (a gene involved in the inhibition of cell-cycle progression) further supports a role for oxidative stress and inflammation in the etiology of these tumors (Belinsky et al. 2002).

In the gpt delta transgenic mouse model, in which point mutations and deletions can be analyzed separately by two distinct selection methods (gpt and Spi – assays), no increases in mutant frequencies were found in the lungs after intratracheal instillation of 0.2 mg Printex 90 per animal (Totsuka et al. 2009).

DNA adducts. Carbon black did not induce DNA adduct formation in the lungs or livers of rats (Wolff et al. 1990; Gallagher et al. 1994; Borm et al. 2005; Danielsen et al. 2010). Borm et al. (2005) analyzed DNA obtained from lung homogenates isolated immediately after 13 weeks of inhalation exposure to up to 50 mg/m^3 of Printex 90 and Sterling V resulting in lung burdens of 4.9 and 7.6 mg, respectively. ^{32}P-post-labeling of lung DNA showed no spots relating to PAH-DNA adduct formation when compared to sham-exposed animals. An increase of adducts in rat alveolar type II cells when compared to the filtered-air controls was reported (Bond et al. 1990), however, the same low-surface area carbon black tested negative in another study (Wolff et al. 1990). The overall interpretation by IARC of these investigations was that carbon black does not cause DNA adduct formation (IARC 2010).

DNA damage and DNA repair. DNA damage and repair was studied in rats in two subchronic studies investigating oxidative damage in lung tissue. One study used intratracheal instillation (Ziemann et al. 2011; Rittinghausen et al. 2013), in the other, inhalation exposure was used (Gallagher et al. 2003). At a clearly inflammatory dose (3 $\times$ 6 mg/rat by intratracheal instillation, once a month for 3 months), Rittinghausen and coworkers found increased levels of certain genotoxic stress markers in pulmonary alveolar lining cells. The authors describe the results as "in line with ongoing ROS production and oxidative DNA damage/repair during inflammatory processes" (Rittinghausen et al. 2013).

A No Observed Effect Level (NOEL) of 1 mg/m^3 could be derived from a 90-day inhalation study, with the Lowest Observed Effect Level (LOEL) for oxidative damage (8-oxo-dG in lung DNA) at 7 mg/m^3 for high-surface area carbon black (Printex 90, 300 m^2/g), and at $\geq$50 mg/m^3 for low surface area carbon black (Sterling V, 50 m^2/g) (Gallagher et al. 2003). An increase in 8-oxo-dG formation was observed at 50 mg/m^3 Printex 90, after 13 weeks of exposure and after 44 weeks of recovery and at 7 mg/m^3 only after 44 weeks of recovery. No increase in 8-oxo-dG was observed at 1 mg/m^3 or for any exposures of Sterling V.

In a comparative study in rats, using single intratracheal or intragastric administrations of 0.64 mg/kg (corresponding to *ca.* 128 μg) of Printex 90, no oxidative damage was reported in the lungs or liver after intratracheal administration; however, increases in 8-oxo-dG (23%) and etheno-adduct levels (54–75%) were reported in the liver after intragastric (gavage)

administration (Danielsen et al. 2010). The intratracheal dose employed was considered by the authors to be "probably below the overload threshold" as inflammatory markers in broncho-alveolar lavage (BAL) were not increased at 24 h after instillation. mRNA levels of genes related to oxidative stress were increased only after intragastric administration. The elevated etheno-adduct levels might have been secondary to macrophage activation and oxidative stress following the bolus (gavage) administration of the particle suspension. Endotoxins contained in the administered formulations could also have further stimulated macrophage activation.

Comet assays. Several studies with carbon black have used the comet assay to investigate effects on DNA.

Inhalation studies (rat, mouse). In rats exposed to 6 mg/m^3 Printex 90 by nose-only inhalation for 14 days (6 h/day, 5 days/week), no DNA strand breaks or oxidative DNA damage was found in BAL cells with the comet assay, measured at day 1 and day 14 post-exposure (Lindner et al. 2017). Also in mice, no DNA damage was found in BAL cells of dams and their offspring after inhalation exposure (whole-body) of the pregnant mice to 42 mg/m^3 (1 h/day, gd8–18); however, an increase in DNA lesions/10^6 base pairs was found in livers of both dams and offspring. The level of oxidatively generated DNA damage in the liver was not increased [only determined in the offspring by the level of formamidopyrimidine DNA glycosylase (fpg) enzyme sensitive sites]. Analysis of BAL fluid cell composition demonstrated the presence of inflammation in the lungs. The observed effects in the liver of dams were most likely due to fur grooming and therefore due to gastrointestinal particle exposure, whereas the effect in the offspring could have been mediated by maternally induced inflammatory cytokines (Jackson et al. 2012a). The observed effects could however also have been within the normal physiological variation (historical controls were not reported). Increased DNA strand breaks in BAL were found 1 h after inhalation exposure of TNF-deficient mice to 4 × 20 mg/m^3 (for 1.5 h each); less damage was found in TNF +/+ mice, which might be explained by the fact that TNF induces neutrophil apoptosis as a mechanism for removal of neutrophils (Murray et al. 1997; Saber et al. 2005).

