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CONTENTS

SUMMARY

Traditionally, many poorly soluble particles have been generally considered to be biologically inert and of low toxicity. However, under conditions of chronic inhalation exposure to high concentrations, such particles have been shown to produce a variety of unexpected toxicological effects, including a decrease in particle clearance from the lung as well as pulmonary inflammation (Lee et al, 1985). The attempt to explain this phenomenon led to the general hypothesis of `lung overload` which was typified by impairment in alveolar macrophage (AM) mediated pulmonary particle clearance and loss of AM mobility (Morrow 1988). Numerous studies focusing on the possible mechanism, especially with regard to observed species differences have been published since then. This culminated in a comprehensive review by an ILSI expert group in 2000, in which a possible rat-specific effect pattern of `lung overload` was discussed. In fact, the noted higher sensitivity of rats to non-neoplastic and uniqueness to neoplastic lung changes had raised questions on the appropriate use and interpretation of the responses of the rat as an animal model for hazard identification or quantitative extrapolation and risk characterisation.

More recently, the phenomenon 'lung overload' regained importance due to the necessary derivation of 'Derived No Effect Levels' (DNEL) under the REACH legislation and the setting of `health based` Occupational Exposure Limits (OEL), as well as for classification and labelling under the Globally Harmonised System of Classification and Labelling of Chemicals (GHS / CLP).

This ECETOC Task Force was convened to examine the current scientific understanding of the `lung overload` hypothesis. In this respect, the review was required to present and discuss new findings on lung toxicity of such low soluble particles of low toxicity and its mechanistic interpretation, as well as to address the progress made concerning the knowledge of the underlying mode of action and its relevance for human risk assessment. Special emphasis was given to the question if the rat is an appropriate model for the extrapolation of `lung overload` related pulmonary effects to humans.

The synopsis of currently available scientific data on `lung overload` allows the Task Force to conclude, that:

- The rat represents a particularly sensitive model concerning the development of pulmonary non-neoplastic lesions and, moreover, a unique model with regard to lung neoplastic responses under conditions of lung overload.
- Lung tumours have to be regarded the final phenotypic `adverse outcome` only in rats, whereas in other species non-neoplastic lesions seem to be the respective `adverse outcome`.
- Humans are less sensitive to `lung overload` as epidemiological studies thus far have not been able to detect an association between occupational exposures to poorly soluble particles of low toxicity and an increased risk for lung cancer.
- The divergence in the largely common mechanistic sequence of the adverse outcome pathway may be related to a biological diversity of detoxification systems, especially in species specific anti-oxidant defences resulting in a more pro-inflammatory environment in rats compared to a more anti-inflammatory environment in other rodent species.
- The measured differences of particle retention, distribution and clearance patterns in the lungs of exposed rats vs. primates or humans, may account for both the greater sensitivity in rats and corresponding differences in pulmonary pathological responses to long-term particle exposures

- Slight differences in the bio solubility of deposited "poorly soluble particles" in biological fluids may influence chemical dissolution and based hereupon accelerate or slow down the process of lung overload development.
- Independent of particle size, inhalation exposure to high concentrations of low soluble particles of low toxicity are eliciting comparable localised pulmonary toxicity via processes that are pro-inflammatory in nature, causing oxidative stress and an persistent pulmonary inflammatory response.
- The mechanisms leading to an oxidative and inflammatory pulmonary status are clearly threshold related.
- There is no "nanoparticle-specific lung overload toxicity" and mechanistic findings for conventional "micro" particles apply also for nanostructured particles.

Finally, there is substantial evidence exemplified in a number of studies that poorly soluble particles of low toxicity, whether nano sized or micro sized, exert their toxicologically relevant non-neoplastic effects and also the neoplastic responses in rats, via a threshold mediated mode of action. Hence, the derivation of DNELs for poorly soluble (nano) particles of low toxicity is toxicologically justified. Due to the higher sensitivity of the rat compared to humans and based on comparable biokinetics, an overall assessment factor of 1 for intra- and interspecies differences is considered sufficient. DNELs based on NOAELs/NOAECs as derived in animal inhalation studies and adjusted for human equivalent concentrations by appropriate dosimetry modelling is recommended.

Since the ILSI 2000 report, there has been a vast amount on in vivo and in vitro work on poorly soluble particles of low toxicity but there have been no compelling studies or a weight of evidence that would allow the Task Force to conclude that the rat lung overload findings is a reliable predictive model, in particular for neoplasia, with regard to hazard or risk assessment for humans who are exposed to poorly soluble particles of low toxicity.

1. INTRODUCTION

1.1 Terms of reference

Although the 'lung overload' phenomenon has been known for decades, it has recently become more prominent for the derivation of 'Derived No Effect Levels' (DNEL) under REACH registrations, setting of exposure limits, and for classification and labelling under the Globally Harmonised System of Classification and Labelling of Chemicals (GHS). In its Guidance on Information Requirements, ECHA recently stated that the interpretation of data obtained following high exposures to poorly soluble particles (PSPs) "should be approached with caution and appropriate discussion should be given to the mechanistic driver behind any pathogenic effects detected" (ECHA 2012a). In this respect ECHA also notes, that "effects occurring secondary to a threshold stimulus such as inflammation could also be considered threshold in nature and as such a DNEL can be derived" and that " a possible example of such a driver is the induction of lung overload in experimental animals exposed to poorly soluble low toxicity (nano)particles leading to inflammation, oxidative stress and culminating in lung tumour formation" (ECHA, 2012b).

The aim of the present document therefore was to examine the current scientific understanding of the "lung overload" hypothesis with regard to the anticipated sensitivity and specificity of the rat lung responses, its implications for hazard identification and human risk assessment. For this a `State of the Science Review` on the relevance of effects, for human health, observed after repeated inhalation exposure in rats to PSPs of low inherent toxicity was prepared based on earlier reviews, including recent information in the field of `lung overload`. Special emphasis was given to the following terms of references:

- Discuss new information on lung effects and mechanistic interpretation of lung overload in humans and animals (rodents and non-rodents).
- Identify and agree on parameters that characterise lung overload (parameters for animals and humans separate).
- Compare effect levels from animal studies to realistic worker exposure (e.g. deposited dose for humans) on a quantitative basis.
- Review relevance of existing/new epidemiology studies with regard to observed experimental and human respiratory effects.
- Elaborate on the possibility to include possible 'lung overload' effects from nanomaterial inhalation studies.
- Give consideration to the organisation of a workshop to discuss the relevance of rat lung overload for humans in terms of e.g. classification and labelling, and the derivation of DNELs.

1.2 Background

Chronic inhalation of poorly soluble, non-fibrous particles of low toxicity (PSPs) can result in pulmonary inflammation; increase in lung weights, epithelial hyperplasia, fibrosis and eventually in adenomas and carcinomas in the peripheral lung of rats (Lee et al, 1985; Mauderly et al, 1987; Saffiotti et al, 1988; Nikula, 2000; Roller, 2009). This cascade of events runs primarily at exposures to high particle concentrations and

thus may be considered a result of the experimental set-up rather than a true reflection of the virtually low intrinsic toxic potential of PSP. The term "high particle concentration" has not been clearly defined but is related to the amount of poorly soluble material deposited daily in the lungs, and thus, the pulmonary clearance rate seems to be a useful indicator to approximate the critical exposure concentration(s) resulting in lung overload conditions. Analysing results from various lung clearance tests in rats and hamsters exposed for several months to a variety of particulate aerosols led to the conclusion that lung clearance is retarded by chronic exposure to respirable particles at concentrations of 3 mg/m^3 or higher (Muhle et al, 1988). A similar concept of a so called "critical deposition rate" was based on mathematical analyses of lung clearance rates by Yu et al, 1989 and was defined as "rate above which the overload condition will be present if the exposure time is sufficient". An alternative definition of "critical deposition rate" may be seen in the threshold dose leading to impaired alveolar macrophage mediated lung clearance, which is equivalent to approximately 1 mg per gram lung tissue (Morrow 1988) or 1 µl per gram of lung (Oberdörster, 1995).

Additionally, it was shown that rats respond more evidently and more intensely to such exposures. Whereas most of the described inflammatory-related pulmonary effects can also be observed in other experimental species, only rats have been shown to respond with lung tumours. Based hereupon, rats are considered to be particularly sensitive towards PSP-induced lung toxicity compared to other rodents, non-human primates as well as humans and that these tumours are rat-specific. Corresponding indications of such species differences following exposures to PSP were shown in comparative studies with different animal species as will be described in more detail hereafter. Consequently, the relevance of the rat as a model for hazard and risk assessments of repeated exposure to PSP for humans is still questioned by a number of critical appraisals. It is now well established that lung effects following chronic inhalation to PSPs of low toxicity occur only at exposures which are concurrently leading to an accumulation of particles in the deep lung as a result of significant impairment of pulmonary particle clearance. This concept of "lung overload" was first introduced by Morrow in 1988 (Morrow, 1988) and the last comprehensive review of available experimental data and a possible rat-specific effect pattern of "lung overload" was developed in the year 2000 by the ILSI Risk Sciences Institute (ILSI 2000).

