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Pulmonary toxicity of silver vapours, nanoparticles and fine dusts: A review

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Abstract

Silver is used in a wide range of products, and during their production and use, humans may be exposed through inhalation. Therefore, it is critical to know the concentration levels at which adverse effects may occur. In rodents, inhalation of silver nanoparticles has resulted in increased silver in the lungs, lymph nodes, liver, kidney, spleen, ovaries, and testes. Reported excretion pathways of pulmonary silver are urinary and faecal excretion. Acute effects in humans of the inhalation of silver include lung failure that involved increased heart rate and decreased arterial blood oxygen pressure. Argyria—a blue-grey discoloration of skin due to deposited silver—was observed after pulmonary exposure in 3 individuals; however, the presence of silver in the discolorations was not tested. Argyria after inhalation seems to be less likely than after oral or dermal exposure. Repeated inhalation findings in rodents have shown effects on lung function, pulmonary inflammation, bile duct hyperplasia, and genotoxicity. In our evaluation, the range of NOAEC values was 0.11 to 0.75 mg/m^3. Silver in the ionic form is likely more toxic than in the nanoparticle form but that difference could reflect their different biokinetics. However, silver nanoparticles and ions have a similar pattern of toxicity, probably reflecting that the effect of silver nanoparticles is primarily mediated by released ions. Concerning genotoxicity studies, we evaluated silver to be positive based on studies in mammalian cells *in vitro* and *in vivo* when considering various exposure routes. Carcinogenicity data are absent; therefore, no conclusion can be provided on this endpoint.

1. Introduction

Occupational inhalation exposure to silver potentially occurs in industries that engage in the following activities: manufacturing and using nanomaterials (Lee et al., 2011), silver smelting and refining and preparation of silver salts (DiVincenzo et al., 1985), silver soldering (Kachru et al., 1989; Vance, 1960), silver brazing (Gan et al., 1995; Mangold and Beckett, 1971), and recovery of silver from recycling sources (Pifer et al., 1989). Another source of silver dusts is the trimming (grinding) of amalgam dies in dental laboratories (Brune and Beltesbrekke, 1979). The occupation of silversmith has also been linked to silver exposure (Aktepe et al., 2015), and silver nanoparticle-containing spray disinfectants are another source of occupational and consumer exposure (Quadros and Marr, 2011; Rogers et al., 2018).

Physical forms of silver relevant to inhalation are ions, particles, flakes, and fibres; the latter 3 includes nanoparticles (up to 100 nm in diameter) and larger particles, nanobelts, and wires. Ions—when present as salts—may act as particle entities when brought into an aerosol in the workplace. Respirable sizes of particles and fibres are up to 5 µm in diameter; thus, powders below this size are most important in the context of alveolar endpoints. However, materials of a larger size can exert toxicity in the upper airways if deposited there, or at sites of deposition upon removal from the upper airways, for example, the gastrointestinal tract. In addition, the potential transfer of silver through the nose to deposit in the brain must be considered.

We have published reviews on oral (Hadrup and Lam, 2014) and skin (Hadrup et al., 2018) toxicity of silver exposure. In this work, we review the adverse effects of silver after pulmonary exposures. We have evaluated studies describing the biokinetics (absorption-distribution-metabolism-excretion; ADME) and toxicity, including genotoxicity and carcinogenicity. Concerning genotoxicity, we evaluated all exposure pathways, and *in vitro* studies because genotoxicity and carcinogenicity are mostly incited as mechanisms inside single cells irrespective of exposure pathway. Notably, the section on genotoxicity and carcinogenicity is an update based on our recent published evaluation (Hadrup et al., 2018). Overall, the objective of this work was to review the kinetics and adverse effects of silver after pulmonary exposure.

61

62 2. Methods

63 Concerning the literature search strategy, we identified all relevant articles in the PubMed database (Pubmed,
64 2020) by using combinations of the following search terms: ‘silver’, ‘toxicity’, ‘pulmonary’, ‘inhalation’,
65 ‘genotoxicity’, and ‘carcinogenicity’. This search yielded 485 articles. We also evaluated reference lists of
66 the retrieved articles to identify possible additional references missed in the aforementioned searches. The
67 inclusion criteria were that the articles included data on kinetics (absorption, distribution, metabolism and
68 excretion), or toxicity after pulmonary exposure of any form of silver. As aforementioned, for genotoxicity
69 and carcinogenicity: all exposure pathways as well as *in vitro* studies were included. Article languages
70 considered were English, German, Dutch, and French. In total,upon an initial screening of abstracts, we
71 evaluated 102 studies; 74 of those studies were included in this article.

72

73 3. Absorption, distribution, metabolism and excretion (ADME)

74 3.1. Absorption and distribution – following pulmonary exposure

75 *Levels of silver in the general population*

76 In 26 individuals living in the Melbourne metropolitan area and having no known occupational exposure to
77 silver, the blood levels were less than 1 µg silver/L (Wan et al., 1991). No silver was detected in the blood of
78 control populations with a detection limit of 6 µg silver/L (Rosenman et al., 1979) or 5 µg silver/L
79 (DiVincenzo et al., 1985).

80

81 *Levels of silver in workers who worked with metals*

Levels of silver in the body of workers reflect absorption from inhalation and from other pathways, for example, through mucosal surfaces. Few studies provided estimates of exposure: Two men involved in the manufacturing of silver nanomaterials were each exposed to 0.00035 and 0.00135 mg silver/m^3, resulting in blood levels of 0.34 and 0.30 µg silver/L, respectively (Lee et al., 2012). Two men worked with the recovery of silver from X-rays and photographic films. One 42-year-old man was exposed to 0.085 mg silver/m^3 (incineration area) and 1 mg/m^3 (pulverising area) and had a blood level of 49 µg silver/L. The other, one 51-year old man, with an estimated exposure between 0.03 and 0.17 mg silver/m^3, had a blood level of 74 µg silver/L (Williams and Gardner, 1995). The same 51-year-old man was monitored for 5 years, and over those 5 years, his initial silver of 74 µg/L decreased to approximately 11 µg/L (Williams, 1999).