Intratracheal instillation studies (mouse). Comet effects were found in BAL fluid of mice at day 1 and day 28 after single instillation of 18 μg Printex 90/mouse (vehicle: physiological saline containing 10% BAL from untreated mice of the same strain), after 28 days when 54 μg were applied, and after 1, 3, and 28 days when 162 μg were instilled. In hyperlipidemic Apo$^{-/-}$ mice, which are particularly sensitive, the comet assay was positive at 3 h after instillation of 54 μg. It was reported that the vehicle used in these studies contained particles >20 nm (Jacobsen et al. 2009; Bourdon et al. 2012b, 2012c); no increase was found at day 1 post-exposure in mice dosed with 54 μg/animal, using the same vehicle (Saber et al. 2012). Comet effects were also found at 3 h and 3 days after a single instillation of 162 μg/mouse, but not anymore after 43 days. In this study, a suspension in nanopure water was used (Husain et al. 2015), which results in smaller particle sizes than the suspension in saline plus BAL. Increases in %tail DNA (the recommended parameter by the OECD test guideline on the *in vivo* comet assay) were found in a recent study only at the first measured time-point (1 day) after a single instillation of low doses (0.67 and 2 μg/animal, as suspensions in nanopure water with hydrodynamic particle diameters of ca. 800 and 900 nm, respectively), whereas no increase was noted at 6 and 162 μg/animal (hydrodynamic diameters around 100 and below 100 nm, respectively). When measured as tail length, DNA damage increased on day 3 in the 2 and 6 μg groups, and on day 28 in the 0.67 and 2 μg groups. A persistent inflammatory response was only seen in the high-dose group (162 μg/animal), but a significant increase in BAL neutrophil count was found at all dose levels on day 1 post-exposure (Kyjovska et al. 2015). In none of the low-dose groups was the percentage of neutrophils in BALF close to the numbers associated with an increased mutation frequency as shown in the study by Carter et al. (2006). It has been reported that smaller particles may be cleared less efficiently than larger sized particles due to impaired phagocytosis (IARC 2010). The time course and magnitude of the observed effects in the low dose range might therefore be explained with the size-dependent efficiency with which macrophages eliminate particles after having been triggered by neutrophil recruitment (Bellingan and Laurent 2008; Hirota and Terada 2012). In phagocytic cells, autophagy and apoptotic pathways have been described for carbon black (Hussain et al. 2010; Stern et al. 2012; Kong et al. 2017), which may lead to typical comet effects (Choucroun et al. 2001) and could explain the observations in the study by Kyjovska et al. (2015). Importantly, faster clearance would hence not result in fewer comet-like structures. In contrast, these structures are likely originating from the phagocytic activity of neutrophils in the first phase after exposure, followed by a second phase dominated by macrophage activity. Because the phagocytic uptake of apoptotic cells is anti-inflammatory (Aderem and Underhill 1999; Aderem 2003; Arandjelovic and Ravichandran 2015) and not associated with significant ROS production unless overload conditions occur, the low-dose findings in the study by Kyjovska et al. (2015) should not be considered as adverse.

In lungs, DNA strand breaks were found after instillation of 18, 54, and 162 μg Printex 90/mouse (in physiological saline containing 10% v/v acellular BAL from C57BL/6 mice). At 54 and 162 μg, the effects were still present at day 28 post-instillation, but fpg-sensitive sites were only found at day 1 (in all dose groups) and at day 3 in the 162 μg group, but no longer on day 28 (Bourdon et al. 2012b). DNA strand breaks were also found at 3 and 24 h after a dose of 200 μg/animal; 50 μg/animal were however without effect (Totsuka et al. 2009). No effects on %tail DNA were found in lung tissue after a single dose of 0.67 μg/mouse, but 28 days post-exposure to 2 μg, and at day 1 after 162 μg (Kyjovska et al. 2015), an effect that is most likely associated with macrophage recruitment from BAL into the lungs (as shown by macrophage counts in BALF reported in the paper) and subsequent phagocytic activity. A dose of 6 μg caused a small increase in tail length only. The apparent lack of a dose–response is well explained by (a) the higher efficiency with which macrophages can phagocytize the bigger aggregates in the 2 μg group as compared to the other groups and (b) the overload situation in the 162 μg group which causes a massive macrophage recruitment into

the lungs. No information on oxidative DNA damage was provided in the paper.

In livers, no DNA strand breaks were found at 1, 3, and 28 days after a single instillation of 0.67, 2, 6, or 162 μg Printex 90/animal (Kyjovska et al. 2015). DNA strand breaks were reported 24 h and 28 days after single intratracheal doses of 18, 54, or 162 μg/animal (Bourdon et al. 2012b) and also in livers of offspring whose mothers were intratracheally treated during pregnancy with 4 × 67 μg/dam (Jackson et al. 2012a). The DNA strand breaks in liver were considered not to be caused by a direct interaction with carbon black particles (Jackson et al. 2012a; Kyjovska et al. 2015).