The main conclusions from this ILSI workshop on `lung overload` can be summarised as follows:

- Hallmark of particle overload is impaired alveolar clearance.
- Precise mechanisms are not known but volumetric inhibition of macrophages and the development of an inflammatory environment seem to be important drivers.
- Differences in potency of various PSPs are obvious and are leading to the need of dosimetric adjustments accounting for differences in deposition and clearance of particles.
- Overload is not a rat specific phenomenon and seems to be generally reversible but may reach conditions where clearance impairment is irreversible.
- Overload contributes to the (species independent) pathogenesis of non-neoplastic lung responses and is a prerequisite for the tumorigenic effects observed in rats. With regard to humans, despite evidence that particle clearance is impaired in many coal workers, no conclusive evidence for increased lung cancer risk exist for workers chronically exposed to coal dust or for workers exposed to other poorly soluble particles.
- For neoplastic lesions, dose-response data from persistent neutrophilic inflammation and cell proliferation can be used as surrogate for risk characterisation

- For non-neoplastic responses, persistent neutrophilic inflammation may also be used as surrogate whereas epithelial cell proliferation is not considered a necessary prerequisite for fibrosis.
- A nonlinear dose-response approach for the characterisation and evaluation of both, neoplastic and non-neoplastic lesions are considered plausible based on the assumed pathogenesis.
- An uncertainty factor of 1 for both neoplastic and non-neoplastic endpoints can be considered sufficient to account for toxicokinetic and toxicodynamic parameters.
- With regard to an appropriate dose metric some estimate/parameter reflecting retained lung burden is recommended together with a full characterisation of the aerosol exposure parameters (e.g. MMAD, particle surface area, density).
- With regard to non-neoplastic responses the rat is considered predictive of a non-neoplastic hazard for humans.
- With regard to neoplastic responses the rat is considered to be more responsive than other species including humans at doses and exposure intervals that result in pulmonary particle overload.
- The mode of action for induced neoplastic responses in rats apparently needs accumulation of particles in lung alveolar and interstitial compartments, persistent inflammation and epithelial cell proliferation.

In the context of the present review the validity of the above conclusions with regard to the observed lung effects of respirable poorly soluble particles of low toxicity are re-assessed based on recent research findings.

1.3 Definition of `poorly soluble particles of low toxicity`

In this document the subject of lung overload of inhaled non-toxic and poorly soluble particles is reviewed. The terms "poorly soluble" and "non-toxic" need clear definitions. We suggest to denote inhaled particles as poorly soluble when their dissolution half-life measured in artificial lung fluids i.e. interstitial fluid (pH 7.4), artificial lysosomal fluid (pH 4.5), and artificial alveolar fluid (pH 7.4) is larger than the macrophage mediated clearance times. This ensures that macrophage clearance, and not dissolution, determines the particle residence time in the lung. Particles are considered to be non-toxic if they are chemically inert and without any known specific toxicity, i.e. there is no (bio)chemical reaction between the molecules at their surface or dissolved from their surface and the embedding lung fluid. It should be noted, that particles exhibiting significant surface related (cyto)toxicity like crystalline silica (quartz) and/or other specific toxic properties do not fall under this definition and are not considered specifically in this document (also Chapter 3 `Biosolubility`).

The concept of lung overload has been developed in the past four decades based on evidence gained primarily from rat inhalation studies carried out with a variety of technical powders. Using current terminology, many of the test substances investigated such as carbon black, titanium dioxide, "baytubes" etc. are classified as nano-powders because their primary building blocks have dimensions in the nanometre range. In reality, aerosols generated from these powders appear mainly as aggregated and/or agglomerated structures with aerodynamic or mobility diameter larger than a few hundred nanometres and this is also how they are administered to the animals and exhibit toxicity. As will be shown in the following discussions, two metrics are concurrently used to correlate the biological effect with the administered dose. These are

particle volume and surface area. Both metrics are integral quantities of the aerosol and require principally no specific consideration of particle size. Particle size enters the dosimetry only via the particle size dependent lung deposition fraction which is required to calculate the administered dose from the exposure concentration. Exposure concentration is usually given in mass concentration. The density and specific surface area of the product, which are preferentially determined in the stage of administration, link the measured quantity mass to the dose metric volume and surface area, respectively. The (envelope) density of a particle can be defined as its mass divided by the displacement volume in a non-wetting liquid. This takes properly into account the particle porosity and represents the volume displacement of a particle when engulfed in a macrophage. The specific surface area is the externally accessible surface area divided by the particle mass. Based on this arguments there is no need to distinguish between nano-powders and micro-powders as far as the lung overload of non-toxic PSPs is concerned.

2. PHYSICO-CHEMICAL ASPECTS ASSOCIATED WITH `LUNG OVERLOAD`

Particle overload (also referred to as `lung overload` or `clearance overload`) describes a condition of slowed/impaired (macrophage mediated) clearance in the lung after prolonged exposure to poorly soluble particles of low inherent toxicity. This condition is further characterised by an increased transfer of particles to lymph nodes, accumulation of particles in the lung, increases in lung weight, pulmonary inflammation, epithelial hyperplasia (proliferation), fibrosis and eventually cancer (in the rat). A rat-specific effect pattern can be assumed as evidenced by greater pulmonary inflammatory -, fibrotic -, hyperplastic – and particularly a unique tumorigenic responses to particle exposures as compared to other species (e.g. mice, hamster, non-human primates, humans). Although a mechanistic understanding in terms of species differences is not yet fully established, factors determining the deposition characteristics of inhaled particles, like particle size, airway geometry, ventilation rate and clearance rate seem to be important while persistent inflammation and epithelial cell proliferation are dominant mechanistic drivers. Additionally, various physico-chemical characteristics like particle density, particle surface area or particle volume, may influence the establishment of an overload of alveolar macrophages. Although doses are normally expressed in terms of particle mass, other metrics have shown stronger associations with the extent of the overload-related events observed during chronic particle exposures.

2.1 Dose metrics

A substantial number of experimental observations have led to the general concept of dust overloading of the lungs (e.g. Klosterkötter and Bueman, 1961, Ferin 1972, 1977, Green et al 1983, Lee et al, 1985). The essence of this experimental finding is bipartite: (a) long-term exposure to relatively high dust concentrations leads to excessive pulmonary dust burdens whereby the pulmonary clearance of persistently retained particles by alveolar macrophages becomes progressively reduced until it essentially ceases: at this time, lung dust burdens increase linearly at a build-up rate approximating the rate of dust deposition; (b) as excessive lung burdens develop, a number of alterations appear in both the disposition of retained particles and their pattern of induced responses and toxic actions within the lungs.

In this context of a more qualitative description, a quantitative definition of the overload condition was still missing; it was not clear whether such an overload theory could be generally applied to all persistent dust and for other species besides rat. In his paper of 1988 (Morrow 1988) Morrow for the first time provided a mechanistic hypothesis and proposed that overload condition was caused and perpetuated by a loss in mobility of alveolar macrophages (AM). He considered the build-up and clearance kinetic of the alveolar region. Assuming first order clearance kinetics, a chronic exposure to certain concentration of a poorly soluble dust would achieve a steady-state condition after a time period corresponding to about five times the retention half time. In this simple model, deposition rate was the factor for build-up of lung burden and retention half time was the factor influencing the clearance. This type of curve had been demonstrated earlier during chronic studies (Leach 1970 and Stöber et al, 1967). It describes what is to be expected during chronic inhalation exposure.

Another aspect of Morrow´s hypothesis was to evaluate factors affecting the mobility of the AM to translocate from the lungs. By reviewing several seemingly isolated highly germane experiments, he considered directed migration by chemotactic factors as the least contentious. The actions of several chemo-attractants, activators and modulators (e.g. macrophage migratory inhibition factor, fibronectin and colony stimulation factors) were mainly examined in vitro. How much they contribute to the cessation of AM mobility is not clear. Scanning electron microscopic examination indicated that volumetric loading-related changes of AM´s cytoskeletal system likely contributed to inability of AM to spreading and locomotion (also Section 4).

Mainly, the following three dose metrics have been used trying to identify and describe the relationship between particle exposure and lung overload conditions.

2.1.1 Particle mass

For isometric particles the mass is and has been the most convenient descriptor. Biological effects have been related to the mass dose, and concentration standards are given in terms of suspended mass per volume of air. Mass can be easily determined by gravimetric and/or chemical analysis. Moreover the measurement techniques themselves are less dependent on a priori assumptions related to factors such as mono- or poly-dispersibility, the particle shape and brief fluctuations of the concentration. An empirical mass-based lung burden of 1-2 mg dust per gram lung was identified early on as a threshold above which relatively benign dusts showed significant prolongation (> doubling) of the pulmonary retention half-time in Fischer F344 rats (Morrow, 1987). More recently, Pauluhn proposed the use of the mass-based pulmonary burden rather than actual mass concentration or surface area concentration as a critical denominator of dose and dose-related pulmonary toxicity (Pauluhn, 2009a).

2.1.2 Particle volume

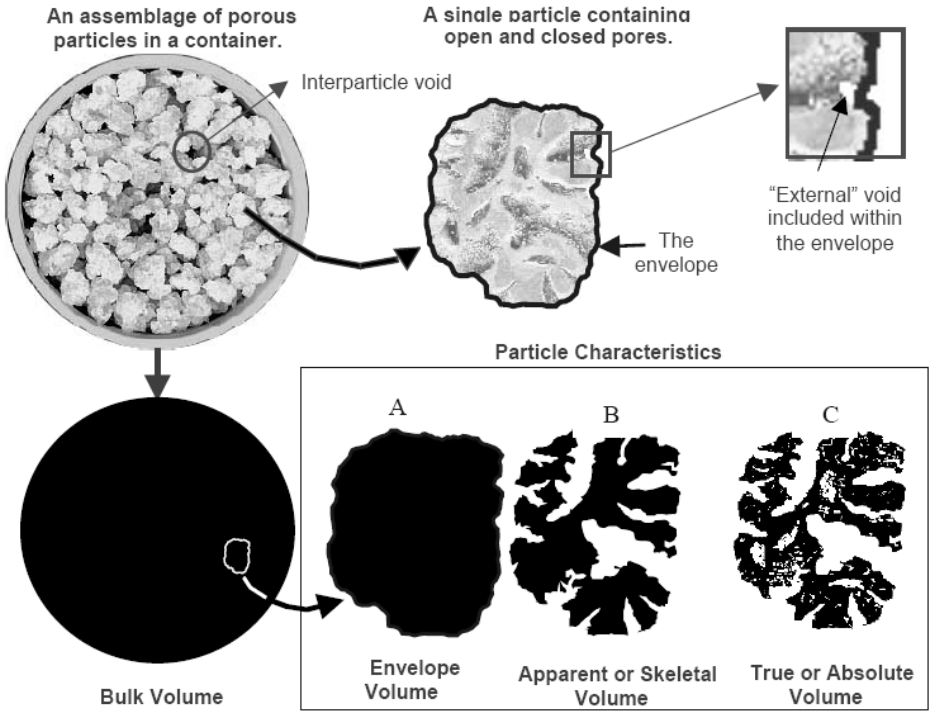
Morrow for the first time considered and calculated particle volume per AM as a quantitative definition of the overload condition. In this context, Morrow mentioned two important issues: 1) particles with higher density would achieve the volume load at respectively higher mass load; 2) the void spaces would increase the volume of phagocytised agglomerates by more than 30%. The volumetric consideration of Morrow was strongly supported by a study performed by Snipes and Clem (1981) with 3-, 9- and 15 µm diameter microspheres simultaneously administered into the lungs of Fischer 344 rats. Although the instilled particle numbers was only 1% of the number of AM in rat, a strongly prolonged retention half time was found for 9 µm particle (580 days) in comparison to 3 µm particles (t½ = 69). The retention half time for 15 µm particles was so long that it could not be determined. A 9 µm particle accounts for 38 % of the volume of a rat AM, it fulfils the overload condition. A 15 µm particle with 1770 µm³ even exceeds the volume of a rat AM. This finding was confirmed by Oberdörster et al (1991) with 3.3 µm (~20 µm³) and 10.3 µm microspheres (~600µm³). The findings of these two studies are consistent with the calculations performed by Morrow and indicate particle volume per cell as an index of overload. Morrow´s hypothesis is appropriate for PSPs with

