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Pulmonary toxicity in rats following inhalation exposure to poorly soluble particles: The issue of impaired clearance and the relevance for human health hazard and risk assessment

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ABSTRACT

Intensive discussions are ongoing about the interpretation of pulmonary effects observed in rats exposed to poorly soluble particles. Alveolar clearance differs between rats and humans and becomes impaired in rats at higher exposure concentrations. Some have doubted the human relevance of toxic effects observed in rats under impaired clearance conditions and have suggested that experimental exposures should stay below concentrations inducing impaired clearance. However, for regulatory purposes, insight in potential health effects at relatively high concentrations is needed to fully understand the hazard. Many aspects of impaired particle clearance remain unclear, hampering human health hazard and risk assessment. For an adequate evaluation of the impact of impaired clearance on pulmonary toxicity, a clear definition of alveolar clearance is needed that enables to quantitatively relate the level of impairment to the induction of adverse pulmonary health effects. Also, information is needed on the mechanism of action and the appropriate dose metric for the pulmonary effects observed. In absence of these data, human hazard and risk assessment can only be performed in a pragmatic way. Unless available data clearly point out otherwise, rat pulmonary toxicity including lung inflammation and tumour formation, needs to be considered relevant for human hazard and risk assessment.

1. Introduction

The term "impaired clearance" is often used in the design and evaluation of (sub)chronic inhalation toxicity studies with poorly soluble particles (PSPs) of low intrinsic toxicity, *e.g.* coal dust, titanium dioxide (TiO_2), carbon black and certain nanomaterials. Impaired clearance is considered to occur at concentrations of PSPs that result in overwhelming the pulmonary clearance (defense) mechanisms of the lung and subsequently lead to a particle retention in the centriacinar regions of the lung that is higher than expected based upon the deposition and clearance kinetics at low concentrations (Borm and Kreyling, 2004). It is associated with a recruitment of macrophages and polymorphonuclear cells (PMN), into the alveolar region (Donaldson et al., 1988) and an increased rate of particle relocation to the interstitium in the lung and the tracheobronchial as well as lung-associated lymph nodes (LANL) (Vincent and Donaldson, 1990). This impaired clearance has been attributed to a reduced macrophage function (Morrow, 1988), although the exact mechanism(s) still remains unclear. Reduced phagocytic as well as reduced chemotactic function of macrophages could be due to excessive particle burdens contained within the individual cell or be influenced by interaction with other immune factors such as neutrophils, pulmonary inflammatory mediators and cytokines (Warheit et al., 1997). It is to be noted that impaired clearance can also be the result of direct damage to the AMs upon chemical-specific toxicity. For example, particles with a reactive surface, specific physical dimensions or toxic chemical composition, such as crystalline silica, synthetic fibres and metal particles, can actively damage alveolar macrophages (AMs) (Borm et al., 2004).

The pulmonary clearance mechanism in rats appears to be different from other species including humans. In rats, PSPs are mainly cleared by AMs whereas in humans, PSPs are, when compared to rats, mainly cleared by translocation into the interstitium and subsequent drainage

Abbreviations: AM, alveolar macrophage; BMC, Benchmark concentration; DNEL, Derived No-Effect-Level; HBGV, Health-Based Guidance Value; NOAEC, No-Observed-Adverse-Effect-Concentration; OEL, Occupational Exposure Level; PMN, polymorphonuclear neutrophil; PoD, point of departure; PSP, poorly soluble particle; RNS, reactive nitrogen species; ROS, reactive oxygen species

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to the lymph nodes. However, it is noted that in humans, still a considerable part of PSPs can be found in the alveoli. Nikula et al. (2001) found that in miners exposed to estimated low doses of airborne coal dust, 70% of coal dust particles in the lung were present in the interstitium (anthracosis), while 30% were found in the alveolar air spaces (referred to as lumens of respiratory bronchioles, alveolar ducts, and alveoli) (Nikula et al., 2001). When in rats the exposure exceeds a certain air exposure concentration and duration at which clearance via AMs becomes impaired, a cascade of reactions leading to adverse pulmonary effects starts to occur (Bevan et al., 2018). These pulmonary effects are considered to be typical for all kind of PSPs, independent of the chemical composition of the particle. Because of the differences in clearance kinetics, adverse effects, in particular lung tumour formation in rats upon inhalation of PSPs have been questioned by some for their relevance for human health hazard and risk assessment (Ilsi Risk Science Institute, 2000; Warheit et al., 2016). Among others, it has been argued that these effects may be merely the result of the physical impairment in clearance of particles from the rat lung which, due to species differences, is considered not to occur in humans (Warheit et al., 2016).

Given that there are differences in clearance kinetics between rats and humans, pulmonary toxicity observed in inhalation studies with rats under impaired clearance conditions has led to discussions in the scientific community on how to interpret this toxicity (both the neoplastic and non-neoplastic effects) as to their relevance for humans. For human health hazard and risk assessment, it is important to understand the type of effects induced by PSPs in experimental animals, their relevance for humans, how these effects are related to exposure and, if considered relevant, how these effects can be extrapolated to humans. The term human health hazard assessment is used to describe the evaluation of the intrinsic hazard properties of a chemical (irrespective of the actual exposure). It is noted that a hazard assessment may include a dose- or concentration response characterisation as well; this can further be used for derivation of health-based guidance values (HBGV). The term human risk assessment refers to a human health assessment of an existing exposure scenario. In addition to this type of human health assessment which is performed *a posteriori*, the term risk characterisation is often used to describe the human health assessment that is performed *a priori* to ensure safe production and use of a material. For instance, *a priori* risk characterisations are performed within the context of biocides and plant protection products or under the European Registration, Evaluation, Authorisation and Restriction of Chemical (REACH) legislation for industrial chemicals. In the remainder of this article, only the terms hazard and risk assessment will be used.

The main question to be discussed here is how the effects induced by PSPs in rats at concentrations resulting in impaired clearance need to be interpreted and weighed in the context of: (a) establishing the intrinsic properties of a chemical in accordance with EU Regulation 1272/2008 on the classification, labelling and packaging of substances and mixtures (EU, 2008a), and (b) assessing risks or setting safe levels for regulatory risk assessment.

2. Impaired clearance

Impaired clearance has been observed or claimed in reports on inhalation studies in various experimental animal models and humans. Decreased pulmonary clearance had already been observed more than 45 year ago by Le Boufant (le Boufant, 1971) who reported decreased alveolar clearance of coal dust from lungs of cats and rats. Paul Morrow proposed the lung particle overload hypothesis based on an analysis of publications on effects and disposition of particles exposure in Fischer F344 rats (Morrow, 1988). He hypothesized that a continuously increasing prolongation of particle lung clearance of PSPs occurs when the retained lung burden exceeds a certain threshold, which was considered to be due to an impairment of the AM clearance function.