Artifacts and "irrelevant positives"

Carbon black, mainly due to its insolubility, adsorption capacity, and optical properties poses particular challenges to toxicological testing and the results are prone to artifacts (Monteiro-Riviere and Inman 2006; Kroll et al. 2009; Kroll et al. 2012). In particular, *in vitro* tests in which cytotoxicity and/or cell viability are measured are readily disturbed by interferences in colorimetric assays. Dispersions of carbon black have been shown to interfere with optical detection systems in several assays, including lactate dehydrogenase (LDH) and ROS measurements and can create both false negative as well as irrelevant positive results (Kuhlbusch et al. 2009; Almutary and Sanderson 2016). Moreover, settling and adsorption of the test substance to the test tubes and plates, and to nutrients, proteins and vehicle constituents during test substance preparation and administration as well as to cells during the incubation step of *in vitro* assays may influence (diminish or enhance) the effect(s) under investigation (Kroll et al. 2011). In only a few of the reported *in vitro* studies is an assessment made of particle interference with the endpoint being measured, e.g. by Cao et al. (2014). Positive *in vitro* findings in cells directly exposed to extreme doses of carbon black in test systems for which it is known that carbon black interferes with assay components should therefore not be considered as an indication of primary genotoxicity.

Indicator tests are useful tools for preliminary screening and for mechanistic studies, e.g. for the detection of oxidative DNA damage. However, they are not suitable to differentiate between primary or secondary genotoxicity mechanisms. For example, in the *in vitro* comet assay, particles remain in close proximity to the virtually naked DNA following the lysis of agarose-embedded cells with the consequence that they may interact with DNA and create artifactual findings. Such a scenario is not expected to occur in intact cells or *in vivo* where the barrier of the nucleus protects the DNA molecule (Rittinghausen et al. 2013). According to the most recent OECD guidance document on genotoxicity testing (OECD 2015) "When evaluating potential genotoxicants, more weight should be given to the measurement of permanent DNA changes than to DNA damage events that are reversible. In general, indicator tests should not be used in isolation and a substance should not be considered mutagenic (or non-mutagenic) on the results of indicator tests alone".

Also, the comet studies in mice with single or multiple intratracheal instillations may be considered useful as preliminary screens to explore a potential hazard. However, because local bolus doses are applied, the physiological defense and internal repair mechanisms are usually overwhelmed and this may induce abnormal or artifactual responses. Jacobsen et al. (2009) report that at similar lung doses, the inhalation of carbon black causes much less inflammation than instillation. In a study in hyperlipidemic $ApoE^{-/-}$ mice, the neutrophils in BALF – a marker for pulmonary inflammation – reached 76% following instillation of 54 μg/animal, whereas only 6% were found following a single inhalation exposure to 60 mg/m^3 for 90 min. High neutrophil counts in BAL are linked to increased ROS production and a secondary genotoxic mechanism.

Furthermore, it is currently not possible with the *in vivo* comet assay to differentiate between different cell types within the investigated organ or tissue (e.g. between macrophages and epithelial cells). This assay is therefore of limited use to differentiate between primary and secondary genotoxicity.

Weight-of-evidence for a primary genotoxicity of carbon black

Primary genotoxicity by particles has been defined as "genetic damage elicited by particles in the absence of pulmonary inflammation". It is characterized by a direct physical interaction or an oxidative attack by ROS at the particle surface on the genomic DNA or its associated components (Schins and Knaapen 2007; DFG 2013). For carbon black, however, a secondary genotoxic mechanism has generally been demonstrated and accepted, as genotoxicity is only observed at concentrations that also cause persistent inflammation. The available experimental *in vitro* data as well as the *ex vivo* and *in vivo* data in rats support such a secondary mechanism for genotoxicity (IARC 2010).

Recently, however, it has been postulated that carbon black also has a primary (direct or indirect) genotoxic effect on DNA (Jacobsen et al. 2007, 2008; Botta and Benameur 2011; Ziemann et al. 2011; Kyjovska et al. 2015; SCCS 2015). However, our weight-of-evidence analysis of all the available genotoxicity data indicates a secondary or indirect genotoxic mechanism for carbon black. Details on the available genotoxicity tests are summarized in Tables 1 and 2 for *in vitro* and *in vivo* studies, respectively.

Carbon black did not induce DNA-adduct formation in the lungs of rats (Wolff et al. 1990; Gallagher et al. 1994; Borm et al. 2005). The lack of DNA-adduct formation strongly supports the view that no direct interaction occurs between carbon black and DNA.

All *in vitro* guideline tests in mammalian cell systems (Mouse Lymphoma and CHO cells) were negative for gene mutations and chromosomal aberrations (summarized in Table 1). A limitation is that carbon black uptake into the cells was not measured, and in some studies, the duration of exposure may be considered too short. It is however difficult to completely wash-off adsorbed carbon black particles from cell surfaces, therefore the exposure duration may in fact be longer than assumed. These studies do not provide evidence for primary genotoxicity. The results of the *in vitro*

micronucleus tests were inconclusive and may have been influenced by different testing conditions. Positive results were found at subtoxic levels in studies using immune cells (mainly mouse peritoneal macrophages); the chromosomal damage therefore was likely caused by phagocytosis. Recently reported DNA strand breaks in BALF and lung cells of mice after single intratracheal bolus doses were not associated with persistent inflammation and therefore assumed to be indicative of a primary genotoxic mode of action by the study authors. Given the differential cell count in BALF and the time course and magnitude of DNA effects, it is however more likely that these findings reflect a functional and adaptive reaction to remove particles by phagocytosis. We find that the reported data are consistent with particle clearance by phagocytic cells with no significant ROS production and that they therefore should not be considered adverse or indicative of a genotoxic effect. In a recent review, comet results were attributed little weight because no clear link has been demonstrated between DNA strand breaks and mutagenesis or carcinogenesis (Møller and Jacobsen 2017).