low general toxicity. The fulfilment of these two requirements is however difficult to define; especially in a quantitative way (Section 1.2).

With the possibility of a higher toxicity of nanoparticles in comparison to the larger chemically-identical counterpart Pauluhn (2011) brought the volumetric overload again into the discussion. He modified the overload hypothesis by introducing agglomerate density (instead of physical density) into the calculation of volumetric loading. For the determination of agglomerate density he refers to Klobes et al (2006). He could demonstrate that volumetric loading nicely correlates to the inflammatory response, as indicated by neutrophil counts in lavage fluid, for several PSPs (e.g. AlOOH 10 nm and 40 nm, Fe_3O_4, and MWCNT).

In a guidance document published by Webb (2001) various definitions of the powder volumes are given. They are illustrated in Figure 1. In the underlying context only the single particle volume is relevant. The envelope volume is given by surrounding the particle by a smooth film. The smaller apparent or skeletal volume takes into account the voids and pores accessible from outside. The true volume of solid material is given by exclusion of the inner pores.

Figure 1: Illustration of the different definitions of particle volume (Webb, 2001)



Regarding the volume-based overload concept and the derivation of human equivalent concentrations (Pauluhn, 2011) the density, r, of the dispersed powder is crucial in relating the deposited dose of particle volume to the administered external mass concentration usually available in inhalation studies. The same applies for the task of the derivation of human equivalent mass concentrations for technical powders based on the NOAEL concentrations which are determined in animal experiments using specific powders (such as

titanium dioxide, or toner particles). Reliable values of the "true" density of the dispersed product are therefore required (Section 2.2.1).

2.1.3 Particle surface area

The correlation of particle surface area dose and biological activity in vivo, suggesting increasing toxicity with decreasing particle size at equal concentrations, has been demonstrated in a large number of studies with various particulate materials.

Oberdörster et al (1994) performed a sub-chronic inhalation study with ultrafine (20 nm) and fine (250 nm) anatase TiO_2 at similar mass concentrations (23.5 and 22.3 mg/m³ respectively). After the exposure period, mass lung burden differed only slightly between the two materials, but ultrafine? **<== CHECK** (nano) TiO_2 caused prolonged retention half-time and macrophage clearance rate (3-and 8 fold, respectively). Moreover, significantly more severe inflammatory responses and changes of lung morphology were found after exposure to nano TiO_2. From the deposited nano and fine particles, 44 % of the nano particles was determined in the interstitial space, whereas there were only 13 % of the fine TiO_2 particles (section on Translocation). When establishing dose-response relationships on the basis of either mass, volume or surface area for these effects, the authors indicate that "surface area" and not volumetric load as the appropriate dose metric for "ultrafine" particles in correlation with the examined endpoints.

In a similar way of establishing dose-response relationships for observed effects, Tran et al (2000) analysed sub-chronic data of TiO_2 and BaSO4 (Cullen et al, 2000). The numbers of polymorphonuclear cells (PMN) in the lung were plotted in relation to the corresponding lung burdens expressed in terms of mass, surface area, and particle numbers. The results indicated that surface-area burden was the most likely of the three measures to explain the difference in the numbers of PMN between the dusts suggesting a threshold for PMN recruitment at a lung burden area of 200 to 300 cm². Tran et al also analysed published data by Driscoll (1996) and Oberdörster et al (1994) concluding to be in line with the results of Cullen et al (2000) and Tran et al (2000). Considering that particle surface area incorporates both particle number and their size, it was postulated that there is a chain of biological responses following deposition of large particle surface area, which may lead via an "activated environment" to immobility of alveolar macrophages, and to the overload condition.

Further comparisons were performed by Sager et al (2008, 2009) who examined the pulmonary response of ultrafine (nano) versus fine titanium dioxide and carbon black (CB) after intratracheal instillation to rats. They compared the dose-response relationship of nano and fine material either on a mass-based or a surface-based dose metric. It was observed that on a mass dose basis nano sized particles gave a 30-100 fold higher response than the fine-sized particles of the same composition in the examined parameters. However, when the dose is normalised to surface area the difference of the same sets of parameters was about an order of magnitude lower (Sager et al, 2008).

In general, a number of studies over the past 15 years, suggest that the smaller the particle (the greater the surface area dose) the greater the induced inflammatory response. Donaldson et al (2008) identified a threshold value for onset of inflammation of 1 cm² particle surface area burden per 1 cm² of proximal

alveolar region (PAR), (Donaldson et al, 2008). The relevance of surface area is discussed particularly in regard to toxicity of nano-particles because at the same mass load surface area increases as particle size decreases. Furthermore, the specific characteristic of engineered nano-particles is very often determined by surface functionalization which may significantly influence their toxicity. Rushton et al (2010) showed good correlation between in-vivo and in-vitro signals when using a surface area (BET-values) based dose metric when testing nanoparticles.

2.2 Particle properties

Considering that different dose metrics exist which might be, depending on the research question, best suited to describe the relationship between particle dose and lung overload-associated effects, it is very important to conduct a thorough physico-chemical characterisation of the material assessed. The particle properties described in the following section have already been identified as being of importance.

2.2.1 Particle density

A density can be related to each volume by dividing the mass by the particle volume. It is clear that for a given particle the envelope density is smaller than the apparent density which is smaller than the true density. Only the apparent density can be considered in view of the volume based overload concept. Which one is of more relevance depends on the wettability of the particle when engulfed by the macrophage fluid. However, for particles in the micron and submicron size range it is reasonable to assume that the envelope volume represents the displacement volume in the macrophage fluid. For these small particles the dimensions of the external voids are in the nanometre range and unlikely to be filled with macrophage fluid. For the definitions of density, see Fig. 2.1., which describes the corresponding definitions of the particle volume.

Among others, pycnometric methods where helium (gas) or a non-wetting liquid such as mercury (liquid) fills the pores are usually employed to measure porosity and hence the density of the material in powder form. The mercury intrusion method allows the determination of the distribution of the pore volume by variation of the pressure imposed on the mercury. The method is applied to an assemblage of (porous) particles in a container. At the low end of the pressure ramp the interparticle voids are filled with mercury. The envelope density is obtained in this pressure regime whereas the apparent powder density is usually calculated from the pycnometer value obtained at the highest pressure value because then even the smallest externally accessible pores are expected to be filled with mercury.

Recently, methods have become available that allow for the determination of the envelope density of individual particles when they are in the airborne state (Park et al, 2004, Liu, et al, 2012). These methods are based on the determination of the mass and the envelope volume of the individual particles using a combination of two well-known aerosol instruments: an electrical differential mobility analyser and a particle mass monitor. In-situ determination of the aerosol surface area can be facilitated by diffusion charging and gas absorption, for example. Gas absorption using the BET method is made on samples of

powders as produced. In a recent study, Lebouf et al (2011) refined the BET method (using krypton instead of nitrogen as the absorbing gas) so that it could be employed to filter samples of material taken after aerosolization i.e. delivering a value of the specific surface area of the aerosol as delivered to the animal.

2.2.2 Particle size (fine, ultrafine, nano)

The term size refers to the linear extension of the particles. In order to take into account density and shape the particle diameter is expressed as so called equivalent diameter: the aerodynamic diameter for particles larger than 0.5 µm and the diffusion equivalent diameter for the smaller size fraction. The term nanoparticle is used for objects with two or three dimensions smaller than 100 nm. Engineered nanomaterials consist of nanoparticles and are produced intentionally and are designed to possess specific properties. In parallel there are ultrafine particles being defined as nanoparticles generated unintentionally such as in combustions processes or by reactions in the atmosphere. Most engineered nanomaterials released into the air occur as agglomerates or aggregates of much smaller primary building blocks (primary/constituent particles). Aggregates are groups of primary particles held together by firm forces such as sinter bridges, whereas agglomerates are loosely tied together by van der Waals forces. The (equivalent) diameter of these agglomerates and aggregates is an important parameter in the estimation of the pulmonary dose (Section 2.3). Importantly, recent experiments have demonstrated that the de-agglomeration of agglomerates consisting of nano-sized constituents is not a significant event in vivo (Creutzenberg et al, 2012, Morfeld et al, 2012, Morfeld et al, 2013, Creutzenberg et al, 2013).