Concerning studies in which exposure levels were not estimated: In 30 workers exposed to silver nitrate or silver oxide, 12 had a measurable blood level of silver (>6 µg/L) (median level: 19.5 µg/L; range: 11 to 84 µg/L). In 25 workers manufacturing silver powders, the mean blood value was 10 µg silver/L (range 0.5 to 62) (Rosenman et al., 1987). In 21 silver reclamation workers, the mean level was also 10 µg silver/L (Pifer et al., 1989). In another study, 98 workers had blood levels of silver between 0.1 and 23 µg/L, silver reclaimers had on average 6.8 µg/L, workers refining silver for bullion coins had 2.5 µg/L, and workers in jewellery production had 1.2 µg/L (Armitage et al., 1996). In 37 American workers involved in processes such as smelting and refining silver and manufacturing silver salts, the mean blood level was 11 µg silver/L (DiVincenzo et al., 1985). A 27-year-old man occupationally exposed to aerosolised silver had a serum level of 154.4 µg silver/L (Cho et al., 2008). In an older study on silver finishers, they had silver in the lung, liver, heart, and spleen (Barrie and Harding, 1947).

Studies with rodent inhalation of silver nanoparticles

Most reports on the distribution of silver after pulmonary exposure to nanoparticles are on the localisation of elemental silver in organs; however, some studies have described the localisation of particles in the body. The question is whether these reports reflect that silver is distributed as particles or whether this is caused by

dissolved silver nanoparticles that have reformed into secondary particles in the tissues. The latter has been demonstrated after oral exposure e.g. in (Aaseth et al., 1981; Juling et al., 2016; Loeschner et al., 2011).

Distribution of silver (measured as elemental silver) after pulmonary exposure occurs in several organs. In 1 study, inhalation of silver nanoparticles (14–15 nm) at 0.05, 0.12, and 0.38 mg/m^3 (6 h/day, 5 day/week for 12 weeks) resulted—at all dose levels—in elevated silver in rat lung, whereas dose-dependent elevations occurred in the liver, kidneys, spleen, testes, blood, and eye, but not the brain. The levels decreased over a recovery period, but silver remained present in the liver, spleen, and eyes at the highest dose at 12 weeks post exposure (Song et al., 2013). In another study, rats were exposed to silver nanoparticles (18–19 nm, at 0.049, 0.133 or 0.515 mg/m^3 6 h/day, 5 days/week for 13 weeks); and silver increased in the lungs liver, kidneys, brain, and olfactory bulb. Blood levels were increased to 0.7 (low dose), 1.8 (mid dose), and 4.3 µg silver/kg tissue wet weight (high dose) compared with 0.09 µg in controls (Sung et al., 2009). In rats exposed to 15 nm silver nanoparticles (6 h at 0.133 mg/m^3), immediately after exposure, silver was detected in substantial amounts in the lung, nasal cavities, lung–associated lymph nodes, and blood; and at low levels in the liver, kidney, spleen, brain, and heart. In the kidney, heart, and lymph nodes, silver was observed 1 day later. The amount of silver in these organs normalised over a recovery period of 7 days (Takenaka et al., 2001). Rats were exposed to silver nanoparticles (18.1–19.6 nm) at mass concentrations of 0.031, 0.082, or 0.116 ,g/m^3, 6 hours/day, 5 days/week for 4 weeks. Silver levels in lungs after 1 day of recovery of 14.7, 6.4 and 1.6 were initially cleared with half-lives of 2 to 4 days, followed by a slow clearance phase with half-lives of 60 to 100 days (Jo et al., 2020). Other studies in rodents demonstrated deposition in the lungs and liver (Braakhuis et al., 2016), heart, spleen, and testes (Kwon et al., 2012).

Other pulmonary exposure pathways

A sufficient number of inhalation studies have directly represented the occupational pulmonary exposure pathway and describe the absorption and distribution of silver nanoparticles. Nevertheless, to describe the

differential distribution of silver ions and nanoparticles, and on nanowires/nanofibers, we also included aspiration and intratracheal instillation studies. Concerning the nanowire form of silver, silver nanowires 2 or 20 µm in length, dosed by intratracheal instillation, were in the lung, localised and enclosed in granulomas or surrounded by only a few macrophages in the terminal bronchiole-alveolar duct junction. No further organs were investigated (Silva et al., 2014).

Concerning the comparison of nanoparticles and ionic silver, mice were administered 20 or 110 nm silver nanoparticles by oropharyngeal aspiration of a single bolus of 0.1, 0.5, or 1 mg/kg body weight (bw); or to 1 mg/kg bw silver ions in the form of $AgNO_3$. After 40 hours of recovery, the deposition of silver in lung tissue was higher with exposure to particles compared with an equal mass amount of ionic silver. By contrast, the amount of silver in the liver increased only after the ionic silver. At 21 days of recovery, silver remained present in the lungs of particle-administered animals, whereas it was absent in the animals dosed with ionic silver (Wang et al., 2014). A similar lung/liver deposition pattern was observed after the intratracheal instillation of 20 nm silver nanoparticles (10.3 µg silver/mouse ~0.5 mg/kg bw) or ionic silver in the form of $AgNO_3$ (7.5 µg silver/mouse ~0.38 mg/kg bw) (Arai et al., 2015). The biodistribution of silver after the dosage with agglomerated 4–10 nm silver nanoparticles (agglomerated particles > 100 nm dominant, but also ultrafine particles present) was compared with that of ionic silver ($AgNO_3$) after intratracheal instillation (50 µg of agglomerated silver nanoparticles/rat, or 7 µg $AgNO_3$/ rat equal to 4.4 µg silver/rat). The amount of silver deposited in the lungs seemed to be more prolonged after the dosage with nanoparticles compared with a more rapid clearance from this organ when silver was dosed as ions (Takenaka et al., 2001). Taken together, the data from these 3 studies suggest that ionic silver is rapidly distributed to the liver, kidney, and spleen, whereas silver in nanoparticles may remain in the lungs for a longer duration, perhaps reflecting a slow release of silver ions from the surface.