2.1. Hypotheses on AM dysfunction

Following chronic exposure, cessation of movement of AMs is thought to be an important step in impairment of pulmonary particle clearance, while reduced phagocytic responses and chemotactic responses have been observed as well (Warheit et al., 1997). Knowing the exact mechanism that leads to reduced macrophage function would increase our understanding why exposure to particles that are thought to have a low intrinsic toxic potential can lead to serious adverse effects. This would allow for more accurate predictions of adverse outcomes after exposure to PSPs. However, the exact mechanism that lead to macrophage dysfunction remains unclarified, but there are several observations. A combination of AM cellular damage and decreased chemotactic ability have been reported after different types of particle exposures. For example, rat alveolar macrophages that were isolated after intratracheal exposure to 0.5, 5 or 50 mg/kg bw of 5 nm TiO_2 induced membrane and ultrastructure damage, while this was not observed for 200 nm TiO_2 particles. In addition, exposure to TiO_2 nanoparticles decreased the chemotactic ability of the macrophages as well as decreased the expression of Fc receptors (immunoglobulin receptor that binds to antibodies) and major histocompatibility complex - class II (MHC-class II found on antigen presenting cells) on the cell surface (Liu et al., 2010). Direct damage to the AMs has also been observed after exposure to particles with a reactive surface, specific physical dimensions or toxic chemical composition, such as crystalline silica, synthetic fibres and metal particles. This also impaired their chemotactic mobility (Borm et al., 2004; Kawasaki, 2015). Phagocytic responses to carbonyl iron particles as well as chemotactive responses to zymosan-activated sera were impaired following a 4-week inhalation to fine TiO_2 at 250 mg/m^3 (Warheit et al., 1997). To what extent and at what dose a PSP will lead to AM dysfunction and whether this is due to cytotoxicity, decreased chemotactic ability, decreased phagocytic function or a combination thereof is difficult to predict beforehand for different types of engineered PSPs. It is likely that particle-induced macrophage dysfunction is dependent on several factors such as particle size, chemical composition, surface properties (see section 2.2.) and physical dimensions. Morrow (1988) has speculated that impairment of macrophage mobility and phagocytic functions by particle overload are likely to be do to competitive cellular requirements for surface membrane and cytoskeletal components.

2.2. Dose metrics in impaired clearance

It was initially concluded that the impaired clearance in rats correlates with the phagocytized volumetric loading of AMs. According to Morrow, the impaired clearance phenomena would start at lung burdens that are roughly 1 mg dust per gram of lung tissue. Considering that volume would be a good dose metric to describe the concept of impaired clearance, this threshold was subsequently described as 60 μm^3/AM (which equals 6% in volume of the total volume of the rat's AM pool) (Morrow, 1988). However, he also indicated that this value is not an exact threshold, and later proposed that a threshold is better reflected by a range of values of about 25–90 μm^3/AM (Morrow, 1992). At lung burdens roughly 10 times higher (*i.e.* 60% of total AM pool or 600 μm^3/AM), clearing by the AMs is observed to cease completely (Morrow, 1988). At present, there are no solid data to support either a specific percentage at which rat AM impaired clearance starts to occur or at which percentage it has reached a maximum, and it is likely that these percentages are chemical specific. After all, Morrow (1992) only used one type of particle to confirm these percentages. In addition, albeit that AM play an important role in removing particles from the lung, it cannot be concluded that impaired clearance of PSPs from the lung is entirely related to immobilization of AMs; lungs of coal miners for example show impaired clearance which is to a large extent related to accumulation of dust in the interstitium. Apart from the volume of PSPs per AM as a valuable dose-metric for impaired clearance, surface

area is also proposed as a dose metric (Morfeld et al., 2015; Pauluhn, 2014).

Oberdörster et al. (1994) have found severely impaired AM clearance of particles following subchronic inhalation of ultrafine TiO_2 in rats at AM phagocytized doses far below the Morrow's 6% AM volume threshold. In this study, rats were exposed by intratracheal instillation to ^{85}Sr-labeled polystyrene test particles following whole-body inhalation to TiO_2; the retained radioactivity of ^{85}Sr was measured up to 200 days postexposure after which retention half-times were assessed. Further, they noticed that the phagocytized surface area correlated well with the observed impaired clearance of both the ultrafine and fine TiO_2 (Oberdörster et al., 1994). Since then, the significance of the particle surface area as a dose metric for impaired clearance and associated particle-induced pulmonary inflammation has been demonstrated by several investigators (Duffin et al., 2007; Elder et al., 2005; Tran et al., 2000) by assessing the total lung burden over time after cessation of the exposure. The clearance kinetics and surface area burden of carbon particles with a low surface area (37 m^2/g) was faster (at an equal mass dose) than that of high surface area carbon particles (300 m^2/g), indicating the importance of particle surface area in particle retention (lung burden). In essence, particle surface area ($4\pi r^2$) can also easily be converted to a volumetric dose of a spherical particle ($4/3\pi r^3$) which would facilitate predictive modelling.

2.3. Mechanism of impaired clearance and adverse outcomes

Based on a conceptual Adverse Outcome Pathway (Borm et al., 2004; ECETOC, 2013; IARC, 2010; Ilsi Risk Science Institute, 2000; Thompson et al., 2016) and a recent literature review on a specific PSP, *i.e.*, TiO_2 (Braakhuis et al., submitted) an overview of the proposed events after chronic exposure to PSPs that lead to impaired clearance is summarized in Fig. 1.

Following chronic exposure to PSPs (box 1), particles will deposit and accumulate in the deep lung depending on their aerodynamic diameter (box 2). Of note, particles deposited in the higher regions of the airways, namely the conducting airways (*e.g.*, nose, trachea, bronchi, bronchioles) are cleared via the mucociliary escalator. In the pulmonary region of the lung, activation of epithelial cells by particles elicit production of chemotactic factors like monocyte chemoattractant protein 1 (MCP-1/CCL2) and interleukin-8 (IL-8/CXCL8) that recruit phagocytic cells, monocytes (that can mature into macrophages) and neutrophils respectively (Takizawa, 1998). AMs are involved in uptake and clearance of particles (box 3). The average life-span of AMs in humans is estimated to be around 81 days (du Bois, 1985). When they die, either at the end of their life-span or due to some cytotoxicity of the PSP, their particle load can be released on the alveolar epithelium and be engulfed by a new wave of macrophages moving to the alveolar surface. Loaded AMs have been hypothesized to either move directly along the bronchiolar surface to the mucociliary escalator via chemotactic signals or lung lining fluid movement (Bowden, 1984; Kreyling et al., 2013; Lauweryns and Baert, 1977; Lehnert, 1992; Lehnert et al., 1990) or clear via the interstitium and lymphatics (Ferin and Feldstein, 1978).

PSPs could either redistribute from the alveolar epithelium to the interstitium in a free form or inside macrophages via the routes mentioned (Lippmann et al., 1980). A relative difference has been postulated between crystalline silica and TiO_2 particles, with crystalline silica redistributing in free form and TiO_2 inside macrophages (Kawasaki, 2019). Impaired AM clearance leads to PSPs retainment on the alveolar surface as well as in the interstitium (box 4).

Pulmonary inflammation is induce by presence of particles and accumulation of particles due to impaired clearance leads to persistent inflammation (box 5). Persistent lung inflammation (either via PMNs or macrophage driven generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS) (box 6/7) and pro-inflammatory cytokines) leads to epithelial cell injury/cell death or (secondary) genotoxicity (box 8). Injury might lead to more inflammation, cell proliferation (box 9) and tissue remodeling which when insufficiently repaired can lead to non-neoplastic events such as fibrosis (box 10) or fixed genetic changes (mutations) to the lung epithelial tissue (box 11) and ultimately cancer (box 12) (Bevan et al., 2018). The genetic damage is caused by oxidative DNA damage through ROS and RNS during the inflammatory process (Schins and Knaapen, 2007), and fixation of the damage leads to tumours (Greim et al., 2001). However, direct particle effects cannot be ruled out. For example, deposited particles might directly generate ROS at their surface (Schins and Knaapen, 2007). Also particles may directly cause cell injury or genotoxic effects. Carbon black is an example of particles with surface-driven ROS formation (Jacobsen et al., 2008) leading to mutations (Jacobsen et al., 2007) *in vitro*. The mutation spectrum is consistent with being caused