The particle size is also directly associated with the surface-based dose metric. Pulmonary toxicity per unit mass of the same material appears to be enhanced with decreasing particle size in a number of studies with various exposure regimes (inhalation and intratracheal instillation) and for a variety of PSPs (Ferin et al, 1991, Oberdörster et al, 1992, Heinrich et al, 1995, Borm et al, 2000, Gallagher et al, 2003, Renwick et al, 2004, Gilmour et al, 2004, Gurr et al, 2005, Sager and Castranova 2009, Kolling et al, 2011). It has also been demonstrated that smaller sized particles exhibit longer residence times in the lungs following exposures compared to larger particles, indicative of slower clearance rates. As small particles may more readily translocate to the pulmonary interstitium, AM-mediated clearance may be hindered which results in prolonged retention times of particles in the lung (Oberdörster et al, 1994; Kreyling et al, 2002) (Geiser & Kreyling, 2010) (Scherbart et al, 2011). The possibility of nano-sized particles originating from inhaled agglomerates and escaping the major particle scavenging pathways is however limited in real-life situations (Landsiedel et al, 2012; Morfeld et al, 2012).

A higher biological activity of smaller particles is however not universal as Eydner et al, did not observe significant changes in elicited effects or translocation behaviour between groups exposed to nano or fine titanium dioxide particles (Eydner et al, 2012). An issue under debate is whether there is truly a universal size limit at which the size-behaviour relationship displays an inflection point A recent report by Hassinger and Sellers stated that neither the experimental data nor the theoretical explanations currently suffice to define the size at which the properties of materials are changed to ‘nanospecific' properties (Hassinger & Sellers, 2012). Regarding the relationship between the "classic” particle toxicology and the nanotoxicology, Donaldson and Seaton stated: ”Nanotoxicology now dominates particle toxicology and for many people nanoparticle toxicology is particle toxicology” (Donaldson & Seaton, 2012).

Comparable findings of no dependency of lung toxicity with particle size and surface area were revealed when comparing TiO_2 nano- and fine particles (25 nm and 100 nm) of different sizes, surface areas, and crystal structure. Although the difference in surface areas were as large as 30 fold, the observed lung inflammatory responses following intratracheal instillation to rats (1 and 5 mg/kg body weight, 24 hours, 1 week and 3 months), the observed lung responses were almost the same for the two particle sizes investigated (Warheit et al, 2006).

A recent critical review article (Donaldson and Poland 2013) makes a well-argued case, based upon a thorough review of the existing data, that conventional particle toxicology is in general, both useful and relevant to the toxicological evaluation of nanoparticle hazards and that there is no clear evidence to show that particles below 100 nm show any kind of step-change in their hazard status and for the onset of any novel nano-specific hazard. For this reason the ECETOC Task Force was of the opinion that there is no need to include any specific section in this report on nanostructured materials and that most of the findings for conventional particles will apply to nanostructured materials. This reasoning is reinforced by the fact that much of the scientific information that has gone into this report are based on studies using carbon black and ultrafine TiO_2, both of which are nanostructured materials.

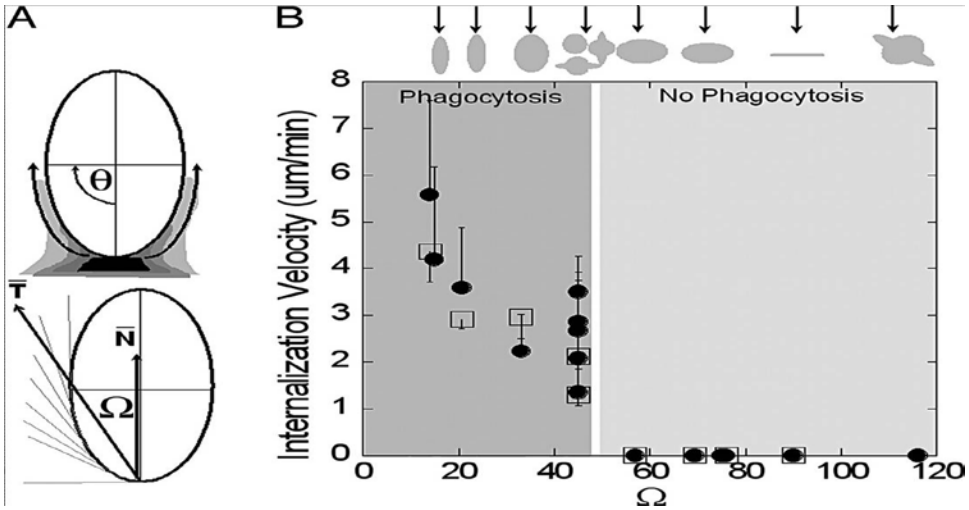
2.2.3 Particle shape

Particle shape can modulate particle properties in several ways. Shape can have an impact on a particle's aerodynamic behaviour. This effect has been well described for fibres which can reach the deep lung despite their very long length because the aerodynamic diameter is proportional to the fibre diameter and not the length (Jones 1993). A similar phenomenon has been described for disc-like particles such as graphene which can also display unusual aerodynamic properties (Johnson & Martonen 1994, Schinwald et al, 2012). In the case of nanomaterials, the particle shape has been listed as one of the factors determining their toxicokinetic behaviour (Landsiedel et al, 2012).

Particle shape has also been identified as having a significant effect on the ability of macrophages to internalise particles via actin-driven movement of the macrophage membrane (Champion & Mitragotri, 2006, 2009). This phenomenon is independent of surface chemistry and depends only on the local particle shape where the cell attaches. Local particle shape, at the point of cell attachment, has been quantified as the angle between the membrane normal at the point of initial contact and tangent lines drawn to the particle contour near the point of initial contact (Figure 2). This angle, Ω, is essentially a measure of the local curvature that has been normalised for size, the length over which that curvature exists. Length-normalised curvature, as defined by the parameter Ω, is the critical shape feature that dictates whether or not phagocytic internalisation will occur. Local shapes that have low length-normalised curvature ($\Omega > 45°$, where 45° is the value for spherical particles) inhibit internalisation while spheres and local shapes that have high length-normalised curvature ($\Omega \leq 45°$) permit internalisation (Champion and Mitragotri, 2006, 2009). If an AM encounters a high aspect respirable particle larger then itself, then it will engage the particle and attempt to engulf it, but it will fail and as a result, the particle will persist in its location and with repeated exposure, may accumulate to reach a toxic dose (Donaldson and Poland 2013). Hamilton et al, showed that the fibrous shape and fibre length of TiO_2 anatase influenced its effect towards primary mouse macrophages; relative to spherical nanoparticle (60-200 nm), fibre structures of greater than 15 µm created

a highly toxic particle (Hamilton et al, 2009). Altered phagocytosis efficiency for a given material in the lung will likely have a significant influence on the clearance efficiency which can ultimately lead to adverse effects, even despite the material's low intrinsic toxic potential.

Figure 2: Definition of Ω and its relationship with membrane velocity[a] (Champion and Mitragotri, 2006)



[a] Legend (A) A schematic diagram illustrating how membrane progresses tangentially around an ED. T represents the average of tangential angles from θ = 0 to θ = π/2. Ω is the angle between T and membrane normal at the site of attachment, N. (B) Membrane velocity (distance travelled by the membrane divided by time to internalise, n ≥ 3; error bars represent SD) decreases with increasing Ω for a variety of shapes and sizes of particles. Non-opsonised particles are indicated by filled circles, and IgG-opsonised particles are indicated by open squares. Each data point represents a different shape, size, or aspect ratio particle. The internalisation velocity is positive for Ω ≤ 45° ($P < 0.001$). Above a critical value of Ω, ≈45°, the internalisation velocity is zero ($P < 0.001$) and there is only membrane spreading after particle attachment, not internalisation. The arrows above the plot indicate the point of attachment for each shape that corresponds to the value of Ω on the x axis.

2.2.4 Surface reactivity

Warheit and colleagues compared the pulmonary toxicity of different sized TiO_2 particles after instillation in rat (Warheit et al, 2006). They found that "toxicity is not dependent upon particle size and surface area". In a subsequent study with different nanoscale TiO_2 test materials, they determined that crystal structure and particle surface reactivity (using the Vitamin C yellowing assay) played important mechanistic roles in facilitating adverse pulmonary effects (Warheit et al, 2007a). Determining the lung toxicity of fine- and nano-quartz in a later study they confirmed previous results (Warheit et al, 2007b). Instead of a correlation between the potency range of toxicity and particle size or surface area, they found consistency between toxicity and the results of in vitro erythrocyte haemolysis studies (as a measure of surface reactivity using a different assay). Silanol groups on the silica surface can react with carbonyl oxygen on the erythrocyte membrane. This interaction may produce ROS in amounts that exhaust the protective mechanisms of the erythrocyte and in the end lead to cell membrane rupture (Razzaboni and Bolsaitis 1990). Thus, Warheit et al, supplemented their first conclusion and claimed that "toxicity is not dependent upon particle size but on surface characteristics. However, based on a comparable surface chemistry per unit of area, the total

amount of charge or reactive groups increased with decreasing particle size (or increasing surface area) but equal particulate mass.

Considering both particle size/surface area dose as well as surface characteristics, Duffin et al (2007) tested the pulmonary toxicity of different particles indicated by neutrophil content in BALF of rats after instillation. Low-toxicity, low-solubility materials (PSP, see also Section 1.3), metal nanoparticles (nickel and cobalt), and quartz as an example of a particle with a highly reactive surface were examined. PSPs revealed a greater inflammatory response than the smaller the particles (i.e. the nominally larger the surface area dose). Compared to these materials, quartz, at the same surface area dose, induced a far greater level of inflammation. The inflammatory response of the metals fell midway between the effect of the LTLS and the quartz particles Duffin et al stated that some materials exhibited a "double hazard", a large surface area per unit mass as well as a specific surface activity and that "for ranking inflammogenic potency of poorly soluble respirable particles" both properties have to be considered.

2.3 Dosimetry

The dosimetry of inhaled particulate matter is determined by:

- transport of material into the respiratory tract and transfer to the surfaces/lining fluids of the different compartments;
- redistribution of the deposited matter in the lung and removal processes from the lung.