3.2. Metabolism of silver formulations following pulmonary exposure

Oral and dermal exposure to silver, in ionic or nanoparticle form, have been demonstrated to lead to the formation of particles within various human and animal tissues—particles that, in addition to silver, contain other elements such as selenium and sulphur (Aaseth et al., 1981; Hadrup et al., 2018; Hadrup and Lam, 2014; Loeschner et al., 2011). However, evidence from pulmonary studies has been scarce and inconclusive. The presence of sulphur in the aforementioned newly formed particles has been explained by the high affinity of silver and sulphur, which could result in a combination of silver ions with biological structures and constituents with high sulphur content. A similar silver and sulphur complex has also been observed in the lungs after a pulmonary exposure by using micro X-ray absorption near edge structure (µXANES). Mice were exposed to a heterogeneous composition of 25 nm spherical and 80–90 nm rod-shaped silver nanoparticles (100 µg/mouse or 4 mg/kg bw) by a single oropharyngeal aspiration. In lungs, silver nanoparticles were mostly present in macrophages, either partially or totally dissolved and chelated by thiol-containing ligands such as cysteine, glutathione, or metallothionein (Smulders et al., 2015). However, the formation of new nanoparticles was not described.

Pregnant and non-pregnant mice were exposed to 18–20 nm silver nanoparticles at 0.54 mg/m^3 for 1 to 4 hours. This exposure resulted in the location of silver-containing nanoparticles in the lungs, spleen, liver and placenta, and the head region of the foetus. In the foetus, the detected silver was almost entirely in the ionic form or as nanoparticles <13 nm. In the placenta, silver nanoparticles were approximately 6% of the total silver, and in the liver and spleen, the percentage was approximately 12% and 14%, respectively. By contrast, 21% of the total silver was still in the nanoparticulate form in the lung at the time of the measurements. Thus, a conclusion was that the silver translocating from the lung was mainly ionic or in the form of small, readily dissolving nanoparticles. This case was also observed for silver translocating through barriers, for example, through the placenta and to the foetus. The authors could not rule out the possibility that some of the nanoparticles identified in tissues were not the original nanoparticles that translocated the lung barrier but instead particles newly formed after interaction with sulphur groups of proteins, and/or selenium or chloride present in tissues (Campagnolo et al., 2017).

Rats inhaled 15 nm silver nanoparticles (0.179 mg/m³) 6 hours per day for 4 consecutive days. Electron-dense structures were observed in the vesicles and nuclei of lung cells on the first day of recovery after employing the silver enhancement technique. The authors did not attempt to prove the presence of silver within the particles. From the size of the silver-enhanced structures (15-20 nm), the authors concluded that the original size of the silver nanoparticles must have been less than 5 nm in diameter. This size was interpreted as an indication of partial dissolution of the nanoparticles (Braakhuis et al., 2014). Davidson and colleagues exposed rats to 20 or 110 nm silver nanoparticles for 6 hours by inhalation. Metallic silver was identified by X-ray absorption spectroscopy as the dominating silver species in the lung throughout a 7-day period. The method could not exclude the existence of small amounts of silver atoms or ions. The results further indicated that the original nanoparticles were transformed to other forms of metallic silver nanomaterials, namely, much smaller silver nanoparticles or a highly porous, zeolite-like nanomaterial, 7 days post exposure. Dissolution of the inhaled silver nanoparticles was considered the most plausible explanation for this observation (Davidson et al., 2015).

3.3. Excretion – following pulmonary exposure

Urinary and faecal excretion of silver Excretion of silver has been described to occur through urine and faeces after oral, dermal, and mucosal surface exposure (Hadrup et al., 2018; Hadrup and Lam, 2014; Skare and Engqvist, 1994). Concerning pulmonary exposure, many human reports of urinary excretion of silver have been provided. Silver was elevated above normal (1.9 µg/L) in urine in 26 of 27 workers involved in the manufacturing of precious metal powders. The mean value was 11.3 µg/L and the range was 0.5 to 52.0 µg/L (Rosenman et al., 1987). In a man occupationally exposed to aerosolised silver, the urinary level was 243.2 µg silver/L (Cho et al., 2008). A man who had for 7 years been working with the manufacturing of silver nanomaterials had a urine level of 0.43 µg/L (Lee et al., 2012). However, other studies have investigated the faecal pathway. Silver was detected in urine in only 1 of 27 silver reclamation employees (≥5 µg/L urine), whereas silver was detected in all faecal samples (n=18) with a mean value of 16.8 µg/g. In controls, the mean value in faeces was 1.5 µg/g (Pifer et al., 1989). In 37 workers exposed to silver and

having a mean blood level of 11 µg/L, urine content was reported to be less than 0.005 µg silver/g, whereas the faecal content was 15 µg silver/g, In controls, the faecal level was 1.5 µg/g (DiVincenzo et al., 1985). These data support that both the urinary and faecal pathways are pathways of excretion in humans; of these the faecal one seems to be most pronounced.

Timeframe for the elimination of silver Rats inhaled 20 or 110 nm silver nanoparticles for 6 hours at 7.2 or 5.4 mg/m^3, respectively. Although there was a reduction in silver in the lungs over a 56-day recovery period, 33% of the delivered dose was still present at this time point (both particle sizes) (Anderson et al., 2015a). Mice inhaled 5 nm silver nanoparticles at 3.3 mg/m³ for 4 hours/day for 10 days. Silver in the lungs was found to be 31 µg/g lung (median value; dry weight) immediately after exposure, decreased to 10 µg/g after a 3-week recovery period (Stebounova et al., 2011). Song *et al.* found that after the inhalation of silver nanoparticles (0.05, 0.12, or 0.38 mg/m^3, 6 h/day, 5 days/week for 12 weeks) the amount of silver in rat lungs decreased over a 12-week recovery period; but was still different from controls at this time point (e.g. following 12 weeks of inhalation at the low dose, the level in lungs was ~100 µg/kg decreasing to ~5 µg/kg after 12 weeks of recovery). Additionally, silver was still present in the liver, spleen, and eyes (in these organs <1 µg/kg) after the 12-week recovery period (Song et al., 2013). Studies in rodents that used intratracheal instillation or aspiration have also demonstrated that after the dosage of silver nanoparticles, silver is only slowly eliminated from the lungs (Anderson et al., 2015b; Smulders et al., 2015; Takenaka et al., 2001; Wang et al., 2014). By contrast, after the dosage of ionic silver, a more rapid elimination occurs in the lungs, accompanied by distribution to the liver (Arai et al., 2015; Wang et al., 2014).