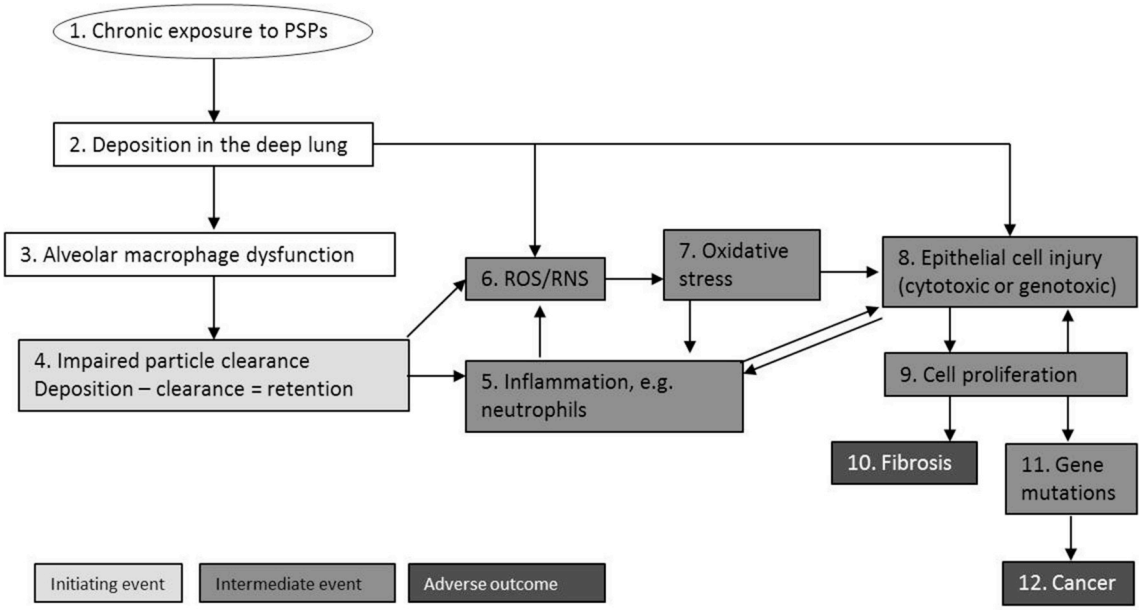

Fig. 1. Proposed sequence of events resulting from impaired clearance following chronic exposure to PSPs (based on (Borm et al., 2004; Braakhuis et al.,; ECETOC, 2013; IARC, 2010; Ilsi Risk Science Institute, 2000; Thompson et al., 2016).

by ROS (Jacobsen et al., 2011). For TiO_2 (nano)particles, several studies are ongoing that investigate the potential for direct genotoxicity (Geiser et al., 2005). for instance, have observed TiO_2 nanoparticles inside rat lung epithelial cells and cell organelles, including the nucleus, 24 h after a 1-h exposure to 0.1 mg/m^3 TiO_2 (4 nm particle). Therefore, a direct interaction with DNA cannot be excluded as was also recently considered by the EU Scientific Committee on Consumer Safety (SCCS) in its opinion on TiO_2 (nano form) as UV-filter in sprays (SCCS, 2018).

2.4. Species differences

For most, but not for all, events that are listed in Fig. 1, species differences have been identified as will be discussed below. These species differences in particle handling, as well as uncertainties related to whether some events take place in humans or not, are relevant for the interpretation and extrapolation of pulmonary effects observed under conditions of impaired clearance.

2.4.1. Anatomical and histological differences

Due to anatomical and histological differences between rodent and mammalian lungs there are differences in particle deposition (box 2), clearance (box 3) and retention (box 4). Rats for example lack respiratory bronchioles and as a result have a more simple functional lung unit (acinus) compared to more complex and larger acini in monkeys and humans (Mercer and Crapo, 1988). Nikula et al. (1997a,b) have shown differences in particle retention in rats versus cynomolgus monkeys (Nikula et al., 1997b). In rats more than 70% of diesel, coal and diesel plus coal particulates were retained in the lumens of the alveolar ducts and alveoli. Approximately 27% of the particulates were retained in the interstitium. In cynomolgus monkeys the same particulate materials were more equally divided between lumenal compartments and the interstitium with around 43% in the alveolar ducts and alveoli and more than 50% in the interstitium. In rats, significant alveolar epithelial hyperplasia, particle associated-inflammation and septal fibrosis have been reported (Nikula et al., 1997b). In monkeys, only some particle associated lung inflammation was found (Nikula et al., 1997a). Mammalian species with fast-clearing lungs, such as rats, retain dust predominantly in macrophages in pulmonary airspaces whereas larger mammals like dogs and monkey, clear pulmonary burdens of dust more slowly and retain dust predominantly within the pulmonary interstitium (Snipes, 1995).

Humans are more like monkeys than like rats, in that particles are primarily retained in the interstitium (Nikula et al., 2001). After chronic exposure of rats to diesel engine exhaust, with increasing dose, 82–85% of the retained particulate material in the form of soot was located in the alveolar and alveolar duct lumens, primarily in macrophages. In lungs obtained from humans with different levels of exposure to coal dust, *i.e.* non miners, coal miners with a low coal dust exposure and coal miners with a high exposure, 57, 68, and 91% of the retained particulate material was located in the interstitium, respectively. In humans, the percentage of particles in the interstitium increased with increasing dose (exposure concentration, years of exposure, and/or lung burden). The assessment of the absolute retained dose in the different compartments of the lung is challenging in experimental animals (depending on the nature of the material) and even more complicated in humans as only less invasive methods can be applied in humans. Alternatively, the deposition, clearance and particle retention in the different species can be modelled, but this requires that critical input values are known (Anjilvel and Asgharian, 1995; RIVM, 2002).

In conclusion, differences among species in the anatomical site of particle retention will result in different lung cells between species to come into contact with the retained particles or particle-containing macrophages. This may account for species differences in response to inhaled particles. Rats respond to PSPs with more chronic inflammation and epithelial type II cell proliferation and relatively less fibrosis. In contrast, humans respond to the same or similar particles with more fibrosis and less inflammatory and epithelial response (Green, 2000; Hahn et al., 1998).

2.4.2. Differences in antioxidant response

Some interspecies differences in antioxidant response have been reported, for example between rat and hamster. Catalase activity is significantly higher in hamster AMs than in rat AMs. The oxidative capacity of hamster AMs appears to be based mainly on the formation of ROS, whereas rat AMs possess an additional oxidative system based on nitric oxide (Jesch et al., 1997) resulting in a 5-fold higher oxidative capacity (Dorger et al., 1997a). Human AMs lack nitric oxide synthetase and consequently human AMs do not produce nitric oxide (Dorger et al., 1997b; Schneemann et al., 1993). After exposure of rats, mice and hamsters to air, 1, 7, or 50 mg/m3 of carbon black for 13 weeks, (inflammatory) cell number and cell types, reactive oxygen and nitrogen species, as well as cytokine levels were determined in the BALF at 1 day, 3 months and 11 months. Rats demonstrated a pro-inflammatory response, whereas mice and hamsters demonstrated an increased anti-inflammatory response (Carter et al., 2006).

It has been proposed that these species differences in anti-oxidative capacity contribute to the differences seen in lung tissue injury in rats and humans after high particle exposures, with rats having a more harsh oxidative environment in the alveoli and react with a pro-inflammatory response.

2.4.3. Differences in alveolar macrophages

With their phagocytic function, AMs are involved in uptake and clearance of particulate material in the lung. There are several species differences with respect to AMs, with rats having slower alveolar clearance with smaller macrophages and smaller numbers of AMs in total and per alveolus as compared to humans (Geiser, 2010). In short, rats have on average 28×10^6 macrophages with a volume of 848 μm^3 and a total number of 1.4 macrophages per alveolus. Humans have 6×10^9 macrophages with a volume of 1474 μm^3 and a total number of 12.5 macrophages per alveolus. The individual morphometric data on alveolar macrophages for two rat strains (Sprague Dawley and Fisher 344) and humans are illustrated in Table 1 (based on the data given in (Geiser, 2010)). From this table, it appears that the total macrophage volume already differs between rat strains.

Given the more prominent interstitial retention and subsequent lymphatic clearance in humans as compared to rodents, it has been postulated that alveolar clearance in humans is independent of the particle load, whereas the clearance in rats is considered dependent on the amount of particles in the alveolar region (Brown et al., 2005).