Consistent with the hitherto generally accepted secondary genotoxicity mechanism, oxidative DNA lesions (8-oxo-deoxyguanosine adducts) in carbon black exposed rats were only found at inflammogenic exposure concentrations and were mainly related to a marked neutrophil influx; similarly, persistent lung inflammation was necessary to induce *hprt* mutations in lung epithelial cells of carbon black exposed rats. Importantly, the mutations could be prevented by treatment with the antioxidant catalase, further supporting the role of ROS, and thus secondary genotoxicity, in the generation of mutations (Driscoll 1996; Driscoll et al. 1996, 1997; Elder et al. 2005; Ziemann et al. 2011; Rittinghausen et al. 2013).

Therefore, particle exposures that do not overwhelm host defense mechanisms and hence do not elicit inflammatory and proliferative responses would not be expected to pose an increased risk of secondary genotoxicity. In the rat, which is the most responsive species under particle overload conditions, an inhalation NOAEL of 1.0 mg/m^3 respirable carbon black has been established in sub-chronic studies, with signs of mild inflammation found at the next higher tested dose level of 7 mg/m^3 (LOAEL) (Driscoll 1996; Driscoll et al. 1996, 1997; Gallagher et al. 2003; Elder et al. 2005; Carter et al. 2006).

In summary, genotoxic effects are not expected to occur under conditions that do not induce persistent and prolonged inflammation.

Evaluating the evidence for reproductive toxicity

To date, there are a number of instillation studies and one short-term inhalation study that have evaluated selected endpoints related to reproduction and developmental toxicity; usually at one time point and/or using a single dose level only. One developmental study via the oral route was also identified [(Ramesh 2012) as cited in SCCS (2015)]. Although the oral route has limited relevance as a potential route of exposure for carbon black in both occupational and consumer scenarios, for the sake of completeness, it is worth noting the conclusions of this developmental toxicity study. The study authors concluded that oral administration of carbon black to pregnant rats at 100, 300, or 1000 mg/kg body weight/day during the sensitive period of organogenesis was well tolerated, and that there were no adverse maternal changes or any effects on embryo-fetal development (Ramesh 2012). However, inhalation represents the main route of exposure in the occupational setting, and a complete guideline developmental toxicity study using the inhalation exposure route is not available. A number of review papers on the reproductive and developmental toxicity of inhaled nanomaterials, including carbon black, also conclude that the available studies are limited and do not allow for definitive conclusions (Ema et al. 2015; Hougaard et al. 2015). An overview of available studies with carbon black on reproductive and developmental endpoints is shown in Table 3, and is discussed below.

Sexual maturation, fertility, and reproduction

In a study evaluating the effects of carbon black on the sexual development and neurofunction of mice exposed *in utero* to carbon black, Jackson et al. (2011) intratracheally instilled pregnant mice on gestational days 7, 10, 15, and 18 to one of three concentrations of carbon black (Printex 90; 2.75, 13.5, or 67 μg in 40 μl water). Final cumulative doses were 11, 54, or 268 μg/animal. Marked inflammation, sustained throughout the lactation period (until weaning) was recorded only in those dams exposed to a total dose of 268 μg/animal carbon black. Gestational and litter parameters were normal for dams and offspring. Sexual development, characterized as anogenital distance in weanlings and as the onset of puberty was unchanged. The results on neurofunction are described below under developmental effects.

In utero exposed mice did not exhibit adverse effects on various reproduction parameters after intratracheal instillation of a total dose of 267 μg/animal carbon black over the gestation period of 7–18 days (instillation on GD 7, 10, 15, 18, and 67 μg/instillation) (Kyjovska et al. 2013). Values for testicular weight, relative testicular weight as well as sperm content per gram testicular tissue and daily sperm production were similar to controls. The male progeny of *in utero* treated males and untreated females displayed slightly reduced daily sperm production in comparison with matched controls. The single dose regimen used in the study precludes any assessment of dose–response relationships. Also, F2 males were analyzed at only one time point as young adults (post-natal day 80). Although the findings (a slight reduction in sperm production) were statistically significant, only one time point and one dose was evaluated, which is a weakness in the study.

In another study, male mice were intratracheally instilled with carbon black (different groups received Printex 90 (14 nm primary particle size), Printex 25 (56 nm primary particle size), and Flammruss 101 (95 nm primary particle size) at a dose of 100 μg given 10 times at weekly intervals (resulting in a total dose of 1000 μg) (Yoshida et al. 2009). Observed effects included increased testosterone with exposure to Printex 90 and Printex 25, changes in seminiferous tubules and decreased daily sperm production with all three carbon blacks. The dose used was very high (totaling1000 μg),

therefore it was not possible to determine the relevance of these effects at more reasonable doses or if there is a dose–response for this reported effect.