4. Toxicity observed in humans

4.1. Acute toxicity

A 27-year-old man, whose job was to melt silver ingots, accidentally inhaled massive silver-containing vapours. Fourteen hours later, he developed a headache, shortness of breath, and difficulty breathing

(moderate dyspnoea). As the dyspnoea intensified, he was hospitalised. His breathing rate, heart rate, and arterial pressure increased, and the oxygen pressure of the capillary blood was depressed. He had markedly livid lips (dark bluish in colour, perhaps reflecting deposition of silver), numerous crackles in the lungs, leucocytosis, and increased transaminase—the latter is an indicator of liver damage. Despite the treatment with oxygen and pharmacological agents, the respiratory inefficiency intensified to include cyanosis (low oxygen saturation leading to bluish colour). There was no verbal contact, and he had narrow, even pupils. He was given artificial ventilation; X-ray photographs suggested *shock lung* (lung failure). He was given further pharmacological treatment, and the next day, he exhibited improvement. On the 14th day of admission, he no longer needed artificial ventilation, and afterwards, complete recovery was observed (Forycki et al., 1983).

4.2. Chronic toxicity

Argyria is a blue-grey discoloration of the skin (and other organs) due to the deposition of silver-containing granules. Argyria can be localised at the exposed area or be generalised covering larger parts of the body—in particular, the parts exposed to sunlight. Argyria has foremost been demonstrated after oral and dermal exposure to silver (Hadrup et al., 2018; Hadrup and Lam, 2014). Notably, some studies have suggested the occurrence of argyria also following pulmonary exposure; however, without testing for silver in the discolorations: A 27-year-old man was exposed to an aerosol reportedly containing silver, alcohol, and acetone. A blue-grey discoloration of the face, eye (sclerae and conjunctiva), and oral mucosa had progressed over 4 months. In a biopsy specimen from the face, granules—proposed to contain silver—were observed in the epidermal basal layer. Granules were also found located in the basement membrane zone of sweat glands. His serum level of silver was 154.4 μg/L (Cho et al., 2008). Of 30 workers exposed to silver nitrate and silver oxide, 6 were reported to have argyria. Based on personal air sampling, time-weighed-average exposure was between 0.039 and 0.378 mg silver/m^3. In addition to argyria, the workers reported other symptoms, for example, the majority reported eye and upper and lower respiratory tract irritation. The presence of silver in the blood was found to be associated with complaints of abdominal pain

255 (Rosenman et al., 1979). A male silver-cleaner aged 37 years was diagnosed with argyria localised to the
256 nasopharyngeal mucosa with sub-epithelial accumulation of black granules (Ferrara et al., 2018).

257 The presence of silver in granules was demonstrated in 1 study but only in the lungs: A 63-year-old man
258 who had worked as a silver finisher for almost his whole working life died of a blood clot in the heart. The
259 pleura covering the right lung was described as being blue-black with a few small denser grey spots. Small
260 areas of fibrosis contained high amounts of pigment; moreover, this was observed in some of the alveoli and
261 in non-fibrotic perivascular aggregates. The pigment contained iron and silver, as demonstrated by chemical
262 analysis of ash. However, the silver content was low (0.036%) compared with iron (3.5%); thus, the
263 discoloration could be due to iron and not silver. In a comparison of the samples, the controls contained iron
264 but no silver. Other signs of toxicity were lung emphysema and a fibrous thickening over 1 lung (Harding,
265 1948). Barrie and Harding reported 4 cases in which silver finishers had excess silver and iron deposition in
266 tissues. By autopsy, all the men were observed to have emphysema and detectable levels of silver in the
267 lungs; in 1 case, detectable levels of silver were observed in the liver and spleen; in another case, detectable
268 levels of silver were observed in the heart (no normal silver level reported). Notably, the men's iron levels in
269 the lung were increased compared with normal values (Barrie and Harding, 1947).

270 Argyria may also manifest in the eyes[1]. Notably, although this condition in theory may develop
271 secondary to pulmonary silver exposure, this condition more likely occurs after direct exposure of the eyes.
272 One case of ocular argyria was in a 51-year-old male who for 7 years had worked at silver refinery and had a
273 blood level of 740 µg silver/L. Over an 18-month follow up period, the blood level of silver decreased to 60
274 µg/L, but the argyria remained. Silver was not chemically proven in the eyes (Williams, 1999; Williams and
275 Gardner, 1995). Thirty workers were exposed to silver nitrate and silver oxide after working for more than 2
276 years at a plant that manufactured precious metal powders, 20 of the workers were reported to have
277 deposition of silver in the eyes (Rosenman et al., 1979).

278

[1] Ocular silver deposition is sometimes referred to as 'argyrosis' (Pifer et al., 1989).

4.3. Human studies in which no toxicity was observed

Twenty-one out of 27 silver reclamation employees had measurable (above detection limit) silver levels in the blood (mean level: 10 µg/L). All 27 controls (matched on sex, age, and race) had silver levels below the detection limit (5 µg/L). No cases of generalised argyria were observed based on the colour of the face and electron microscopy of skin biopsies. Additionally, no general signs of adverse effects of the silver were observed (Pifer et al., 1989). In 2 workers recovering silver from X-rays and photographic films, and having blood levels of silver of 49 and 74 µg/L, no signs or symptoms of toxicity were observed (Williams and Gardner, 1995).