The first group of transport processes links the airborne particle concentration to the internal dose rate (D_R) defined as the amount of material deposited in the lung per time. Influencing parameters are the distribution of mass concentration among the different particle size intervals covering the exposure aerosol, as well as the respiratory minute volume (RMV) and the (pulmonary) deposition fraction, $f_{d,i}$ of the species under consideration. The particle size is measured as aerodynamic or mobility diameter, respectively; i, is the index of the size class ranging from 1 to N , which is the total number of size classes used to characterise the size distribution. The deposition fraction quantifies the fraction of the inhaled mass of the particles in the i-th size range that is deposited in the lung.

In most of the reported inhalation studies the total dose rate is not directly measured but is calculated by adding the contributions of each of the N size classes

$$D_R = RMV \cdot (c_1 \cdot f_{d,1} + c_2 \cdot d_{f,2} + ...+ c_N \cdot f_{d,N}) \quad \text{(Eq 1)}$$

The units of the dose rate are given in µg/h, resulting from l/h for the respiratory minute volume and µg/l for the concentrations. The deposition fraction is dimensionless.

Comparison of studies carried out with different animal species and calculation of human equivalent concentrations require quantitative information on the respiratory minute volume and the deposition fraction. RMV-values are usually obtained from allometric relationships to the body weight, if not measured directly.

The particle size dependence of the regional deposition fraction is primarily controlled by physical mechanisms such as sedimentation, inertial impaction and diffusion; the first two being relevant for particles larger than 0.5 µm, the latter governing lung deposition of nanoparticles (< 0.1 µm). The anatomy of the respiratory system and the respiratory parameters together with the particle size determine in which part of the respiratory tract and to which percentage of the inhaled particles are deposited.

The deposition fraction for experimental animals is taken either directly from experimental data (Raabe et al, 1988) or they are calculated using computer models. Almost all recent inhalation studies rely on the so called multiple path particle dosimetry model (MPPD-model (CIIT Centers for Health Research, 2004) that calculates the retention of inhaled insoluble particles in rats and humans. Dose extrapolations from rats to humans are based on the deposition fractions in the pulmonary compartment calculated by the MPPD-model. New experimental data on the deposition fraction in mice and rats are generated by Kuehl et al (2012) using radio-labelled aerosols of 0.5, 1.0, 3.0 and 5.0 µm MMAD and nuclear imaging by SPECT/CIT. The MPPD modelling results for rats agree quite well with the data obtained for the micron sized aerosols but significantly underpredict the pulmonary deposition fraction of the 0.5µm aerosol: 3 % modelled versus 12.5 % measured. This could be of importance for the interpretation and extrapolation of inhalation studies carried out with aerosols having a substantial mass fraction in the submicron range.

In humans, regional particle deposition is mainly assessed by using the HRT-model (Human Respiratory Tract Model; ICRP, 1994) which is a semi-empirical model based on a large amount of experimental data carried out with humans under well controlled conditions. The deterministic MPPD model is also used for the calculation of the deposition fraction in humans. These models estimate a deposition fraction averaged over the entire lung compartment under consideration. In the past decade a large number of papers appeared where computational fluid dynamics and realistic data on airway structure were used to investigate regional particle deposition. The results brought up the issue of so called hot spots in the deposition pattern. This means that particularly in the tracheo-bronchial airways the average deposition fraction does not adequately describe local doses on the epithelial cells. It was demonstrated that the particle deposition is inhomogeneous over the surface leading to enhancements of local doses by factors up to several hundred (Phalen et al, 2010).

The second group of material transport processes involves mechanisms such as dissolution, translocation into epithelial cells and interstitial tissue, migration of particles caused by mucociliar motion, and migration after incorporation in AMs. These mechanisms determine the fate of particulate matter after deposition, particularly particle retention in the lung, the key quantity with regard to lung overload. One of the early mechanistic approaches brought up by Morrow (1988) to explain lung overload is to assume an impairment of macrophage related clearance as the particulate volumetric lung burden exceeds a certain minimum value. The critical displacement volume of particulates is 6 % of the available volume of the population of macrophage on the alveolar surface. The concentration that leads to this steady state lung burden in rats for chronic exposure can be calculated from physiological data such as for example the respiratory minute volume, the deposited aerosol fraction as calculated by the MPPD-model using the size distribution properties of the challenge aerosol, and the first order clearance constant. The NOAEL concentration calculated from the overload criteria compare reasonably well with values found in inhalation studies with different powders (Pauluhn, 2011). Human equivalent concentrations are derived by assuming the same overload threshold value for steady state volume load per macrophage and adjusting for the differences in the relevant physiological parameters i.e. clearance half-time, deposition fraction, total macrophage volume

etc.. From this, the human equivalent volume concentration of respirable particles is calculated to be 0.5 µl/m³ which can be translated into mass concentration by multiplication with the particle density (measured in g/cm3). However, this result depends considerably on the values used for translation from rats to humans, e.g. the half-time of alveolar clearance in humans was set to 400 days by Pauluhn (2011) but, the most recent and probably best estimate available was derived by Gregoratto et al (2010). They estimated the alveolar half-time in humans to be 250 days. Using this estimate, the volume concentration changes to 400/250 × 0.5 ul=0.8 µl/m³

The above derivation is based on volumetric lung burden. However, the issue of the metric used for dose quantification i.e. the physical quantity to which the biological effect is related, is still under discussion. For isometric particles the mass has been the most convenient descriptor. Historically, biological effects of isometric particle have been related to the mass dose, and concentration standards are given in terms of suspended mass per volume of air. Mass can be easily determined by gravimetric and/or chemical analysis. Besides the mass, the surface area has been suggested as a parameter that sometimes correlates better with biological endpoints than mass or volume (Saager and Castranova, 2008, Rushton et al (2010)).

2.4 Conclusion

Several dose metrics have been tried to describe the relationship between particle dose and lung overload associated-effects. Particle mass is the most widely used dose metric for inhalation studies because of its practical convenience, but it has been shown to be less suitable to describe the relationship with lung overload effects. Dose metrics which have been proposed as being suitable are primarily the particle volume and the particle surface. Albeit that multiple studies have been published supporting the adequacy of these dose metrics, no one universal dose metric has been identified thus far, and it is unlikely that any metric will be singled out because the best metric might depend heavily on the investigated substance and the type of effect investigated.

Other particle characteristics have been shown to play a significant role in the way the respiratory system responds to particle exposure. The particle density is directly linked to the volumetric overload concept, and the way it is determined is a critical parameter. The particle size is directly related to the surface-based dose metric. The particle shape has been demonstrated to have a significant impact on the particle's aerodynamic behaviour and on how lung cells interact with particles. Also differences in surface reactivity of chemically similar particles can result in different biological effects.

The successful inclusion of studies on nanomaterials in both the models relying on the volume-based dose metric and the surface-based dose metric have demonstrated the relevance and applicability of the concept of lung overload also for nano-particles.

The parameters determining the dosimetry of inhaled particles both in laboratory animals and humans are well understood, and models allowing the estimation of the deposited dose have been developed and validated. When using these models, it is of critical importance that the methodologies used to obtain the input parameters are well developed and understood.

3. BIOSOLUBILITY

Solubility of inhaled particles has great implication on its mode of action and subsequent risk assessment thereof. The term "poorly soluble particles", is often used without being specified. Further to the definition given in Section 1.3, we aim to give some guidance for the applicability of this term in this chapter.

3.1 Definition for (bio)solubility

According to an IUPAC definition (IUPAC 1997) solubility is the analytical composition of a saturated solution expressed as a proportion of a designated solute in a designated solvent. In text books of chemistry, a substance is said to be soluble if more than 0.1 g of that substance dissolves in 100 ml solvent. If less than 0.1 g dissolves in 100 ml solvent, the substance is said to be insoluble or, more exactly, sparingly soluble (Fundamentals of Chemistry; http://www.chem.wisc.edu/deptfiles/genchem/sstutorial/FunChem.htm). For the definition of "poorly soluble particles" in the framework of `lung overload` see also Section 1.3.

Inhaled particles are processed by biological systems upon deposition, and are exposed to complex mixtures of biogenic solvents. Thus, biosolubility means solubility in a biological system e.g. cell system, biological fluid (or its simulants), in an animal. Biosolubility may differ significantly from the solubility in water and varies depending on biological systems.

3.2 Impact of biosolubility on clearance of deposited particular matter

Several defence mechanisms, located in all three main regions of the respiratory tract (nasopharyngeal, tracheobronchial and alveolar) exist to prevent and clear the mucosal surfaces from contaminant deposition. Classical clearance mechanisms of deposited particles are following two basic pathways, physical translocation of particles and chemical dissolution processes. When inhaled particles are deposited, soluble particles (e.g. NaCl salt) will dissolve in mucus or lung-lining fluid, get access into blood stream, distributed in the body and finally excreted in urine and faeces depending on its physical chemical properties (e.g. molecular weight). In the airway, deposited particles which do not readily dissolve, will be cleared rapidly by mucociliary transport moving particles from the nasal passage and tracheobronchiolar region to the oropharynx, where they are swallowed and are excreted in faeces and, if systemically available, in the urine. Excretion of systemically available substance via bile into faeces may be another possible route, which however cannot be differentiated from those swallowed. In the respiratory unciliated airways and alveoli, particles will get phagocytised within hours by alveolar macrophages (AM) under normal conditions. AM-derived transport is slower than the mucociliary escalator. Phagocytised particles can either move toward the ciliated airways or to lymphatic vessels and the pulmonary lymph nodes. Many studies have shown that excessive dust burdens progressively reduce AM-mediated clearance until it is completely inhibited, a phenomenon known and described earlier in this report as overload (Ferin 1972; Ferin and Feldstein 1978; Lee et al, 1985). Reduced clearance rate are supposed to be related to the loss of macrophage mobility (Morrow 1988). Thus, the potential for particles to dissolve can effectively influence their persistence in the

non-ciliated airway and alveoli; and act as a critical control on their biological response. If the solubility and dissolution rate are sufficiently high, a high lung burden leading to overload cannot be built up. In this case, the toxicity will be triggered by dissolved chemical, an "overload"-mediated toxicity as proposed by Morrow (1988) is not applicable for this type of material.