In a subsequent study, pregnant mice were intratracheally instilled with carbon black at 200 μg/mouse on GD 7 and 14, resulting in a total dose of 400 μg (Yoshida et al. 2010). In this study, male offspring were evaluated at 5, 10, and 15 weeks after birth. They showed histological changes in the testes, as well as decreases in daily sperm production. There were no changes in body weight, testicle weight, epididymis weight, or serum testosterone levels in male offspring. Only one high dose was used, therefore it was not possible to determine if these effects are relevant at lower doses that may be more representative of human exposures or to determine any possible dose-response relationship.

Developmental effects

An oral (gavage) prenatal development toxicity study in rats was conducted with carbon black following OECD Guideline No. 414 (Ramesh 2012). The carbon black used in this study had a very low PAH content [total PAH <0.5 ppm and benzo(a)pyrene <0.005 ppm] and is permitted for use in cosmetics. In this study, rats were administered doses of 0, 100, 300, or 1000 mg/kg body weight/day carbon black by oral gavage on days 5 through 19 of gestation. Maternal evaluations and measurements included daily clinical signs and body weight/food intake measured at designated intervals. No deaths were observed. Dark colored feces were observed in all animals given carbon black which is related to the color of the test substance. This finding is therefore considered to be non-adverse. There were no changes in litter parameters, and there were no fetuses with major malformations. Minor fetal anomalies and normal variants observed were of the type and incidences commonly observed in rats of this strain and age and hence were considered to be incidental. The study authors concluded that oral administration of carbon black to pregnant rats at 100, 300, or 1000 mg/kg body weight/day during the sensitive period of organogenesis was well tolerated. There were no adverse maternal changes or any effects on embryo-fetal development. Accordingly, under the conditions of this study, NOAEL for maternal toxicity and the NOEL for developmental toxicity were both set at 1000 mg/kg body weight/day.

Jackson et al. (2012a) conducted an inhalation and instillation study with carbon black to examine its effects on the development of *in utero* exposed offspring. In the inhalation part of the study, carbon black was administered to pregnant mice at a single concentration of ca. 42 mg/m^3 for 1 h/day from days 8 through 18 of gestation. DNA damage and lung inflammation were examined 5 and 24 days after cessation of exposure in dams. On post-natal days (PND) 2, 22–23, and 50, livers of offspring animals were subjected to DNA analyzes. Treatment resulted in the generation of an increase in polymorphonuclear neutrophil (PMN) associated inflammation in the lungs of dams, which was detected 5 days after exposure and sustained at the 24 day post-exposure observation time point. The level of DNA strand breaks was increased in the liver cells of dams at both sampling time points: 5 and 24 days after exposure and in liver of weanlings (PND 22–23) and in adolescent (PND 50) offspring when compared to matched controls. However, in BAL cells, DNA damage was not evident. Neither the inhalation or instillation exposure routes resulted in effects on gestational and lactation parameters assessed in dams, or developmental effects in the offspring (Jackson et al. 2012a). DNA strand breaks on its own cannot be considered as reproductive toxicity as there is no indication that this would result in heritable chromosomal changes.

In the instillation part of the Jackson et al. study, carbon black, suspended in water, was administered intratracheally to three groups of time-mated pregnant mice on gestations days 7, 10, 15, and 18. The final doses over the four instillation time points were 11, 54, and 268 μg carbon black/animal, respectively. DNA damage and lung inflammation were examined in dams 3–4 and 26–27 days after cessation of exposure. In the offspring, DNA analyzes were performed on post-natal days (PND) 2, 24–25, and 47 to probe for DNA damage. Intratracheal instillation did not result in DNA damage (Jackson et al. 2012a).

In a separate publication, the toxicogenomic effects after treatment were evaluated for dams, 26–27 days; and for neonates, 4 days after cessation of exposure (Jackson et al. 2012b). There was no effect on gestational and lactation parameters assessed in dams or developmental effects in the offspring. Histological analyzes indicated retention of carbon black particles in lungs of dams from both the medium and the high dose groups. Nevertheless, persistent neutrophil-marked inflammation, measured in BALF, was confined to the high dose animals only. Results from histological analysis of lung tissue correlated well with BAL findings and indicated a thickening of the alveolar septa with simultaneous interstitial infiltration of macrophages and neutrophils in the high dose animals. However, despite the evidence of sustained inflammation, collagen deposition, a hallmark feature of fibrosis, was not evident. The exposure to carbon black also did not affect the level of DNA strand breaks in BAL cells of dams or in liver cells of dams and offspring. Toxicogenomic analysis revealed altered expression of several inflammatory regulators such as cytokines and chemokines in dams, both at the transcriptional and tissue protein levels, which was significant only in the high dose group (Jackson et al. 2012b).