5. Toxicity findings in animal studies

5.1. Lung function endpoints

Decreased tidal and minute volumes were observed in rats during inhalation of 18 nm silver nanoparticles ($LOAEC_{lung\ function}$ 0.05 mg/m^3, 6h/day for 13 weeks) (Sung et al., 2008). The same endpoints and peak expiratory flow were decreased in rats that inhaled 14–15 nm silver nanoparticles (6 h/day, 5 days/week for 12 weeks) at 3 mass concentrations: 0.05, 0.12 and 0.38 mg/m^3. The effects were persistently observed during exposure and over 12 weeks of recovery at all dose levels ($LOAEC_{lung\ function}$ 0.05 mg/m^3) (Song et al., 2013). Inhalation of 13–16 nm silver particles increased tissue elastance in 1 of 2 rat stains ($LOAEC_{elastance}$: 0.6 mg/m^3 for 12 h) (Seiffert et al., 2016). Increased pulmonary elastance was also observed in mice 1 day after intratracheal instillation of 0.05 mg/kg bw silver nanoparticles 20 and 110 nm) (Botelho et al., 2015). Additionally, following intratracheal instillation of 15 nm silver nanoparticles (0.5 mg/kg bw), a decrease in tissue stiffness and resistance was accompanied by increased surfactant protein D in mice (Botelho et al., 2018), and increased pulmonary resistance and decreased dynamic compliance was observed in rats (Seiffert et al., 2015). Concerning negative studies, no effect on tidal volume and minute volume was observed after 4 hours of inhalation of silver nanoparticles (18–20 nm) at up to 0.75 mg/m^3 (Sung et al., 2011).

5.2. Inflammation and cytotoxicity measured in bronchoalveolar lavage fluid

Inhalation studies Rats that inhaled silver nanoparticles of various sizes had increased neutrophil numbers, total protein, and LDH in bronchoalveolar lavage (BAL) fluid at all exposure periods (34 min–6 h/day for 4 consecutive days, 1 dose level per particle). The following dose descriptors were observed: 18 nm particle: LOAEC 0.055 mg/m^3; 34 nm: NOAEC: 0.041 mg/m^3; 60 nm: LOAEC: 0.043 mg/m^3; 160 nm: NOAEC: 0.555 mg/m^3 (Braakhuis et al., 2016). In the same animal species, inhalation of 15 nm silver nanoparticles was compared with inhalation of 410 nm particles (0.179 mg/m³ and 0.167 mg/m³, 6 h/day for 4 days). Exposure to 15 nm silver nanoparticles resulted at 1 day of recovery in increased neutrophil, lymphocyte, and monocyte numbers and increased cellular damage markers in the lungs. Only increased BAL protein was observed in response to exposure to the 410 nm silver particle (15 nm silver $LOAEC_{neutrophils}$: 0.18 mg/m^3; 410 nm silver $NOAEC_{neutrophils}$: 0.17 mg/m^3)(Braakhuis et al., 2014). The inhalation of 13–16 nm silver nanoparticles (0.6 to 0.8 mg/m^3, 3 or 12 h) resulted in increased numbers of neutrophils in rat BAL fluid and changes in IL-1β, KC, IL-17, CCL2 and CCL3, phospholipid levels, surfactant protein D across doses, and time points ($LOAEC_{neutrophils}$: 0.62 mg/m^3, 12 h) (Seiffert et al., 2016). A $NOAEC_{neutrophils}$ in BAL fluid was 0.082 mg/m^3 in rats exposed to silver nanoparticles 18.1–19.6 nm in diameter (6 hours/day, 5 days/week for 4 weeks) (Jo et al., 2020). Mice were exposed to 5 nm silver nanoparticles (3.3 mg/m³, 4 h/day for 10 days), and increased neutrophils in BAL fluid were observed both at exposure end and at 3 weeks of recovery. No effects were observed in the other endpoints ($LOAEC_{neutrophils}$: 3.3 mg/m^3) (Stebounova et al., 2011).

Other studies have shown no effect on neutrophil numbers. Neutrophil numbers were not increased in rats exposed to silver nanoparticles (0.01 or 0.1 mg/m^3; 5 h). The mean aerodynamic diameters were 33 to 39 nm ($NOAEC_{neutrophils}$: 1 mg/m^3) (Roberts et al., 2013). Rats were exposed to 18 nm silver nanoparticles at 0.05, 0.13 mg/m^3, or 0.5 mg/m^3. The duration was 6 hours/day 5 days/week for 90 days. There were no

differences in polymorphonuclear cells[2] in BAL ($NOAEC_{polymorphonuclear\ cells}$: 0.5 mg/$m^3$) (Sung et al., 2008). Mice were exposed to 20 nm silver nanoparticles at 2.9 mg/ for 6 hours. There were no changes in neutrophil numbers in BAL fluid. Total protein in BAL increased immediately after exposure but normalised after 24 hours of recovery ($NOAEC_{neutrophils}$: 2.9 mg/m^3) (Kwon et al., 2012).

Intratracheal instillation or oropharyngeal aspiration studies A substantial number of studies have investigated pulmonary inflammation following inhalation of silver nanoparticles, and a less important role for intratracheal instillation/aspiration studies in the hazard assessment is suggested. However, these studies are crucial for the comparison between silver nanoparticles and ionic silver and nanowires/fibres, as these have not been investigated by inhalation. Concerning nanowires, rats were instilled with 2 or 20 µm silver nanowires (0.1, 0.5, or 1.0 mg/kg bw). After 1 day of recovery, exposure to the short wire was associated—at the 2 highest doses—with increases in polymorphonuclear cells in BAL. The long wire only increased inflammation and eosinophilia at the highest dose. Protein in BAL was increased at all doses and both lengths at Day 1. Frustrated phagocytosis was reported for the long nanowires. After exposure to the 20 µm nanowires, alveolar and bronchial inflammation was observed by histopathology (2 µm nanowires $NOAEL_{neutrophils}$: 0.1 mg/kg bw; 20 µm nanowires $NOAEL_{neutrophils}$: 0.5 mg/kg bw (Silva et al., 2014).

Concerning intratracheal instillation studies comparing silver nanoparticles to ionic silver: Mice were exposed through single oropharyngeal aspiration to silver nanoparticles 20 or 110 nm, coated with citrate or polyvinylpyrrolidone (0.1, 0.5 or 1 mg/kg bw) or silver ions ($AgNO_3$ at 1 mg/kg bw). Exposure to the 20 nm particle resulted in increased neutrophils in BAL at all doses, whereas the 110 nm particle only had an effect at the highest dose. $AgNO_3$ also increased neutrophil numbers after 40 hours of recovery. At 21 days of recovery, there was still increased neutrophil numbers in BAL at the highest dose of the 110 nm particle, but not after exposure to the 20 nm particle or $AgNO_3$ (20 nm particle $LOAEL_{neutrophils}$: 0.1 mg/kg bw; 110 nm particle $NOAEL_{neutrophils}$: 1 mg/kg bw; $AgNO_3$ $LOAEL_{neutrophils}$: 1 mg/kg bw) (Wang et al., 2014). Mice were exposed to 20 nm silver nanoparticles (10.3 µg silver/mouse ~0.5 mg/kg bw) or ionic silver in the form of

[2] Polymorphonuclear cells (PMN) is the collective term for neutrophils, basophils, and eosinophils.