In addition, next to the phagocytic function, AMs have a regulatory function in the immune response. They secrete antimicrobials like ROS and RNS, nitric oxide and several chemo-/cytokines depending on their specific subtype. Classically activated macrophages "M1″ are known to release pro-inflammatory and cytotoxic mediators, having anti-proliferative, anti-microbial and tumoricidal activities (Gao et al., 2018). "M2″ macrophages are involved in down-regulation of inflammatory processes, initiation of cell-proliferation and wound repair. Similar to dysregulation of pro-inflammatory M1 macrophages, overstimulation of M2 macrophages can lead to excess production of cytokines, bioactive lipids and growth factors (like TGF-beta) and a pathogenic fibrogenic response (Mosser and Edwards, 2008).

Depending on the need, macrophage activity is adapted to a mix of M1 and M2 phenotypes, rather than having only M1 or M2 phenotypes (Bazzan et al., 2017). The involvement and interplay with other cell types of the immune system seems to be less investigated. There is a role for PMNs that are recruited following particulate exposure in persistent inflammation. Of note, in relative comparison to other cell types, rodent broncho alveolar lavage (BAL) consists of ≥95% macrophages, few lymphocytes and neutrophils, whereas human BAL consists of ≥80% macrophages, 18% lymphocytes and few neutrophils (ECETOC, 2013), which in itself may result in differences in the

Table 1
Morphometric data on alveolar macrophages in rats versus human (Geiser, 2010).

	Body weight (kg)	Lung volume (ml)	Surface area of alveolar region (m^2)	No. of macrophages (x 10^6)	Macrophage volume (μm^3)	Surface area per macrophage (μm^2)	No. of alveoli (x 10^6)	No. of macrophages per alveolus
Fischer 344 rat	0.290	8.6	0.41	29.1	639	14089	20.1	1.45
Sprague Dawley rat	0.363	10.6	0.40	26.9	1058	14870	19.7	1.37
Human	69	4777	102	5990	1474	17062	480	12.5

biological response to PSP including inflammation and immune responses.

2.4.4. Differences in inflammatory response

Inflammation is usually a protective response to eliminate an eliciting agent and to promote the repair of injured tissue. However, when inflammation is excessive or persistent it may cause tissue injury or organ dysfunction itself and may contribute to the pathogenesis of disease. Persistent lung inflammation characterised by an increased number of PMNs is seen as the driver for carcinogenesis in rats (Kolling et al., 2011; Oberdorster, 1997). In the lung, neutrophilic granulocytes are considered to be a source of ROS/RNS. Driscoll et al. (1997) showed that a significant increase in the level of DNA damage in the rat type II epithelial cells was seen only if the number of neutrophils went up to 40–50% of the total cell number after quartz, carbon black or TiO_2 exposure. In the absence of lung inflammation, rat lung tumours were not induced. In mice however, inhalation of carbon black particle-induced increased levels of DNA damage in absence of neutrophil influx (PMID: 15798890), suggesting that persistent inflammation is not required for DNA damage induced by poorly soluble particles (Saber et al., 2005). There is also recent evidence that not in all cases of lung inflammation, this leads to lung tumours in the rat. After 2 yr inhalation to 0.1 mg/m^3 of CeO_2 NM-212, an inflammatory reaction was observed which persisted until 6 months after cessation of the inhalation (month 30), however no subsequent tumour induction was found (Ernst et al., 2019).

For coal workers heavily exposed to particulates, no consistent evidence of an increased risk of lung cancer has been demonstrated, whereas an increased risk of non-malignant effects in the lung, such as restrictive and obstructive lung disease, has been identified for this specific population (IARC, 1997; Mauderly et al., 1994). Increased particle lung retention and pulmonary inflammation in workers exposed to coal dust or crystalline silica have also been observed (Castranova and Vallyathan, 2000; Kuempel et al., 2001; Lapp and Castranova, 1993). Therefore, lung inflammation after PSP exposure does not necessarily lead to tumour formation in humans. Fibrosis is however a seen response upon exposure to PSP both in rats and humans, though not in mice.

2.4.5. Differences in neoplastic lung lesions

Adverse outcomes to inhaled particles depend on the species investigated, with lung tumours being reported in rats, but not in mice, hamsters, monkeys and humans (Ilsi Risk Science Institute, 2000). For example, inhalation exposures of long durations in hamsters or mice to talc (103 or 104 weeks exposure of mice), titanium dioxide (13.5 months exposure of mice with a 6 months follow-up period), or diesel soot (2 year exposure of mice and 18 months exposure of hamsters) have not resulted in lung tumours, even though the lung particle burdens achieved in these studies were similar to or greater than those producing lung cancer in rats (Heinrich et al., 1995; Heinrich et al., 1989; Heinrich et al., 1986; Heinrich et al., 1982; Mauderly et al., 1996; Mauderly et al., 1994; NTP, 1993).

The type and location of lung tumours are thought to be influenced by particle size, airway anatomy, and site of particle deposition. The majority of lung tumours are found in the regions of maximum particle deposition (which is a function of particle aerodynamic behaviour including size aspects and particle density) i.e., the conducting airways in humans and the alveolar ducts in rats. Most lung tumours in rats exposed to PSPs are adenocarcinomas or squamous-cell carcinomas of the alveolar ducts, including a range of squamous-cell proliferative responses that do not appear to have human analogies (Green, 2000). In humans, lung tumours are typically bronchiolar in origin with four different cell types: squamous cell carcinoma, adenocarcinoma, small cell anaplastic carcinoma, and large-cell anaplastic carcinoma (Green, 2000). The latter two tumour types have not been seen in rats. In case there would be a relation between chronic PSP exposure and increased

lung tumour incidence in humans, the specific types of tumours expected in humans would most likely be not directly comparable to those found in rat studies.

3. Hazard and risk assessment of PSPs

Given the differences in clearance kinetics between species and their potential impact on pulmonary toxicity, the question is how to deal with adverse pulmonary effects observed in rats after inhalation exposure to PSPs in regulatory human health hazard and risk assessment. This question has been intensively discussed (ECHA, 2017c) or is still topic of ongoing discussions (Bevan et al., 2018; Borm and Driscoll, 2019; ECHA, 2017c). Fundamentally, these discussions focus on whether the pulmonary effects observed in rats following PSP exposure are relevant to humans, and whether a distinction should be made between exposures under conditions of impaired clearance and exposures below these levels. However, in these discussions several arguments are being brought forward that also are topics of broader discussions outside the context of PSPs, *e.g.*, on lowering the top dose or concentration in animal experiments. For instance, it is argued that animal experiments should only be performed at exposure concentrations that are relevant for human exposure in practice or at concentration ranges where kinetic processes show linearity. An additional argument brought forward for PSP exposure is that hazard assessment should be restricted to the intrinsic properties of a chemical, thus excluding particle-related toxicity. These discussions also touch upon the required study design to obtain information that is relevant for hazard assessment. In these discussions, the term hazard assessment and the term risk assessment are sometimes mixed up which obscures the discussions.

This section will deal with several issues directly or indirectly related to the interpretation and extrapolation of pulmonary toxicity observed under conditions of impaired clearance. First, it will be summarized what guidance is provided for human health hazard and risk assessment in regulatory frameworks. Next, the regulatory information needs are discussed for human health hazard assessment and for human health risk assessment. This is followed by a discussion from a regulatory perspective on human relevance of toxic effects observed in experimental animals and what this means for extrapolation of pulmonary toxicity observed in rats to humans. An important topic that will be addressed is the choice of an appropriate dose metric.