Neurodevelopmental parameters in the offspring were part of the intratracheal mouse study performed by Jackson et al. (2011) and described above in more detail (Jackson et al. 2011). Behavioral testing, performed only with animals of the control and high dose groups (268 μg/mice), revealed a different pattern of habituation in the female offspring only. Contrary to the males, females moved differently in open field tests during the first 2 min compared with control; total ambulation decreased significantly during the first minute of observation and increased significantly in the second minute of observation compared with that of controls. The authors speculate that the aberrant movement pattern is more likely a result of maternal inflammation than a direct particle effect on offspring. No effects on gestation and lactation were observed in this study (Jackson et al. 2011).

Several studies have been conducted that evaluated different endpoints in the offspring of *in utero* treated animals, including effects on the brain, kidney, genotoxicity, and immune system. Pregnant mice were intranasally instilled with Printex 90 at 95 μg/kg/day on GD 5 and 9, and brains were collected from male offspring at 6 and 12 weeks after birth (Onoda et al. 2014). The brains showed enlargement of granules of perivascular macrophages and changes in astrocyte phenotypes. The authors state that these changes indicate increased risk of dysfunction and disorder in the offspring brain although a treated but intact satellite group was not included in the study to further investigate and support or refute this claim. In the only primate study available for developmental endpoints for carbon black, pregnant rhesus macaque monkeys were injected intradermally with carbon black suspended in 0.1% Tween 80 at a dose of 10 mg/ml 4–6 times at intervals of 10–12 days. It is not clear whether 1 ml was injected each time; therefore, the total dose injected is uncertain. Diesel exhaust particulates and titanium dioxide were also used as test materials in different animals. The brains collected from newborn infants showed higher levels of hemoglobin compared to control animals. The study showed that diesel exhaust particulates caused the highest levels of hemoglobin. The authors state that the altered hemoglobin was likely due to responses to oxidative stress and/or hypoxia in the fetal brain. Maternal adverse effects were not evaluated in the study, so that it is not possible to determine whether the effects on the fetus are a secondary non-specific consequence of overall maternal toxicity. The authors also state that hemoglobin upregulation may be a consequence of oxidative or inflammatory stress, or hypoxia; therefore, at least initially, the increased hemoglobin is a protective mechanism. The authors state that higher levels of hemoglobin are reportedly neurotoxic; however, the level of increase necessary to cause neurotoxicity is not stated (Mitsunaga et al. 2016).

In a study evaluating the kidney as an endpoint, pregnant mice were intratracheally instilled with Printex 90 at 50 μg/mouse on GD 5 and 9, resulting in a total dose of 100 μg (Umezawa et al. 2011). There was increased expression of collagen type VIII in the kidney of 12-week-old offspring mice but not in 3-week-old offspring mice. There was no difference in levels of serum creatinine or blood urea nitrogen. The relevance of this finding is not clear because no changes were noted in kidney function.

In mice, oral doses of carbon black administered during pregnancy did not increase the number of eye-spots in the offspring (Reliene et al. 2005). Eye-spots are the results of somatic reversions or deletions at the pink-eyed unstable mutation site (p^{un} site). Twenty days old offspring of treated dams were sacrificed, eyes removed, and retinal pigment epithelium (RPE) slides prepared for eye-spot analysis.

Pregnant mice were intranasally instilled with Printex 90 (95 μg/kg/day) on GD 9 and 15 (El-Sayed et al. 2015). The thymus and spleen were collected from the offspring on postnatal days 1, 3, and 5. Increases in thymocyte and lymphocyte counts were seen in male offspring. Another study using a similar exposure regimen showed that prenatal intranasal instillations of carbon black on GD 5 and 9 induced immunosuppression in newborn mice, which was characterized by the depletion of splenic cells in newborn mice (Shimizu et al. 2014). El-Sayed et al. (2015) has noted the apparent contrast in the results obtained by Shimizu et al. (2014). It is therefore difficult to ascertain the relevance of these findings.

Fedulov et al. (2008) investigated whether neonatal susceptibility to asthma is elevated following *in utero* exposure to particles such as diesel exhaust particles, carbon black or titanium dioxide. They administered a single intranasal dose of 50 μg carbon black to pregnant mice (Balb/c) on day 14 of gestation. After birth (post-natal day 4), offspring of treated dams were sensitized by an intraperitoneal injection of ovalbumin (OVA) and challenged on post-natal days 12–14 with aerosolized 3% OVA. Post-challenge, mice were subjected to pulmonary function and pathologic analysis. Airway responsiveness, analyzed using whole body plethysmography, was a measure for allergic response. The offspring showed increased susceptibility to allergy. However, there is a scientific debate on whether the applied plethysmography technique accurately and reliably measures lung mechanics in small laboratory animals such as mice. Some authors have reported theoretical and practical problems with the technique (Petak et al. 2001; Albertine et al. 2002; Adler et al. 2004), whereas others have reported good correlation between enhanced pause (Penh) measurements and other assays of airway responsiveness (Hamelmann et al. 1997; Finotto et al. 2001; Lee et al. 2008).

Evaluation of studies

Table 3 summarizes studies that evaluate reproductive and developmental toxicity endpoints for carbon black. There is one oral gavage study that was conducted using a standard OECD protocol (OECD 414; prenatal developmental toxicity study). The other studies were conducted using non-standard or novel approaches, and typically evaluated very limited and specific endpoints. Because each of these studies has limitations, such as test material characterization, test material administration using non-physiological routes (such as intratracheal instillation), use of high or single doses etc., a weight-of-evidence approach is used to assess the available studies for the reproductive and developmental toxicity of carbon black.