351 $AgNO_3$ (7.5 µg silver/mouse ~0.38 mg/kg bw) by intratracheal instillation. Exposures to both forms of silver
352 were associated with increases in neutrophil numbers at 4 and 24 hours of recovery. However, the $AgNO_3$
353 exposed animals had higher neutrophil numbers at 24 hours compared with silver nanoparticles. The
354 interleukin-1β concentration in BAL fluid was, by contrast, higher for silver nanoparticles than for ionic
355 silver (20 nm particle $LOAEL_{neutrophils}$: 0.5 mg/kg bw; $AgNO_3$ $LOAEL_{neutrophils}$: 0.38 mg silver/kg bw) (Arai et
356 al., 2015).

357 Concerning other intratracheal studies, and investigating only nanoparticles, the NOAEL/LOAEL
358 values of increased neutrophil/polymorphonuclear-cell numbers in BAL fluid are as follows. In rats,
359 NOAELs of 0.1 mg/kg bw (Seiffert et al., 2015) (Silva et al., 2015) to 0.2 mg/kg bw (Haberl et al., 2013)
360 were observed. In mice, NOAELs of 0.05 and 0.5 mg/kg bw (Botelho et al., 2018, 2015) and of 6.4 mg/kg
361 bw (Gosens et al., 2015) have been reported. LOAEL values of 0.25 and 4 mg/kg bw have been reported by
362 (Scoville et al., 2017; Smulders et al., 2015, 2014).

363 5.3. Inflammation evaluated by histology

364 Increased alveolar accumulation of macrophages and alveolar chronic inflammation were observed in rats
365 after inhalation of 14–15 nm silver nanoparticles (NOAEC: 0.12 mg/m^3 6 h/day, 5 days/week for 12 weeks)[3]
366 (Song et al., 2013). Abnormal inflammatory cell infiltrates, chronic alveolar inflammation, and small
367 granulomatous lesions were observed in rats exposed to silver nanoparticles (18–19 nm) ($NOAEC_{histology}$ 0.13
368 mg/m^3 for 390 h) (Sung et al., 2009). In addition, intratracheal instillation studies with silver nanoparticles
369 produced LOAEL values of 0.1 mg/kg bw (Seiffert et al., 2015) and 1 mg/kg bw (Silva et al., 2015). After
370 instillation of 20 µm silver nanowires, histological scores showed that 0.5 and 1.0 mg/kg bw produced
371 inflammation (NOAEL 0.25 mg/kg bw). Additionally, frustrated phagocytosis was observed—the
372 insufficient ingestion of long fibres into BAL fluid cells (Silva et al., 2014).

[3] No statistics were reported, but we applied Fisher's exact test

5.4. Respiratory sensitisation and irritation

Eye irritation and skin sensitisation after silver exposure have been described in some human case studies (Hadrup et al., 2018). However, following pulmonary exposure, we found no studies in the open peer-reviewed literature describing a sensitisation or irritation caused by silver.

5.5. Other toxicity endpoints

Liver toxicity Bile duct hyperplasia was observed in rats exposed to silver nanoparticles (18-19 nm) at 0.5 mg/m^3 for 13 weeks (390 h) in a study conducted under the OECD 413 guideline (NOAEC 0.133 mg/m^3) (Sung et al., 2009).

Renal toxicity Creatinine clearance was lower in workers involved in the manufacturing of precious metal powders and exposed to silver (26 of 27 workers had elevated urinary silver concentrations). However, no firm conclusion could be drawn on the role of silver because the effect of cadmium could not be excluded (Rosenman et al., 1987).

Cardiovascular effects Decreased stimulation-induced dilation of the tail artery and elevated heart rate were observed in rats after inhalation of 0.1 mg/m^3 silver nanoparticles (5 h), but not at a higher exposure level (1 mg/m^3). Other pulmonary and vascular endpoints were not affected by silver (Roberts et al., 2013). Concerning blood coagulation, activated partial thrombin time was decreased at all dose levels in rats exposed to silver nanoparticles (18.1–19.6 nm) at mass concentrations of 0.031, 0.082, or 0.116 mg/m^3 (6 hours/day, 5 days/week for 4 weeks) (Jo et al., 2020).

Reproductive toxicity Pregnant and non-pregnant mice were exposed to 18–20 nm silver nanoparticles at 0.54 mg/m^3. Four hours of exposure resulted in an increased number of foetal resorptions. The oestradiol serum level was decreased and a range of cytokines was increased after exposure to silver nanoparticles ($LOAEC_{foetal\ resorptions}$: 0.54 mg/m^3) (Campagnolo et al., 2017).

Animal studies in which no toxicity was observed We have described studies in which no effect on lung function or inflammation occurred. In addition, 1 study in which rats were exposed to a low dose of silver nanoparticles did not cause any effects on body weight, or biochemical or haematological parameters (NOAEC: 0.061 mg/m^3, 120 h of exposure) (Ji et al., 2007).

6. Genotoxicity and carcinogenicity

Concerning genotoxicity and carcinogenicity, all pathways of exposure and also *in vitro* incubation have often been described in articles otherwise with a focus on 1 specific exposure pathway. We have also done that for the oral and dermal/mucosal surface pathways of silver exposure (Hadrup et al., 2018; Hadrup and Lam, 2014). The reason was the severity of this endpoint, combined with genotoxicity and carcinogenicity, often developing through mechanisms inside single cells. The result of our previous review was that the genotoxic potential of silver seemed likely, but the data was insufficient to provide a firm conclusion on the carcinogenic potential (Hadrup et al., 2018). However, since our previous review, new studies have been published, and we present these in the following section. Notably, carcinogenicity studies of silver were not observed. First, we describe genotoxicity studies with the exposure pathway of inhalation.