In the sense of being able to build up particle overload, poorly soluble particles are inhaled particles that are removed mainly by AM-mediated clearance, not by dissolution. Whether biosolubility contributes significantly to lung clearance depends strongly on exposure concentration and duration.

3.3 Clearance classes

The International Commission on Radiological Protection (ICRP) recognised the impact of particle solubility on their toxicological profile. ICRP considered solubility and dissolution rate in its human respiratory tract dosimetry model (HRTM). Assuming that AM-derived clearance of particles in the deep lung was constant for a given species, the first HRTM (ICRP 1979) proposed three default clearance classes with different biosolubility (class D for $t_{1/2}$ < 10 days; class W for $t_{1/2}$ between 10 and 100 days; class Y $t_{1/2}$ > 100 days, $t_{1/2}$ was referred to pulmonary clearance in human). In the second model (ICRP 1994) absorption classes were introduced (class F; M and S). The classification is linked to default constants for calculating the dose and clearance of a respective radioactive particles. As ICRP recommended the clearance classes to assess internal dose of inhaled radiation, it is reasonable to employ the existing solubility classes in course of assessing the toxicological potential of particular matter. In the following table, clearance classes in line with recommendations of ICRP are proposed. A few examples are listed, and described in details as case studies.

Table 1: Clearance classes for particular matter (ICRP, 1979)

Clearance class	Pulmonary clearance in humans ($t_{1/2}$, days)	Proposed solubility class	Example
D (days)	< 10	Soluble	ZnO, CuO
W (weeks)	10 - 100	Partly soluble	SiO_2
Y (years)	> 100	Poorly soluble	Co_3O_4, TiO_2, CeO_2

3.3.1 Case study on zinc oxide (ZnO)

The solubility of ZnO varies strongly in different media. nano-ZnO as well as micro-ZnO dissolves in water (pH about 7) up to 13 µg/ml. Up to 4.5 mg/ml ZnO dissolved in artificial lysosomal fluid (pH 4.5) during 24 hours at room temperature, while little was dissolved in artificial interstitial fluid (Gamble´s solution, pH 7.4) under the same experimental condition (Cho et al, 2012). Cho et al (2012) also examined ZnO particle and Zn^{2+} ion induced pulmonary toxicity after intratracheal instillation. The authors found qualitatively comparable effects after instillation of ZnO particles and dissolved Zn^{2+} ion. In vitro, lysosomal instability and cell death was increased in macrophage culture after treatment with nano ZnO. In a rat instillation study, Hirano et al (1989) reported a clearance half-time of 14 hours after intratracheal instillation of ZnO to rat (100 µg Zn/rat)

and examination of Zn content in lung after 8 h, 1, 2, 3, 5, 7, 14 and 21 days. The instilled Zn was recovery solely in the supernatant of the bronchoalveolar lavage fluid.

These data reported by two research groups demonstrate biological solubility of ZnO. Based on the short half-time, ZnO is considered to be biologically soluble.

3.3.2 Case study on amorphous silica

Roelofs and Vogelsberger (2004) determined solubility of pyrogenic, precipitated and gel forms of synthetic amorphous silica (SAS), including surface-treated forms. At 37°C and pH values near 7 the solubility for different forms of SAS was between 138 to 162 mg/l in simulated biological medium, close to the solubility in water. Roelofs and Vogelsberger (2004) also confirmed that silica has a tendency to supersaturate, i.e., the dissolution rate is more rapid than the precipitation rate. Hence, the different forms of SAS dissolve both in water and in simulated biological systems beyond the equilibrium concentration. Total dissolution can be expected in biological systems where dissolved SAS is quickly removed, such as in the lungs.

In a rat 4-week inhalation study, Warheit et al (1991) tested the pulmonary toxicity of colloidal silica Ludox. Treatment-related effects were observed at 50 and 150 mg/m³, which were reversible within 3-month recovery period (Warheit et al, 1991, Lee and Kelly 1992). The exposure led to inhaled doses of 489 µg/lung (10 mg/m³ group), 2418 µg/lung (50 mg/m³), and 7378 µg/lung (150 mg/m³), respectively. Although high lung burden were achieved at 50 and 150 mg/m³, half-times of 40 to 50 days were determined in rats (Lee and Kelly, 1992). The half-time at such high lung burden was shorter than physiological AM-derived clearance half-time of approximately 60 days. Therefore, contribution of dissolution is suggested. This view is strongly supported by the solubility of 138 to 162 mg/l in simulated biological medium. Based on the half-time of 40 to 50 days, amorphous SiO_2 is partly soluble.

3.3.3 Case study on cobalt oxide (Co_3O_4)

In a human study, Forster et al (Foster et al, 1989) examined clearance of radiolabeled $^{57}Co_3O_4$ by external counting of lung burden and urine and faecal collections over 230 days. The fast clearance during the first few days was interpreted as tracheobronchial clearance which was followed by a slow clearance phase. The investigators reported that the measured pulmonary retention half-times in their subjects ranged between 150 and 250 days. Taking into account the urinary and faecal excretion, which they associated with dissolution and AM-mediated (mechanical) clearance, respectively, they concluded that about ¾ of the pulmonary clearance was due to dissolution. Morrow (Morrow 1992) calculated 231 day as the half-time for dissolution and 640 day half-time for AM-mediated clearance. According to the criteria mentioned above, Co_3O_4 is a poorly soluble particle.

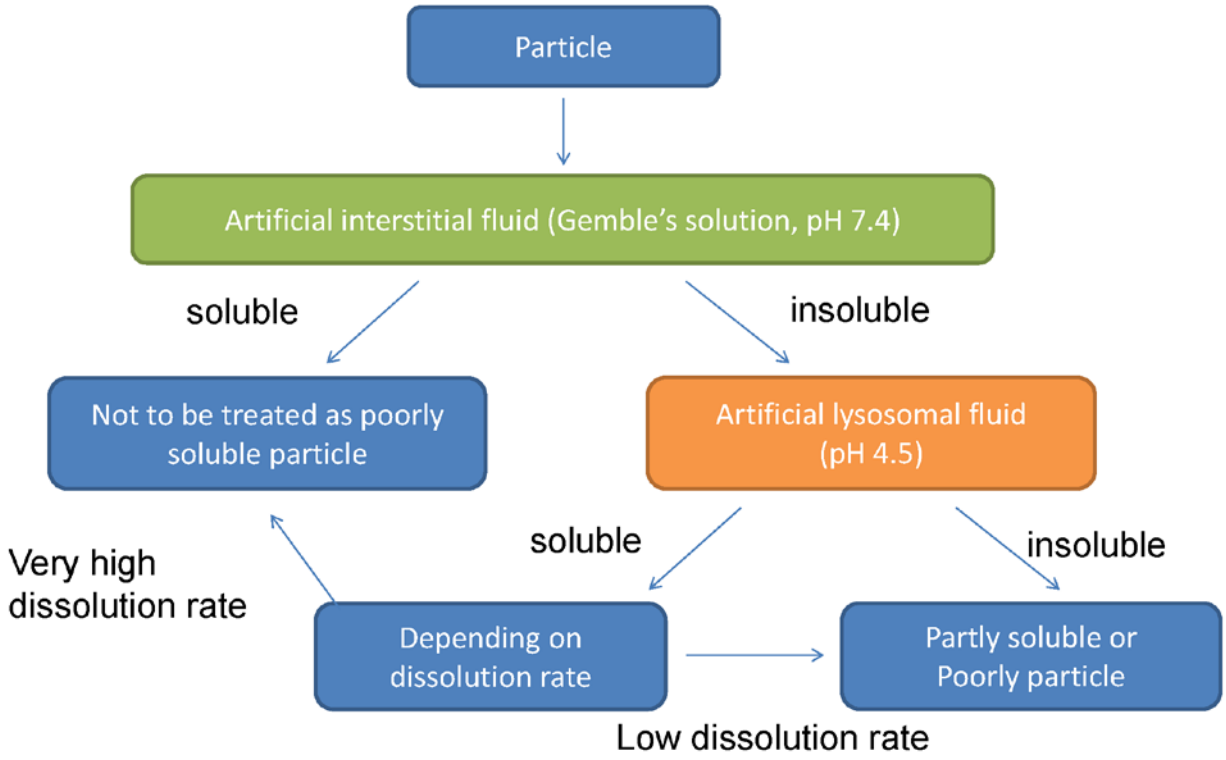
3.4 Guidance on assess bio-solubility

The classes given by ICRP were based on the pulmonary clearance half-time, which is often not available. Mercer (1967) proposed a mathematical model to predict half-time of substances retained in the respiratory tract. This model is not widely applied because the required constants can only be determined from in vivo measurements. In vitro approaches cannot provide the in vivo clearance half-time, but can give an indication for the potential solubility classes. Therefore, abiotic assays have been used to assess potential bio-solubility in vivo.

The abiotic solubility assays consider that constituents of solvent have strong impact on solubility. Early simulant dissolution studies focused on the fate of aerosol particles deposited in lung extracellular fluid. The most common simulants used were based on measurements of the ionic composition of lung extracellular fluid by Gamble (Ansoborlo et al, 1999). However, particles deposited in the lower respiratory tract are most likely to be phagocytised and will be finally enclosed in secondary lysosomes or phagolysosomes. Phagolysosomes contain proteolytic enzymes, oxygen radicals, chelators, precipitators. The pH values in phagolysosmes range between 4.35 and 4.95 for baboons, dogs, guinea pigs and rabbits (Kreyling et al, 1991). Lundborg (Lundborg and Camner 1984) determined the dissolution rate of MnO_2 in vitro with and without AM. They found a higher dissolution rate in the presence of AM than without. Kreyling et al (Kreyling et al, 1986; Kreyling et al, 1990) examined the parameters that influence intracellular particle dissolution using mainly Co_3O_4 as model substance. They considered that intracellular particle dissolution was controlled by phagolysolomal pH and chelate agents within the phagolysosomes. Based on this information, Stefaniak (Stefaniak et al, 2005) refined and characterised a potassium hydrogen phthalate (KHP) buffered solution with pH 4.55, termed phagolysosomal simulant fluid (PSF), for use in a static dissolution technique.