Test material preparation. Most of the reported studies evaluating reproductive toxicity endpoints administered the test material via intranasal or intratracheal instillation. Such an administration method requires that the test material be suspended in water or other solution. Some of the test methods involved aggressive means of suspending carbon black, such as sonicating for up to 30 min, filtering to exclude larger aggregates/agglomerates, and use of dispersing agents such as Tween 80 to achieve and maintain a homogenous distribution of test particles in solution. It has been noted, however, that exposure to extraneous surfactants can disturb the homeostasis of endogenous surfactants of the lungs. Additionally, such dispersing agents can also generate adverse effects themselves (Driscoll et al. 2000). These dispersion methods might have resulted in breaking down carbon

black agglomerates into aggregates, such that the size of the carbon black may be substantially different from the agglomerated form that is typically encountered in the workplace environment.

Dosing. Most of the reproductive toxicity studies with carbon black use single or multiple instillations, which may be useful preliminary screens to explore a potential hazard, develop hypotheses or, compare differences between test materials. However, ECETOC (2013) noted that "A major concern regarding the use of intratracheal instillation is that the introduction of a large bolus dose of the test substance into the lung in a short period of time will overwhelm the normal lung response and defense systems to the extent that it may produce responses that are pathophysiological artifacts that would not be seen if the same dose was delivered over a longer period of time via an inhalation exposure. This can thus produce serious problems for the interpretation of both hazard identification and risk assessment." If the instillation method with high doses produces no adverse effects then it is unlikely that adverse effects would be observed at lower doses given over a longer period of time. However, if the instillation method shows adverse effects, it would be reasonable to then conduct inhalation studies to see if these effects are still observed with physiological exposure routes and doses that are more relevant for human exposures.

Many of the studies used single doses and/or very high doses of carbon black. The one inhalation study available for evaluating reproductive and developmental toxicity endpoints used an aerosol concentration of 42 mg/m^3 administered 1 h/day to pregnant mice during gestation days 8–18 (Jackson et al. 2012a). Jackson et al. justify this high aerosol concentration by stating that the dose of 1 h exposure to 42 mg Printex 90/m^3 corresponds to only one-and-a-half day exposure that Danish workers might experience at the time-weighted average occupational exposure limit (3.5 mg/m^3 as an 8-h time-weighted average for carbon black). However, this extrapolation does not account for the large differences between humans and mice in factors such as body weight, breathing rates, lung surface area etc. For example, Erdely et al. (2013) developed mouse inhalation doses for a test material by extrapolating from human to mouse alveolar depositions, which accounted for the difference between the human alveolar surface area of 102 m^2 compared to the mouse alveolar surface area of 0.05 m^2. This extrapolation exercise shows that there are orders of magnitude differences in physiological parameters between humans and mice. It is also noted that mice were dosed with 42 mg/m^3 carbon black for 1 h/day over 11 days (gestation days 8–18), which is a very high exposure that is likely to overwhelm normal lung defense responses.

Many of the instillation studies also use a single dose, rather than a range of doses thus precluding evaluation of a dose–response effect. Some of the instillation doses used are also quite high; for example, Jackson et al. (2012a, 2012b) use a high dose of 268 μg/mouse, and Yoshida et al. (2009) instilled 100 μg/mouse at 10 weekly intervals resulting in a total dose of 1000 μg/mouse. In contrast, researchers at the US National Institute of Occupational Safety and Health (NIOSH) typically use doses under 100 μg/mouse for pharyngeal aspiration studies of nanoparticles as these doses usually fall below lung overload in the mouse model. At a carbon black (Printex 90) dose of 40 μg/mouse, Roberts et al. (2016) found increased but transient inflammation in the lung. While the use of a high dose indicates that it may be possible to evoke certain adverse effects, the interpretation of such findings for hazard identification and human risk assessment is not clear because of the generation of potential artifactual effects.

Screening level studies looking at specific endpoints. As noted in a review of reproductive and developmental toxicity of carbon-based nanomaterials (Ema et al. 2015), none of the rodent studies evaluating airway exposure of carbon black used a standard guideline testing approach. Many of the studies looked at very specific post-natal endpoints, such as lymphocyte count in thymus and spleen, perivascular macrophages in brain, collagen in the kidney, etc. It is not always clear what are the overall effects on the offspring of changes in these specific endpoints. Many of the studies also evaluated post-natal effects at only one time point so that it is not clear if there was recovery from some or all of the observed changes.