3.1. Guidance on impaired clearance in regulatory frameworks

The relevance for humans of adverse effects at impaired clearance conditions in rats is a subject of scientific debate noted within regulatory frameworks like the EU Regulation 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP) (EU, 2008b), the EU Regulation 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EU 2006) and Occupational Safety and Health (OSH). This is partly due to the fact that there is no clear guidance on how to deal with this phenomenon. For example, in the CLP-guidance, it is stated that "*relevance of lung overload in animals to humans is currently not clear and is subject to continued scientific debate*" (ECHA, 2017b), whereas the ECHA Guidance on nanomaterials states "*In the case of adverse effects observed in animals under overload conditions, the relevance for humans has to be assumed a priori; any claimed non-relevance for humans must be* supported *by data*" and "*Lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans unless it can be clearly substantiated otherwise*" (ECHA, 2017a). Further, with respect to selection of exposure concentrations for inhalation testing, some guidance is presented in OECD guidance document GD 116 on the conduct and design of chronic toxicity and carcinogenicity studies as follows: "*For substances likely to accumulate in the lung over time due to poor solubility or other properties, the degree of lung-overload and delay in clearance needs to be estimated based on adequately designed pre-studies; ideally a 90-day study with post-exposure periods long enough to encompass at least one elimination half-time. The use of concentrations exceeding an elimination half-time of approximately 1 year due to lung-overload at the end of study is discouraged*" (OECD, 2012). It is however not clear what the basis of the 1-year criterion is, as was for instance, recently acknowledged by the Committee for Risk Assessment (RAC) of ECHA in its opinion on titanium dioxide (ECHA, 2017c). Finally, the recently revised OECD Test Guidelines TG 412 and TG 413 and the accompanying GD 39 for subacute and subchronic inhalation toxicity testing describe that "*measurements of lung burden, which inform on pulmonary deposition and retention of particles in the lung, should be done when a range-finding study or other relevant information suggests that inhaled test particles are poorly soluble and likely to be retained in the lung*" (OECD, 2018a; OECD, 2018b; OECD, 2018c).

Poor understanding of the process, onset and interpretation of adverse effects observed under conditions of impaired clearance impacts the hazard assessment of chemicals, including nanomaterials, within regulatory frameworks. An incorrect interpretation of the data for hazard assessment might result in an under- or over classification and in subsequent risk management measures that are too limited or too stringent, or in too high or too low HBGV (*e.g.* derived no-effect levels (DNELs) under REACH or occupational exposure levels (OELs) under OSH). For example, a carcinogenicity classification (especially in category 1A/B, *i.e.*, known or presumed to have carcinogenic potential for humans, respectively) warrants additional regulatory actions, not only within the REACH framework, but also within other regulatory frameworks such as those concerning cosmetics, toys and the protection of workers. Therefore, a careful evaluation of the available data is needed as well as an accurate decision on the relevance for humans of effects found at impaired clearance conditions and the way of extrapolating these animal findings to humans.

3.2. Purpose of toxicity studies: hazard versus risk assessment

The purpose of a toxicity study determines the study design, *i.e.*, the design should be such that the required regulatory information can be obtained from that study. Since a toxicity study may meet multiple information requirements, as will be discussed below, it should be considered beforehand what information actually is needed for human health hazard assessment and for human health risk assessment. In the discussions on human relevance of the PSP-induced pulmonary effects, in particular in relation to classification for carcinogenicity, the relevance is debated for multiple reasons. One reason is that the biological response observed in the rat lung itself is considered as not relevant, because observed pulmonary effects result from the specific clearance processes in the rat which are considered to be different from that in humans (Kuempel et al, 2001; Jarabek et al., 2005; Bevan et al., 2018). Another reason is that tumours appeared to be observed at concentrations considered to be extreme to what humans would experience (Borm and Driscoll, 2019). However, the latter argument reasons from possible human exposures and mixes up hazard and risk assessment. From a regulatory perspective, it is important to distinguish between the different regulatory information needs and the purpose of the animal experiments aimed at meeting these needs.

Toxicity testing can be performed to meet different regulatory needs, including (1) prioritization, (2) classification (*i.e.*, hazard evaluation), (3) derivation of a HBGV such as a DNEL under REACH or an OEL under OSH and 4) risk assessment of exposure situations of concern. Each purpose requires information that is specific for that goal and therefore, each purpose may require its own optimal study design, including an appropriate selection of test concentrations or doses.

Prioritization (ranking) can be based on the level of exposure, on hazard characteristics or a combination of both. Selection of the concentration(s) to be tested is determined by the origin of concern and the aim of the prioritization.

A toxicity study performed for classification purposes (within the

United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS)) aims at deriving information on the intrinsic hazard of a chemical. The choice of exposure concentrations is therefore not determined by expected human exposures in practice but whether a chemical has the potency to induce a specific toxic effect. Therefore, the endpoint of carcinogenicity, as an example, is relevant, independent of the exposure conditions. As the magnitude of a particular effect will in general be greater at higher doses, testing at too low concentrations would result in a higher probability of *not* significantly detecting an effect (Slob, 2014), which contradicts with the regulatory purpose of the toxicity study, *i.e.*, determination of relevant effects for classification purposes.

For the purpose of HBGV derivation, the basic need includes a reference point for an adequate critical adverse effect (Benchmark concentration (BMC), No-observed adverse effect concentration (NOAEC)), so a toxicity study should be designed such that at least the critical effect can be identified and an appropriate point of departure (PoD) can be determined. Here, it also applies that study designs resulting in relatively high incidences at the top dose perform better with respect to the BMC precision (Slob, 2014) and thus for the determination of an adequate point of departure for the regarding critical effect.

Finally, when performing a human health risk assessment in an occurring exposure scenario for which a concern has risen, quantitative information on the exposure-response relationship is needed. This applies not only to the critical effect but also requires insight in the occurrence of additional adverse effects at higher exposure levels for an adequate risk communication to meet societal concerns and to make appropriate decisions on risk reduction measures.

Also for PSPs, it has been argued that high animal exposure concentrations are not relevant for humans since human exposures will be much lower, sometimes orders of magnitude, than the exposure levels in an animal experiment. However, it is noted that human exposure is often not well known at the time of testing, and may well be close to or similar to exposure concentrations applied for animal inhalation testing. This is illustrated by the example of synthetic amorphous silica (SAS) showing that occupational exposure to respirable particles may be at concentrations (*i.e.*, up to 3.4 mg/m^3 respirable SAS) (ECETOC, 2006) that are comparable to or higher than concentrations inducing severe pulmonary effects in rats (*i.e.*, 1, 6 and 30 mg/m^3) (Reuzel et al., 1991).

It is noted that, because of animal welfare principles, a repeated dose toxicity study will seldom be performed anew, even if the study observations do not (fully) meet the requirements for a specific purpose. Therefore, from the perspective of human health hazard or risk assessment, it is preferable that a repeated dose toxicity study is adequately designed beforehand to meet multiple, if not all of the mentioned purposes for hazard and risk assessment; this requires an appropriate study design, including an adequate selection of the top dose or concentration. With regard to testing of PSPs, this means that the choice of test concentrations should thus not be restricted beforehand to concentrations below those that induce impaired clearance.

3.3. Selection of top dose or concentration

The previous section discussed the need for an appropriate dose selection for inhalation exposure to PSPs from a regulatory point of view. At present, arguments used in broader discussions on study design are also brought into the discussions on the selection of top dose or top concentration for PSP exposures.

One argument is that the top concentration should be determined by the kinetically-derived maximum dose (KMD), *i.e.*, concentration ranges should lie within the range of linear kinetics. Since alveolar clearance is a kinetic process, this argument has also been introduced for PSPs. For instance, Borm and Driscoll (2019) recently introduced the term maximum functionally tolerated dose (MFTD) in the context of PSP exposures and stated that "*chronic studies should be designed to minimize overload of normal clearance processes at the maximal exposure concentration*" (*i.e.*, the MFTD) (Borm and Driscoll, 2019). However, this statement is not clearly substantiated other than by the general viewpoint that health effects observed in rats under conditions of impaired clearance are not relevant for humans.