Two studies report genotoxicity following inhalation. One study assessed DNA strand breaks in lungs in male rats after 12 weeks of inhalation of silver nanoparticles (0.05, 0.12, or 0.38 mg/m^3, 6h/day). An effect was observed only at the highest dose level (Cho et al. 2013). The other study investigated micronuclei formation after rats inhaled silver nanoparticles at 0.7×10^6, 1.4×10^6, or 2.9×10^6 particles/cm^3, 6 hours/day for 90 days. The exposure to silver did not induce micronuclei, however, the treatment schedule (90 days) is unusual for the *in vivo* micronucleus assay, and the calculation of the dose into mass units is not readily performed (Kim et al. 2011). Only 1 study reported genotoxicity after intratracheal instillation. Double strand breaks (γ-H2AX) were induced in rat lungs after a dosage of polyvinlyprrolidone-coated silver nanoparticles (300 µg/rat, ~1.2 mg/kg bw) (Wiemann et al. 2017).

Concerning other exposure pathways, 2 oral studies that investigate if silver nanoparticles induce micronucleus in bone marrow have reported contradictive results. The first study observed no effect in rats after 28 days of exposure up to 1000 mg/kg bw/day (Kim et al. 2008). The other study reported an effect of both coated and uncoated particles at the highest tested dose of 250 mg/kg bw/day in both male and female mice after 28 days of exposure (Wang et al. 2009). After the intraperitoneal exposure of nanoparticles, micronuclei formation was induced in bone marrow cells of mice, whereas DNA strand breaks results in the same cell type were negative (Ghosh et al. 2012). No induction of sister chromatic exchanges were induced in lymphocytes in the peritoneal cavity in mice after intraperitoneal injection of silver iodide (up to 100 mg/kg bw, 48 h) (Eliopoulos and Mourelatos, 1998). Intravenous exposure of nanoparticles has induced different effects: oxidised damage to DNA (8-Oxo-2'-deoxyguanosine; 8-OHdG) in pregnant rats and foetuses, DNA strand breaks (rabbit liver and mice spleen), and micronuclei formation in rat bone marrow (Salim et al. 2019; Kim et al. 2019; Dobrnzynska et al. 2014; Ordzhonikidze et al. 2009). Silver iodide also induced DNA strand breaks in mice after iv exposure (Ordzhonikidze et al. 2009). In erythrocytes from the African sharptooth catfish (*Clarias gariepinus*) with water as the exposure route, micronuclei formation was induced by nanoparticles (Ogunsuyi et al. 2019). One study investigated *in vivo* genotoxic effects of silver nitrate. DNA strand breaks were induced in fruit fly (*drosophila melanogaster*) haemocytes by both silver nitrate and nanoparticles (Alaraby et al. 2019). One study investigated jewellery workers exposed to metallic silver through inhalation and skin contact, and DNA strand breaks were observed in circulating mononuclear leukocytes (Aktepe et al., 2015).

The deposition of silver nanoparticles in the nuclei of lung cells has been demonstrated in lungs of rats exposed to 15 nm silver nanoparticles (Braakhuis et al., 2014). Whether the silver entered the nucleus as whole nanoparticles or as ions is unknown. Whether the silver can interact directly with DNA to induce genotoxicity is also unknown.

We reviewed a range of *in vitro* assays and present the details in Tables S1 and S2. The general summary of all the *in vitro* studies is that silver does not induce mutations in bacteria but induces DNA strand breaks and chromosomal aberrations in different mammalian cell lines. The addition of new *in vivo*

studies, compared with (Hadrup et al., 2018; Hadrup and Lam, 2014), does indicate that silver is genotoxic by different exposure routes. However, we still find no data on carcinogenicity and therefore cannot provide a conclusion on this endpoint. The references presented in tables S1 and S2 are as follows: (Akram et al., 2013; Castro-Gamboa et al., 2019; Clark, 1953; Demerec et al., 1951; Eliopoulos and Mourelatos, 1998; Foldbjerg et al., 2011; Guo et al., 2016; Hackenberg et al., 2011; Kanematsu et al., 1980; H. R. Kim et al., 2013; J. S. Kim et al., 2013; Li et al., 2012; Mei et al., 2012; Nishioka, 1975; Park et al., 2011; Rossman and Molina, 1986; Roszak et al., 2017; Wang et al., 2019); And in vivo: (Aktepe et al., 2015; Alaraby et al., 2019; Cho et al., 2013; Dobrzyńska et al., 2014; Eliopoulos and Mourelatos, 1998; Ghosh et al., 2012; Katsnelson et al., 2013; Kim et al., 2011, 2019, 2008; Ogunsuyi et al., 2019; Ordzhonikidze et al., 2009; Salim et al., 2019; Wang et al., 2019).

Conclusion The addition of new studies indicates that silver is genotoxic in mammalian cells, and *in vivo* when considering various exposure routes. Carcinogenicity data are absent; therefore, no conclusion can be provided on this endpoint.

7. Comparison of ionic and nanoparticulate silver

A question of silver toxicity is whether the effect is mediated by the particle size and shape or by ions released from the particle surface. In oral and dermal studies, it was difficult to distinguish a nanoparticle-specific effect of the particle form because there were only limited differential effects and differential organ deposition (Hadrup et al., 2018; Hadrup and Lam, 2014). Only a few studies on pulmonary exposure have compared effects of silver nanoparticle preparations directly to those of free ions. Ionic silver was rapidly distributed from the lung to the liver compared with silver nanoparticles for which silver was retained in the lung for a longer period (Arai et al., 2015; Takenaka et al., 2001; Wang et al., 2014). Arai *et al.* found that silver ions induced a higher effect on neutrophil cell numbers in BAL compared with silver nanoparticles; this, taken together with the difference in deposition of equal doses, suggests that silver nanoparticles do not rapidly dissolve in the lungs (Arai et al., 2015). Wang and colleagues found neutrophil numbers increased

after similar doses of both 20 nm silver nanoparticles and $AgNO_3$ but not after 110 nm silver nanoparticles, also suggesting a similar toxic mechanism of silver ions and nanoparticles in the lungs (Wang et al., 2014). Coccini *et al.* investigated changes in mRNA levels in rat liver and testes after a silver nanoparticle or silver nitrate instillation. The mRNA profiles differed between the 2 formulations, suggesting dissimilar mechanisms (Coccini, 2014). By contrast, in another study by the same group, silver nanoparticles at 50 µg and a dose of 7 µg $AgNO_3$ (4.4 µg Ag)/rat) given by intratracheal instillation exhibited similar effects in the kidney, including histopathological effects and dilation of Bowman's space (Roda et al., 2017).