Weight-of-evidence for reproductive toxicity of carbon black

It is important to consider the weight-of-evidence from all the available studies to determine whether carbon black is likely to act as a reproductive toxicant. Various regulatory agencies recommend the use of weight-of-evidence in data assessment. For example, the European Chemicals Agency (ECHA 2015) states the following regarding reproductive toxicity: "the weight given to the available evidence will be influenced by factors such as the quality of the studies, consistency of results, nature and severity of effects, the presence of maternal toxicity in experimental animal studies, level of statistical significance for inter-group differences, number of endpoints affected, relevance of route of administration to humans, and freedom from bias. Both positive and negative results are assembled together into a weight of evidence determination." As discussed above, many of the short-term studies evaluating reproductive toxicity of carbon black have limitations, such as test material characterization, test material administration using non-physiological routes (such as intratracheal instillation), use of high doses, evaluation of very limited and specific endpoints, and lack of recovery period in the studies. The one study that was conducted using a standard OECD protocol for developmental effects is an oral study that reported no adverse maternal changes and no effects on embryo-fetal development at the highest dose tested of 1000 mg/kg body weight/day. Based on the chemical characteristics of carbon black, toxicokinetic behavior, relevant exposure levels, and understanding of mechanism of toxicity, the overall weight of evidence indicates that carbon black should not be considered a reproductive toxicant.

Summary and conclusions

Carbon black has been extensively tested for its genotoxic activity both *in vitro* and *in vivo*. The totality of available data

is consistent with the interpretation that carbon black does not directly interact with DNA. Increases in *hprt* mutation frequencies were only noted at concentrations that were clearly associated with marked pulmonary inflammation (Driscoll et al. 1996, 1997). Further evidence supporting that these mutations are due to a secondary mechanism and not due to a direct interaction of carbon black with DNA can be obtained by the negative results in DNA adduct studies. These studies have demonstrated the inability of carbon black to produce DNA adducts in the lungs of rats and in human lung epithelial cells (Wolff et al. 1990; Gallagher et al. 1994; Borm et al. 2005; Danielsen et al. 2010). Clearly, genetic damage might occur by ROS generated as a consequence of impaired particle clearance, i.e. under lung and macrophage overload conditions leading to pulmonary inflammation. In the rat, the most sensitive species with regard to lung overload, a threshold below which no genetic damage is expected to occur has been derived from subchronic inhalation studies at 1 mg/m^3. This value was the NOAEL for any inflammatory effects including any increases in pro- or anti-inflammatory markers in a well-conducted 90 day inhalation study (Driscoll et al. 1996; Elder et al. 2005). Biological responses described after exposure to carbon black *in vitro* investigations tended to be non-specific effects such as cytotoxicity and DNA strand breaks due to often unrealistic and high exposure levels; in only a few instances, were assay interferences controlled or even considered. DNA strand breaks observed at sub-toxic doses in macrophage cell lines and *in vivo* in BAL and lung cells are considered to be due to particle clearance without significant ROS production, and therefore should not be considered as indicative of genotoxicity or as an adverse toxicological effect.

For the reproductive toxicity endpoint, no adverse maternal changes or any effects on embryo-fetal development were seen at the highest dose tested of 1000 mg/kg body weight/day in an oral developmental toxicity study. However, an oral study has limited relevance for interpretation into human situations where inhalation is the most likely exposure route. There are a number of short-term instillation studies and one inhalation study available for carbon black that evaluate endpoints related to reproductive toxicity in mice. In the inhalation study, which was performed only at a single, very high dose level (42 mg/m^3) during the whole sensitive period of gestation, the gestational and post-gestational parameters were not affected, and there were no developmental effects observed in the offspring.

Various other short-term intratracheal instillation studies looked at very specific post-natal endpoints; usually at one single time point and with high dose instillations. The relevance of the reported findings (altered habituation pattern, decreased sperm production, intracerebral macrophage accumulation, changes in immune parameters) to reproduction and the overall development of the offspring cannot be fully interpreted due to the lack of reported historical control data for these endpoints and inconsistent results. It is also possible that the effects observed in the instillation studies may be the result of non-specific inflammatory effects caused by high exposure doses.

Toxicokinetic studies on carbon black and similar carbonaceous materials indicate that industrially produced carbon black is unlikely to be absorbed or distributed in the body. Therefore, carbon black is unlikely to reach reproductive organs and tissues and have a direct effect on reproductive functions or the developing organism.

Thus, based on an overall weight-of-evidence evaluation of the available published studies, industrially produced carbon black is neither considered to be a primary genotoxicant nor a reproductive or developmental toxicant.

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Declaration of interest

IC and YN are employees of Cabot Corporation and Orion Engineered Carbons, respectively, both of which are carbon black manufacturing companies. IC, YN, and LL are members of the Scientific Advisory Group (SAG) to the ICBA (http://www.carbon-black.org) who funded this review paper. ICBA is a scientific, non-profit corporation originally founded in 1996. The purpose of the ICBA is to encourage and develop international communication, cooperation, and research concerning carbon black environmental, health, and safety matters and related regulatory matters. The ICBA is a seven member association of carbon black manufacturers with global operations and is funded by the member companies. LL and CF are both independent consultants to the SAG/ICBA. This review article was prepared during the course of employment (IC and YN) or as compensated consultants by SAG/ICBA (LL and CF). In the past 5 years, none of the authors have appeared in any legal or regulatory proceedings related to the content of the paper. The paper has not been reviewed by either in-house or outside legal counsel. The review, synthesis, and conclusions reported in this paper are the exclusive professional work product of the authors and may not necessarily represent the views of their employers or funding sources.

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