A second argument brought forward is that hazard or risk assessment should be based on intrinsic chemical properties. Effects observed under conditions of impaired clearance are then considered to be particle-specific, not chemical-specific, and therefore not relevant for hazard or risk assessment. Although it is acknowledged that in case of PSPs, the retained particles and not the chemical structure can be assumed to be responsible for the induction of the observed toxicity, the adverse effects still result from exposure to the chemical. This, in principle, merits further consideration for human health assessment, irrespective the fact that in advance it is often not known if a particle should be considered to be a PSP. Whenever an exposure to a chemical, in whatever form and at whatever concentration, causes adverse health effects in humans, there is a need to limit the exposure to a level at which these health effects will no longer occur, *e.g.*, to derive an appropriate HBGV. A well-known example of toxicity related to specific particle-characteristics is asbestos-induced mesothelioma, which is attributed to the specific dimensions of asbestos fibres. Only asbestos fibres thinner than 3 μm, longer than 5 μm and with a length-width ratio above 3 are considered of relevance for asbestos-induced mesothelioma. Nowadays, all biopersistent fibres, irrespective of the chemical composition, that meet these dimensions are presently considered to have similar carcinogenic properties.[1] This example shows that an adverse effect that is related to specific particle characteristics in itself cannot be considered irrelevant for human hazard assessment without further (mechanistic) explanation.

In addition, a recent consultation of experts with various backgrounds revealed great hesitation to approach PSPs of low toxicity as a group for hazard assessment. It was stated that some of the consulted experts "*believed any PSPLT* [poorly soluble particle of low toxicity] *grouping strategy needed to allow for differentiating members based on potency of effect*" (Borm and Driscoll, 2019). Important factors mentioned included surface area and surface reactivity. This indicates that there is no consensus as to whether PSPs should be considered as one group by definition. The evaluation of PSPs therefore preferably needs to be performed case-by-case rather than by general principles of particle-related toxicity.

For the reasons mentioned, the possibility of occurrence of impaired clearance in itself should not be used beforehand to decide on lowering the top dose or exposure concentration for PSPs when aiming at a study design for an inhalation toxicity experiment in rats with the most optimal relevance for humans.

3.4. Human relevance of PSP-induced pulmonary toxicity observed in rats

The use of experimental animals to investigate potential health effects resulting from exposure to a chemical inevitably involves a number of uncertainties. The basic assumption is that the animal species selected is an appropriate model. From a hazard assessment perspective, the animal model is presently considered the best predictive model for human health effects and thus the effects observed should be considered relevant for humans, unless an adequate mechanistic explanation can be provided to conclude otherwise. Rats are the most frequently used species in toxicological experiments for human health hazard and risk assessment. According to OECD TG 412 and 413, the rat is the preferred species of choice for a 28-day or 90-day inhalation

[1] The World Health Organization (WHO) characterised the properties of bio persistent fibers. This refers to inorganic fiber dusts (except asbestos fibers) with a length > 5 μm, a diameter < 3 μm and a length-to-diameter ratio of > 3:1.

toxicity experiment, but another species may be used if a justification is provided (OECD, 2018b; OECD, 2018c). If the rat is chosen or has been used for toxicity testing of a PSP and pulmonary effects have been observed, several aspects need to be considered in rat-to-human extrapolation.

In general, if a difference in response to a chemical exposure between humans and experimental animals is suspected, the underlying cause needs to be clarified. Such a difference may result from a number of causes that may have a toxicokinetic and/or a toxicodynamic component. Understanding the underlying cause is essential to adequately interpret the data in view of human relevance and to extrapolate the results to human exposure situations. If toxicodynamic of origin, it may be that a specific mechanism of action observed in rats will not occur in humans, which is for instance the case with renal tubular tumours in male rats mediated via α-2 μ globulin nephropathy (IARC, 1999). In such a case the effect concerned can be considered not relevant for humans and can be disregarded for hazard assessment and hence for human risk assessment. However, a difference in response may also be due to a difference in kinetics, leading for instance to a different target organ or tissue concentration at similar external exposure levels. In such a case, the effect concerned can occur in humans and is thus considered relevant for classification purposes. For the purpose of risk assessment, the question then is under which exposure conditions this will happen. So, the challenge is to determine the most appropriate way to extrapolate the animal concentration-response curve to a human exposure situation.

As discussed in section 2.4.1, there appears to be a clear difference in clearance kinetics of PSPs in the lung between rats and humans (Bevan et al., 2018). The alveolar clearance in the rat via AMs may become impaired at high exposure concentrations after which the concentration of free particles in the alveolar space will show a steep increase. In contrast, humans will show higher concentrations of particles at interstitial sites than rats, although still a significant number of particles may be present in the alveolar space (Nikula et al., 2001). However, the appropriate dose metric often is unknown and the level of clearance impairment (*e.g.*, slight, significant, complete) is seldom assessed which makes it difficult to relate effects observed to an impaired clearance. Therefore, although differences in clearance kinetics do exist, the relevance of the pulmonary toxicity observed in rats as a consequence of this impairment to humans is difficult to evaluate.

This section so far focused on the human relevance of effects observed in the rat. In addition, it is noted that the reverse may also need to be considered. Is it possible that specific health effects can occur in humans, *e.g.*, as a result of the high particle sequestration to the human pulmonary interstitium, that will not be picked up in rats? This may especially be of importance at relatively low exposure concentrations below the level at which clearance significantly becomes impaired in the rat but that may be high enough to lead to a considerable amount of PSPs in the interstitium in humans. In other words, can adverse health effects occur in human interstitial tissues of the lung below respirable concentrations that induce adverse pulmonary effects in rats? Nikula et al. (2001) described higher particle retentions within the interstitium with higher exposure concentrations and working years in coal workers, related to the occurrence of pneumoconiosis (Nikula et al., 2001; Warheit et al., 2016). It is not clear whether these effects are adequately covered by animal experiments.

3.5. -Extrapolation of rat toxicity data to humans

As abovementioned, health effects observed in experimental animals are considered to be relevant to humans unless data are available that point out otherwise. If such data are absent, an appropriate dose metric needs to be determined to serve as a PoD for the assessment of a safe exposure level for humans. However, this may turn out to be problematic for PSPs. The precise mechanism for pulmonary effects observed in rats are not fully understood yet and several particle characteristics have been proposed to play a role in the induction of these effects (Bevan et al., 2018; Borm and Driscoll, 2019).

3.5.1. Choice of dose metric

Because of the differences in clearance kinetics, a comparable external exposure concentration of PSPs will lead to a different pulmonary distribution of particles in rats versus humans. This difference may change with increasing exposure concentrations (Nikula et al., 2001), especially once the level of impaired clearance in rats has been reached. How to deal with these differences in hazard and risk assessment depends on the mechanism of action for the adverse effects observed, and subsequently on the identification of the appropriate dose metric. In general, if an appropriate (internal) dose metric can be identified, the next step is to establish a quantitative relationship between this dose metric and the observed effect(s). An appropriate reference value for this internal dose metric can be extrapolated to humans (whether or not with application of assessment factors) and subsequently be translated into an external exposure concentration for humans. This provides a human equivalent concentration, *i.e.*, an external human exposure concentration that is expected to lead to a level of the internal dose metric that will induce comparable biological effects in humans as in rats. However, the question is whether such a dose metric can be determined for PSPs.