Overall, we evaluate that the effects of silver nanoparticles after pulmonary dosage exert effects similar to those of silver ions/salts, suggesting that the effect of the nanoparticles is mediated by ions released from their surface. Concerning kinetics, there seems to be a more rapid distribution of silver from salts compared with silver from nanoparticles, likely reflecting the time necessary to release silver ions from the particle surface.

8. Hazard assessment

The dose descriptors of animal studies we deemed most relevant are summarised in Table 1. Based on this overview, we could suggest that a NOAEC value be set in the range of 0.11 to 0.75 mg/m^3. A previous suggestion was (Weldon et al., 2016) that the bile duct hyperplasia observed in a subchronic study (Sung et al., 2009) was a critical effect, suggesting a NOAEC of 0.133 mg/m^3, and this is in accordance with our aforementioned range.

Endpoint	**NOAEC**	**LOAEC**
Lung function	18–20 nm particles did not affect pulmonary tidal or minute volume at up to 0.75 mg/m^3 (Sung et al., 2011) Not available for studies showing an effect at the lowest tested concentration.	14–15 nm particle 0.05 mg/m^3 (360 h) (Song et al., 2013) 18 nm particle, 0.05 mg/m^3 (390 h) (Sung et al., 2008)

Inflammation (BAL neutrophils)	18 nm particle, rat, 0.5 mg/m^3 (390 h) (highest tested dose level) (Sung et al., 2008) 34 nm particle, rat, 0.041 mg/m^3 (24 h) (only dose level tested) (Braakhuis et al., 2016) 160 nm particle, rat, 0.555 mg/m^3 (24 h) (only dose level tested) (Braakhuis et al., 2016) 410 nm particle, rat, 0.17 mg/m^3 (24 h) (Braakhuis et al., 2014)	15 nm particle, rat, 0.18 mg/m^3 (24 h) (Braakhuis et al., 2014) 18 nm particle, rat 0.055 mg/m^3 (24 h) (only dose level tested) (Braakhuis et al., 2016) 60 nm particle, rat, 0.043 mg/m^3 (24 h) (only dose level tested) (Braakhuis et al., 2016)
Inflammation (histology)	14–15 nm particle, rat, 0.117 mg/m^3 (420 h) (Song et al., 2013) 18–9 nm, rat, 0.133 mg/m^3 (390 h) (Sung et al., 2009)	14–15 nm particle, rat, 0.381 mg/m^3 (420 h) (Song et al., 2013) 18–19 nm, rat, 0.515 mg/m^3 (390 h) (Sung et al., 2009)
Bile duct hyperplasia	18–19 nm particle, rat, 0.133 mg/m^3 (390 h) (Sung et al., 2009)	18.2 nm particle, rat, 0.515 mg/m^3 (390 h) (Sung et al., 2009)
Reproductive toxicity (foetal resorptions)	Not available	18–20 nm particle, mouse, 0.54 mg/m^3 (4 h) (Campagnolo et al., 2017)

Table 1 Suggested dose descriptors from inhalation studies in rodents. No descriptors were reported for cardiovascular effects because the data were deemed to scarce. Genotoxicity was not included in this table because it is not an endpoint but a mechanism for cancer and reproductive effects.

We identified no inhalation studies in animals using silver ions (e.g. as silver nitrate); however, in a direct comparison by intratracheal instillation, there is an indication on neutrophil numbers in BAL that ionic silver has a higher effect compared with that of silver nanoparticles (Arai et al., 2015). This finding indicates that lower exposure limits should be considered if also covering silver salts/ions.

Concerning particle size, if the limit is set at 100 nm, some studies compare particles that are smaller and larger than this. Neutrophil numbers were increased in BAL, and a 160 nm particle had a higher NOAEC of 0.56 mg/m^3 compared with the LOAECs and NOAECs of 18–60 nm particles (0.05 mg/m^3) (agglomerated sizes) (Braakhuis et al., 2016). In another study, a 15 nm particle resulted in a LOAEC of 0.18 mg/m^3, whereas this level was a NOAEC for a 410 nm particle (agglomeratated sizes), and a similar picture was observed after intratracheal instillation (Braakhuis et al., 2014). These comparisons indicate that fine dust

(larger than nanoparticles but below 2.5 µm) silver particles are less toxic compared with silver nanoparticles.

Finally, we evaluated that silver is genotoxic in mammalian cells and *in vivo*. However, there was insufficient data to determine whether silver has a carcinogenic potential, warranting that additional data on this endpoint be produced.

9. Summary

In 1 acute toxicity case, respiratory inefficiency and lung failure were reported, but the patient recovered. Concerning chronic effects in humans, argyria was only observed in 3 cases in which silver was not proven in the skin, and in 1 case, argyria was observed localised to the lungs but not the skin of 1 patient. The occurrence of argyria after pulmonary exposure seems less likely than after dermal or oral exposure probably due to lower total exposure levels of silver. In animal studies using inhalation, based on our evaluation, the NOAEC values should be set in the range of 0.11 to 0.75 mg/m^3. Ions are likely more toxic than nanoparticles, but that could reflect that differences in biokinetics; and silver nanoparticles have a similar pattern of toxicity to that of silver ions, probably reflecting that the effect of silver nanoparticles is mediated by released ions. We evaluated silver to be genotoxic *in vitro* in mammalian cells, and *in vivo* when considering various exposure routes. Carcinogenicity data are absent; therefore, no conclusion can be provided on this endpoint.

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Highlights

Inhaled silver is distributed to several organs. Few cases of argyria are reported

Excretion of silver includes faecal and to a lesser extent urinary pathways

Most affected endpoints in rodents are lung function and pulmonary inflammation

Ions show a higher effect, but the toxicity pattern is similar to nanoparticles

Silver was evaluated to be genotoxic based on *in vitro* and *in vivo* studies

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

On behalf of all co-authors,

Yours Sincerely

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