Based on these published data, we suggest determining the solubility of particles in Gamble´s solutions and/or PSF. The following decision tree (Figure 3) illustrates how to assess the biosolubility of a test substance based on the results of the abiotic solubility assays.

Figure 3: Decision tree to assess biosolubility of particles



However, abiotic solubility assays may underestimate in vivo biosolubility for the following reasons:

1) Solubility is defined as the concentration in the liquid at equilibrium, when dissolution rate equals precipitation rate. In vivo within the respiratory tract, renewal of the lining fluid is continuous, and therefore particle dissolution does not reach equilibrium.
2) Dissolution is a dynamic process in which constituent molecules of the dissolving solid migrate from the surface to the bulk solution through a dissolution layer (Borm et al, 2006). Dissolution rate increases, when particles become smaller during the course of dissolution.

Thus, when using abiotic solubility data to assess in vivo solubility, these uncertainties have to be considered.

3.5 Conclusion

Besides physical translocation of deposited particles, chemical dissolution is one of the basic clearance principles of the respiratory tract. In this respect (bio)solubility is of importance, because chemical dissolution is especially targeted at biosoluble particles which are dissolved in intracellular or extracellular fluids and may then be cleared into blood or lymphatic circulation. Thus, chemical clearance of biosoluble particles can happen within all three major regions of the respiratory tract, i.e. the nasopharyngeal,

tracheobronchial and/or alveolar region. In contrast, pathways for physical translocation of inhaled particles of low (bio)solubility are not only limited and arranged differently in these three regions, but some of them show also significant particle-size dependent differences. As the most prevailing clearance mechanism for solid particles in the alveolar region is mediated by alveolar macrophages, differences in the biosolubility of particles can considered to be a major determinant with regard to the establishment of `particle lung overload' conditions.

Summarising all available facts, biosolubility may differ significantly from solubility determined in water. It is influenced by different physical-chemical properties of the materials and is also influenced by the biological system. In vivo, biosolubility may therefore contribute significantly to the clearance rate of particular matter deposited in the deep lung and influence directly the retention rate of particular matter, thus becoming an important driver at which doses lung overload develops.

4. PATHOBIOLOGY OF 'LUNG OVERLOAD'

4.1 Relevance of alveolar macrophages

Alveolar macrophages (AM) represent primary phagocytes of the innate immune system and are of fundamental importance in mediating the removal of inhaled particles from the lung. Phagocytic and cytotoxic activity of AMs along with mucociliary transport provides an effective, nonspecific pulmonary defence. Consequently a rapid increase of phagocytic active cells like polymorphonuclear leucocytes (PMNs), neutrophils and AMs is observed in the lung following particulate exposure. In fact, AM accumulation can be considered an early indicator of pulmonary particle deposition. Several studies have shown that AMs can be differentiated based on their anatomical location and biological activities. They consist of several functionally distinct subpopulations which differ from each other by their phagocytic activity as well as their biochemical, cytotoxic/cytostatic cellular functions, and in their ability to synthesise and release soluble mediators. Species differences in composition, localisation and function between the different AM subsets may therefore account for observed differences in responses to inhaled particulates. Depending on their localisation, intravascular, interstitial, pleural and surface associated AMs can be distinguished. Because surface macrophages participate primarily in innate immune responses like phagocytosis, differences in this subpopulation may be responsible for differences in clearance rates of particulates. Surface macrophages can easily be obtained by bronchoalveolar lavage (BAL). Rodent BAL cells consist of ≥95% macrophages, few lymphocytes and neutrophils whereas human BAL cells normally consist of ≥80% macrophages, about 18% lymphocytes and few neutrophils. This is in line with findings that the shortest macrophage clearance times are reported for rodents where deposited micrometre-sized particles remain on the epithelial surface of the lung (Lehnert et al, 1989; Ellender et al, 1992). Longer clearance times are found in humans, monkeys, dogs and guinea pigs (Snipes 1989; Kreyling 1990).

Based on reported retention kinetics of poorly soluble particles, generic elimination half-times of 60 days for rats and approximately 1 year for humans can be assumed (Oberdörster 1995). According to Pauluhn (2011), the roughly 7-fold greater AM pool of humans relative to rats results in a proportionally longer clearance of approximately 420 days making the anticipated clearance half-time in humans plausible. Whereas the alveolar clearance rate in humans seems to be independent on the particle load, the clearance rate in rats depends on the amount of particles in the alveolar region which may contribute to a more pronounced impairment of macrophage mediated alveolar clearance and thus the higher susceptibility of rats to lung overload (Brown et al, 2005).

Marked species differences exist also in the cell size of alveolar macrophages with AMs from humans being significantly larger than those from rats, hamster or monkeys. Based hereupon, number and size range of phagocytised particles may also differ among species (Krombach et al, 1997).

Beside phagocytosis, macrophages are also one of the most active secretory cell types releasing a multitude of mediators. In this respect they are involved in the regulation of nearly all aspects of host defence, inflammation, wound healing and homeostasis (Murray and Wynn 2011, Laskin et al, 2011). This diverse biological activity of macrophages is mediated by two phenotypically distinct subpopulations of macrophages, classically activated "M1 macrophages" and "alternatively activated "M2 macrophages". Classically activated macrophages (M1) are produced during cell-mediated immune responses and exhibit

microbicidal and tumouricidal activity. By releasing reactive oxygen/nitrogen species (ROS/RNS) and pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12, IL-23, TNF-α), they also exert strong anti-proliferative and cytotoxic activities. Although M1 macrophages are vital components of host defence, their activation must be tightly controlled because the produced cytokines and mediators can promote host-tissue damage. In contrast to pro-inflammatory "M1" macrophages, alternatively activated "M2" macrophages exhibit strong anti-inflammatory activity. Consequently, M2 macrophages play an important role in wound healing. This activity is mainly related to the release of cytokines, bioactive lipids and growth factors like TGF-ß. However, similar to classically activated pro-inflammatory M1 macrophages, anti-inflammatory M2 macrophages can also be detrimental to the host when dysregulated. Excessive release of mediators and growth factors then can lead to pathologic fibrogenic responses (Mosser and Edwards, 2008).

Based on all the available experimental data it can be concluded that AMs respond to exposure against toxicants normally through a carefully balanced system consisting of "M1"macrophages which release pro-inflammatory and cytotoxic mediators important in host-defence and "M2" macrophages which are involved in down-regulating inflammatory processes and the initiation of wound-repair.

However, prolonged exposure to high levels of particles may provoke an imbalance resulting in hyper-responsive AMs followed by the dysregulated release of mediators that promote acute tissue injury and the progression of chronic diseases like fibrosis and eventually cancer; in the case of the rat. Differences in the quantitative activation of M1 or M2 macrophages as well as qualitative functional changes of AM may thereby account for the observed species differences in pulmonary responses following particle induced lung overload. Following such exposures, AMs with an inflammatory phenotype ("M1" macrophages) release pro-inflammatory cytokines as well as reactive oxygen (ROS) and reactive nitrogen (RNS) species that can be severely damaging to surrounding cells and tissue and may initiate abnormal inflammatory reactions.

ROS and RNS, e.g. superoxide anion, hydrogenperoxide, hydroxyl radical, nitric oxide, peroxynitrite, are generated via enzyme-catalysed reactions. Produced in large quantities by classically activated macrophages during inflammatory pulmonary responses, the resulting oxidative and nitrosative stress can lead to severe tissue injury. There are ongoing discussions that species differences in the oxidative capacity of AMs may be important for the different observed pulmonary responses to particulates. Data from Dörger et al (1997a) suggest that such species differences exist. The oxidative capacity was about 5-fold higher in rat AM than in hamster AM. Whereas the oxidative capacity of hamster AM appeared to be based mainly on the formation of ROS, the authors suggested that rat AM possess an additional oxidative system. In this respect an observation made by Schneemann et al (1993) may be of interest that in contrast to rodent AMs, human mononuclear phagocytes lack nitric oxide synthetase. Additionally, Dörger et al (1997b) and Jesch et al (1997) also reported that nitric oxide formation could only be observed by rat AMs, but not in AMs from hamsters, monkeys or humans. The authors concluded that distinct regulatory mechanisms of the nitric oxide pathway in alveolar macrophages from these four different species seem to exist. Taking into account that nitric oxide rapidly reacts with superoxide anion to form the relatively long-lived strong cytotoxic oxidant peroxynitrite, the higher sensitivity of rats towards both, inflammation driven non-neoplastic and unique neoplastic responses may be plausible. When protonated, peroxynitrite will decompose into NO_2, as well as nitrate. These species may then interact with each other, as well as with O_2 or ROS to form higher oxides of nitrogen which may oxidise thiols and a variety of amino acids including methionine and cysteins. It therefore can be concluded that species differences in the formation of RNS, as revealed between

rats compared to other species, may contribute to the observed differences in pulmonary tissue injury following chronic exposure to high concentrations of poorly soluble particles.

In contrast to M1 macrophages, alternatively activated M2 macrophages exhibit strong anti-inflammatory activity; play an important role in wound healing and when hyper-responsive also in the development of fibrosis (Wynn & Barron 2010). This activity is mainly related to excessive release of cytokines, bioactive lipids and growth factors like TGF-ß, a known mediator of fibrosis by stimulating the production of extracellular matrix proteins (Pulichino et al, 2008). Species differences in the pulmonary micro-environmental conditions, responsible for the activation of "M1" and "M2" macrophages and/or differences in generating mediators as well as ROS/RNS, may therefore play an important role in some of the observed species differences in lung injury following chronic exposure to PSPs of low toxicity.