As discussed in section 2, the appropriate dose metric for the pulmonary effects observed in rats has not been determined yet. The total particle volume in relation to the volume of the AM pool has been mentioned as dose metric for specific effects in the alveoli (Morrow, 1988, 1992; Pauluhn, 2011, 2014). However, also total surface area has been proposed in relation to total lung weight (Elder et al., 2005; Morfeld et al., 2015; Tran et al., 2000). Brown et al. (2005) described that multiple parameters come into consideration when defining a dose metric for inhalation toxicology (Brown et al., 2005). These include 1) particle characteristics such as mass, volume, surface area, particle number, particle distribution, density and shape, 2) the respiratory region (*e.g.*, alveoli, macrophage, interstitium) and 3) the type of dose (either being for example total/average/maximum, or deposited/retained). There are many options to combine these parameters, and for a specific effect observed in rats it is often not clear which dose metric is the most appropriate to serve as PoD for extrapolation to humans. It might be that different health effects associate with different dose metrics (Brown et al., 2005). Next to particle volume and surface area, also particle size may determine the behaviour of particles in the lung and hence their toxicity (Borm and Driscoll, 2019). This is further complicated by the fact that the parameters mentioned under 1) are interrelated. For instance, at constant mass, an increase in particle number is associated with an increase in surface area. In this context, the evaluation of TiO_2 by NIOSH is of interest. NIOSH concluded that the appropriate dose metric in risk assessment of TiO_2 was particle surface area. Because of the greater surface area for a given mass of ultrafine TiO_2 compared to fine TiO_2, a lower airborne exposure limit was recommended for ultrafine TiO_2 than for fine TiO_2 (0.3 mg/m^3 and 2.4 mg/m^3, respectively) (NIOSH, 2011).

As to the third set of parameters, it has been mentioned that lung deposition may be altered in various pathological states, such as bronchitis, emphysema, and fibrosis (Newton et al., 1991; Sweeney et al., 1987, 1995). Therefore, induction of an inflammatory state or initiation of pathogenesis during exposure may alter actual particle deposition. Hence, deposition may be different depending on the exposure period and on the exposure concentration. This introduces an uncertainty when modelling lung burden estimates instead of measuring in an *in vivo* animal study (OECD, 2018a).

Despite the uncertainties involved in modelling lung burden, it can provide insight in differences between rats and humans as to the relation between external exposure and internal exposure estimates for different dose metrics. Brown et al. (2005) performed extensive modelling of human and rat exposure to PSPs (Brown et al., 2005). Three

particle classes were distinguished, coarse-mode particles (larger than about 1 μm in mass median aerodynamic diameter), accumulation-mode particles (about 0.1–1.0 μm in diameter) and ultrafine particles (< 0.1 μm in diameter). For a number of internal dose metrics, they calculated the ratios of external 6-h exposure concentrations for rat versus human that would provide equivalent normalized doses for both species for the respective dose metrics. A rat-to-human ratio smaller than 1 indicates that humans must be exposed to higher concentrations than rats for an equivalent internal dose. The ratios for the respective dose metrics varied largely depending on the particle size distributions and the level of exertion for humans (including oral breathing with moderate exertion as might occur in workers). Moderate differences were obtained when humans and rats were exposed to one atmospheric mode. For resting humans, higher exposure concentrations were required to reach a comparable normalized deposited or retained dose in the alveolar area (expressed as $\mu g/m^2$) as in resting rats (ratio of 0.16–0.20 for ultrafine and accumulation mode particles; 0.54 for coarse mode particles). However, when simulating human exposure under conditions of exertion or oral breathing, higher concentrations were required for rats (ratio up to 4) to reach an equivalent internal dose. Similar results were obtained with mass per macrophage as dose metric. Modelling particle surface area or particle number per alveolar area or per macrophage as dose metrics revealed comparable results as for particle mass as dose metric (Brown et al., 2005).

Perhaps of more interest are the calculations by Brown et al. for a more practical situation, *i.e.*, rats exposed to resuspended particulate matter (PM; MMAD = 2 μm, $\sigma_g = 2$) versus humans exposed to a combination of the three atmospheric modes (Brown et al., 2005). With particle mass per alveolar area or per macrophage, the modelling results were comparable to the calculations performed with the individual atmospheric modes, although the variation was smaller for the combined exposures. However, for dose metrics based on surface area, the external concentrations required for rats to reach a comparable normalized internal dose as in humans, were one to two orders of magnitude higher than for humans. For dose metrics based on particle number the required external concentrations for rats appeared to be even four orders of magnitude higher than in humans. This was considered to be due to the fact that resuspended PM is lacking in smaller particles (Brown et al., 2005). These modelling results indicate that a human equivalent concentration may differ orders of magnitude, depending on the dose metric. This stresses the importance to identify the appropriate dose metric for the extrapolation of pulmonary toxicity data in rats to human exposures.

Further, the simulations by Brown et al. (2005) underline the complexity of extrapolating an internal dose metric derived from a rat study to humans and a subsequent translation into an external human exposure estimate. This may especially be the case if human exposure is to PSPs with a particle size distribution that deviates significantly from that in the rat experiment. Further, an additional factor to be dealt with in occupational exposures is the level of exertion that often is accompanied by an increase in oral breathing next to nose breathing. The dose reaching the lower respiratory tract will increase with an increasing contribution of oral breathing to the respiration.

3.5.2. Particle size distribution

Furthermore, whatever the appropriate dose metric might be, the particle size distribution will play an important role in the rat-to-human extrapolation. For rat studies with repeated exposures, test guidelines limit particle size to an MMAD of ≤2 μm with a σ of 1–3 (OECD, 2018a). It may be difficult to ascertain the deposition pattern in the respective regions of the respiratory tract and thus the precise amount (in whatever unit) of a PSP that will reach the alveoli. On the other hand, to perform a risk assessment in human exposure situations, the airborne particle size distribution needs to be adequately measured in order to estimate the deposited dose. The particle size distribution of a powder may be well-known but the particle size distribution in environmental air may be different. For instance, if bagged powder is handled in an occupational setting, it can be expected that the fraction of respirable particles in air will be higher than in the bag due to the fact that once in air, large particles will be more subject to deposition by gravity than small particles. The particle size distribution of the bagged powder then will not be representative for that in air. This is especially of importance in occupational settings where inhalation exposures generally represent the highest human exposure scenarios. This is illustrated by the previous mentioned example of occupational exposure to SAS. Occupational exposure levels could reach up to 10 mg/m^3 total silica, of which approximately 20–40% appeared to be respirable (*i.e.*, up to 3.4 mg/m^3 respirable SAS) (ECETOC, 2006). The respirable fraction of SAS in air was considerably higher than what was expected from the particle size distribution in bags or drums. It appeared that the exposure concentrations of respirable SAS at the workplace were similar to the concentrations inducing pulmonary fibrosis in a 13-week study in rats (Reuzel et al., 1991). Hence, the determination of the airborne particle size distribution of PSPs may be relevant for hazard identification (as dose metric related to effects) as well as for the risk assessment (rat-to-human extrapolation; determination of the respirable fraction). Identification of its dustiness may be of aid to adequately predict exposure to (respirable) PSPs under occupational conditions (where in general the highest exposures occur), although the relationship between dustiness and exposure remains complex (Ribalta et al., 2019).

4. Conclusions on human health hazard and risk assessment of PSPs

Within the EU, there are regulatory information needs to assure a safe production and use of chemicals, including PSPs. At present, animal experiments are required to meet these needs for human health hazard and risk assessment. Exposure concentrations should be sufficiently high to adequately identify the intrinsic hazard of a chemical for classification purposes, to provide sufficient precision for the determination of an appropriate PoD to derive an HBGV and to determine potential adverse effects for risk assessment of unintentional and unforeseen future exposures at relatively high concentrations.

An important limitation in the present is that the occurrence of impaired pulmonary clearance in rats often is not substantiated by actual measurements. It can generally not be determined if and how pulmonary effects observed (including carcinogenic effects) are related to the level of impaired clearance. One question to be answered is whether these effects already start to occur at first signs of impaired clearance, or not before a significant impairment has been achieved or even not before complete clearance impairment has been reached. A quantitative determination of the relationship between pulmonary effects and the level of impaired clearance requires a clear definition of when alveolar clearance becomes impaired to a level that is relevant for human hazard or risk assessment. Also, it should be verified how this impairment can be adequately measured. What is more, defining a level of impaired clearance is hampered by the fact that the size of the AM pool of the rat lungs may be altered by the alveolar deposition of dust particles (*e.g.*, increased up to a factor of 10 (Morrow, 1988)) and that clear interspecies differences in total volume of AMs exist but also between rat strains (Geiser and Kreyling, 2010). Further, it is still under discussion whether the volumetric load is the main trigger for impaired clearance or that other particle characteristics play a more important role in the pulmonary response to PSP exposure (Bevan et al., 2018; ECETOC, 2013). The choice of dose metric can have a large impact on the estimation of the human equivalent concentration (Brown et al., 2005).

At present, it is noted that the discussion on impaired clearance is applied to PSPs in general which implies that all insoluble particles behave similarly with respect to the deposition in the lung, the uptake by AMs, and subsequently the clearing behaviour of the macrophages.

However, this is not self-evident. Recently, a group of experts expressed differing opinions on the possibility to classify PSPs as one group. Among others, it was considered that differences in the inherent properties may lead to different behaviour or toxicity of PSPs (Borm and Driscoll, 2019).

Summarizing the information discussed, a pragmatic approach is proposed if a rat study forms the basis for human hazard and risk assessment for a PSP. Because of the many uncertainties mentioned, the relevance of neoplastic lung lesions in rats can only be considered on a case-by-case basis for hazard assessment. It should be verified whether the observed neoplastic effects clearly can be attributed to a rat-specific mechanism and whether or not these tumours only occur under conditions of impaired clearance that will not occur in humans; these issues are relevant for both hazard and risk assessment.

A generic mode of action has been described for the induction of lung tumours in rats (Bevan et al., 2018; IARC, 2010) and shown in Fig. 1 in section 2. As mentioned, lung tumours in rats are considered to only occur under conditions of impaired clearance that lead to persistent inflammation. However, the cascade of reactions leading to inflammation are comparable in rats and humans (Bevan et al., 2018; ECETOC, 2013). The observation that the adverse effects in rats occur only under impaired clearance conditions is thus insufficient to conclude that these effects are not relevant for humans. As long as the appropriate dose metric cannot be assigned to specific toxicity in a concentration- or dose–effect relationship, it will be difficult to quantitatively extrapolate the observed effects to humans and therefore, to clearly conclude whether specific pulmonary effects in rats will never occur in humans.

Further, although no conclusive epidemiological evidence for an increased risk of lung cancer in humans was found, some steps in the development of lung cancer in rats (retained mass lung burden and decreased lung clearance) have also been observed in coal workers (Green, 2000). Therefore, ILSI and IARC both concluded that the cancer data in rats obtained under conditions of impaired lung clearance are relevant to identify potential carcinogenic hazards to humans (IARC, 2010; Ilsi Risk Science Institute, 2000). Tumour induction by PSPs can be caused by direct or indirect genotoxicity, as was mentioned in section 2 for TiO_2, one of the most extensively discussed PSPs. IARC concluded that the available human occupational epidemiological studies do not provide evidence for an association between occupational exposure to TiO_2 and risk for cancer, but further stated that all available human studies had some limitations (IARC, 2010). RAC recently concluded that the epidemiological studies were insufficient to conclude that TiO_2 was not carcinogenic to humans: the exposure data were inconclusive and the epidemiological data could not overrule the outcome of the animal studies (ECHA, 2017c). RAC stated that TiO_2 lung cancer potency in rats is already comparatively low. If in addition there is low exposure to TiO_2, then TiO_2 lung cancer potential in humans would not be expected to be shown in the available epidemiology studies (ECHA, 2017c).

At present, it is not clear whether high lung burdens of PSPs can also lead to lung cancer in humans via mechanisms similar to those in the rat. Therefore, from a regulatory point of view, the relevance of lung tumours observed in rats exposed to PSPs needs to be assessed on a case-by-case basis. Unless the available data clearly point out otherwise, lung tumours need to be considered relevant for human health hazard and risk assessment and thus for classification as a human carcinogen.

As to non-neoplastic effects, a similar pragmatic approach is required. In order to be able to account for possible differences in pulmonary response to PSPs between rats and humans, the study design should be such that sufficient information is generated to clearly decide upon the occurrence of impaired clearance, the appropriate dose metric and a threshold level for the regarding dose metric. From the perspective of hazard assessment, it does not suffice to make assumptions about the occurrence of impaired clearance on theoretical grounds, considering the many factors and uncertainties involved. For an adequate derivation of a human threshold of exposure to PSPs, insight in the toxicity profile with sufficiently high test concentrations is needed. Preferably, the highest concentration tested should clearly induce impaired clearance and at least one concentration should be below levels that induce impaired clearance. This will help to verify how pulmonary effects are quantitatively related to levels of impaired alveolar clearance. Also, sufficiently high test concentrations will provide insight in the concentration-response relationship and improve the estimation of a BMC (reduction of uncertainty), and will also provide information to decide upon risk reduction measures in case of unexpected (accidental) high exposures. Since toxicity studies will seldom be performed anew for reasons of animal welfare and available resources, it is of importance from a regulatory perspective, that animal experiments with repeated exposures will be performed such that they can serve multiple purposes as mentioned in section 3.2.

There is no approach or strategy available yet that has been validated and approved, and that has proven its value in human health hazard and risk assessment of PSPs, taking into account issues related to impaired clearance. Information that may be helpful though, are lung burden measurements as presently included in OECD TG 412 and 413, that enable to estimate the retention half time of a PSP. However, it is noted that clearance estimates based on lung burden measurements in general, may not necessarily relate well with alveolar clearance. Information about particle size distribution and adequate modelling data of the deposition throughout the respiratory tract may aid to estimate deposition and retention of PSPs and provide insight in the alveolar clearance, impairment thereof and its relationship with pulmonary effects observed. *In vitro* test systems such as the recently published *in vitro* assay on interaction of particles with AMs (Wiemann et al., 2016), may provide a valuable tool but still await validation. Inflammation markers present in BALF sampled at different exposure concentrations may, in combination with information on alveolar clearance, provide information about a correlation between inflammation and a possible impaired clearance, if present. For this purpose, a stepwise approach, including a well described and robust testing strategy, needs to be developed to provide clear answers to the many unsolved questions. It is again noted, that first impaired clearance needs to be clearly defined such that it can be used in a quantitative hazard and risk assessment and that the mechanism of action (including the appropriate dose metric) needs to be clarified.

As long as adequate information is lacking, the most pragmatic way forward in risk assessment is to determine a threshold for the most appropriate dose metric in the rat and extrapolate this concentration to a concentration for humans that is considered safe. It is acknowledged that this will require data that go beyond the requirements of the present OECD Test Guidelines (TG 412, TG 413 and TG 451/453), including determination of the exposure level at which impaired clearance has been reached, information on the particle kinetics and mechanism of action in both rats and humans (to determine the relevance for humans), the appropriate dose metric and a quantitative relationship thereof with the observed effects. In addition to a good dose metric, it is important to have a well defined and well defended (based on clinical relevance) adverse response metric. Acute inflammation alone is often not a good early indicator of a long-term adverse effect like cancer or fibrosis, whereas sustained cell proliferation (*e.g.* PCNA, Ki67) in tissue is a better biomarker.

Furthermore, one might consider whether the relatively high concentration of PSPs in the human interstitium at this threshold level still might lead to adverse effects that are not covered by the rat experiment. This can be considered in the choice of appropriate assessment factors. Modelling the deposition of particles in the rat and human respiratory tract at the respective exposure conditions can be helpful to obtain a better insight in and understanding of the differences in the processes of deposition and clearance between rats and humans. This might help to refine the rat-to-human extrapolation.

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Declaration of competing interest

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.

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