4.2 Relevance of inflammation

As evidenced by numerous experimental studies, pulmonary inflammation has to be regarded as a key driver in the cascade of pathogenic events following chronic inhalation of PSP including the neoplastic lung effects observed in rats. However, it remains unclear why only rats respond with the development of neoplasms of the lung but other animal species chronically exposed to PSPs do not. In fact, lung tumours were never found in rats when pulmonary inflammation was absent (Levy 1995, Oberdörster 1997; ILSI 2000; Greim et al, 2001; Kolling et al, 2011). In addition, a direct relationship between chronic inflammation and carcinogenesis in exposed rats has been established (Oberdörster, 1997; Kolling et al, 2011). Using a qualitative scoring system for specific non-neoplastic endpoints of lung toxicity such as inflammation, epithelial hyperplasia and fibrosis, a correlation between tumour frequencies and increased scores could be established (Kolling et al, 2011). Based hereupon it is hypothesised that the mechanism of tumour development presumably involves the severe, persistent inflammatory reaction which induces marked cell proliferation which has been demonstrated for several so-called inert particles (Driscoll, 1996). Increased epithelial cell proliferation may influence the number and survival of transformed cells, thus increasing the chances for lung tumour development (Oberdörster, 1995). According to Driscoll et al (1995, 1996) such a persistent inflammatory reaction can lead to an elevated influx of reactive oxygen species releasing activated neutrophils into the lung, which in turn can induce mutagenic effects in epithelial lung cells. It is speculated that the greater sensitivity of the rat lung with regard to oxidative stress and subsequent epithelial cell responses is due to a more pro-inflammatory environment compared to other species. This hypothesis, that lung tumours in rats following chronic exposure to PSP are induced by such an indirect mechanism, is supported by results of various other experiments. Inflammatory cells and activated alveolar macrophages, which are found in large numbers in animals exposed to PSP, can release ROS and other mediators of inflammation which in turn can then cause DNA damage by a secondary mechanism (Driscoll et al, 1997; Jackson et al, 1989; Weitzman and Gordon 1990; Oberdörster 1997). The following mechanistic findings were experimentally established:

1. Activated inflammatory cells (macrophages, granulocytes) from the rat lung cause DNA damage at the HPRT locus in vitro (Driscoll 1996)
2. The amount of DNA damage is directly dependent on the level of activation and on the capacity for generating ROS (Driscoll et al, 1997)

3. Exposure to concentrations which do not induce inflammatory reactions in the rat lung causes neither DNA damage nor lung tumour development (Driscoll, 1996; Dricoll et al, 1997)

Based on the outcome of a workshop on the toxicity of fibres and particles, it was concluded that the tumourigenesis of PSP involves a mechanism of secondary genotoxicity at doses that induce inflammation (Greim et al, 2001). Schins and Knaapen (2007) defined this secondary genotoxic effect as a pathway of genetic damage resulting from the oxidative DNA attack by reactive oxygen/nitrogen species, generated during particle-elicited inflammation. Measurable biological endpoints had been mutations in the HPRT gene of isolated alveolar cells in ex vivo assays (Drisoll et al, 1997), changes in markers of cellular injury and/or inflammation in bronchoalveolar lavage fluid (BALF), expression of mRNA for chemokines and detection of oxidative DNA damage (Johnston et al, 2000).

The hypothesis of secondary genotoxicity is based on findings that various PSP are carcinogenic in rat lungs, irrespective of their chemical composition, but obviously only after prolonged high exposures that are associated with persistent inflammation and lung overload (Miller, 2000; Greim et al, 2001; Borm et al, 2004). Importantly it has to be stressed that these substances do not present a carcinogenic hazard in rats at doses/concentrations not producing concurrently severe lung inflammation reactions. Based hereupon it can be concluded that the observed lung tumours in rats are not due to a substance specific toxicity but the result of generic particle toxicity in a highly sensitive species. Exposure to concentrations of inert particles which do not induce inflammatory reactions in the rat lung causes neither DNA damage nor lung tumour development (Driscoll et al, 1996) indicating that this mechanism has a threshold.

In the lung, neutrophilic granulocytes are considered to be a source of ROS/RNS. A significant increase in the level of DNA damage in the epithelial cells of the rat lungs exposed to non-genotoxic particles was found only if the number of neutrophils had increased to 40-50% of the total cell population (Driscoll et al, 1997). Other studies performed to detect any increases in the rate of proliferation of pulmonary epithelial cells after inhalative exposure of rats to an inert particle revealed an early onset of increased cell proliferation of pulmonary epithelial cells, assayed by means of bromodeoxyuridine (BrdU) indices distinguishing between cells labelled in the S-phase (BrdU positive) and unlabelled cells (BrdU negative). In all cases, exposed animals exhibited an epithelial cell labelling index significantly above control indicating increased proliferation of pulmonary epithelial cells (IPA 1999 cited by MAK 1999).

In conclusion, particle induced pulmonary inflammation is a key driver in the hypothesised cascade of PSP induced events leading to non-neoplastic as well as neoplastic changes in the rat lung. Thus, they are not driven by a substance specific toxicity but due to generic particle responses for which thresholds can be established. In a dose-dependent manner, the level of pro-inflammatory cellular responses (e.g. release or ROS/RNS) is increased and antioxidant defences are weakened. This in turn increases oxidative stress and the susceptibility of airway epithelium towards both, genetic damage in alveolar cells as well as advancing cell proliferation and tissue remodelling. One major conclusion to be drawn from the synopsis of existing data is that the tumourigenesis of PSP in rats apparently involves mechanisms of secondary genotoxicity only at doses that induce also inflammatory pulmonary responses in vivo. This is of significant importance for hazard and risk assessments, because such secondary mechanisms involve thresholds to be considered for the derivation of DNELs in risk characterisation as well as for risk management decisions.

4.3 Relevance of cell proliferation

Repeated inhalation exposure to particulates, whether inherently acutely toxic or not, represent one of the key drivers for the development of toxicological relevant proliferative lesions in the respiratory tract. Cellular damage resulting from repeated exposure to toxicants, induces patho-physiological repair processes during which damaged tissue may proliferate (hyperplasia) and/or undergo metaplasia to a different, more resistant cell type (e.g. fibrosis) if return to normal cell morphology is not complete. The site of these changes is heavily dependent upon the nature of the toxicant and the type of tissue exposed. In case of particulate matter, physical-chemical properties such as particle size (Section 2) and (bio)solubility (Section 3) are also of importance. Ciliated columnar and olfactory epithelia are the most fragile respiratory epithelia and thus the most susceptible to damage from inhaled toxicants. Cuboidal epithelium is more resistant, and squamous epithelium is the most resistant to damage from direct contact with toxic or irritant materials (Renne et al, 2009).

Increased epithelial cell proliferation and lung tumour formation following long-term exposure of rats to different PSPs have been reported (Miller and Hook 1990, Muhle et al 1990, Heinrich et al 1995, Nikula et al 1997, Mauderly 1997).

Repeated inhalation exposure of rodents to cytotoxic or irritant materials, may result in degeneration and necrosis of epithelial surfaces, inflammation, interstitial fibrosis, metaplasia and hyperplasia of damaged bronchiolar and alveolar epithelium. Whereas bronchiolisation occurs rarely spontaneously in rats, it is frequently observed in centriacinar regions of the lungs of rats following chronic inflammation due to repeated exposures (Brix et al, 2004). However, three types of alveolar-bronchiolar hyperplasia (an alveolar type, a bronchiolar type and a mixed type) as potential preneoplastic lesions have been observed in the rat lung following repeated exposures to relatively inert particulates (Mohr et al, 1990; Muhle et al, 1995). In rats, continued severe epithelial hyperplasia and metaplasia may then progress to malignant epithelial neoplasia. However, in contrast to rats, bronchiolisation was not observed in mice or hamsters exposed to comparable concentrations of TiO_2 (Bermudez et al, 2002) or carbon black (Elder et al, 2005).

In conclusion, a synopsis of available data suggest that the ability of particles to induce persistent lung inflammation is key in the initiation of cell proliferation and tissue remodelling as necessary prerequisites for non-neoplastic lung lesions (e.g. fibrosis) as well as the fixation of secondary caused mutations in affected target cells and, in the rat, the progression of these cells also to neoplastic lesions. Hence it follows, that the induction of cell proliferation and all subsequent events resulting hereof are threshold related.

4.4 Relevance of oxidative stress

The development of rat lung tumours in response to chronic inhalation of PSP is a well-established phenomenon. However, most if not all of these particulate materials are not considered to be inherently genotoxic. This lack of association implies the involvement of a secondary mechanism leading to the genetic changes necessary for neoplastic transformation of cells. Evidence shows that excessive and persistent formation of ROS and RNS is a significant prerequisite in particle induced fibrosis and also in rat lung tumour formation. During particle exposure, ROS and RNS are mainly generated from the oxidative burst of

pulmonary inflammatory cells, i.e. neutrophils and AMs. The importance of inflammatory-mediated ROS/RNS for the development of secondary driven genotoxicity and subsequent mutagenesis was initially provided by Driscoll and coworkers (1995, 1997).

A comprehensive overview of potential mode of actions of particle-induced genotoxicity is discussed in Knaapen et al (2004). In this respect, it has been hypothesised that particle induced persistent inflammation in the rat lung and heretofore related release of ROS can provoke indirectly genetic damage in epithelial cells (Weitzman and Stossel, 1982; Driscoll 1995). During concurrently enhanced epithelial cell proliferation such mutations may then be fixed in dividing cells and clonally expressed. Implicit in such an inflammatory/proliferative driven mechanism is the existence of a threshold for such responses, i.e. that particle exposures not eliciting persistent pulmonary inflammation should also not pose a lung tumorigenic hazard. Driscoll et al (1996) demonstrated for the first time a dose-dependent increase of mutation frequency in alveolar epithelial cells following exposure of rats for 13 weeks against 1.1, 7.1 and 52.8 mg/m^3 carbon black particulate including 3 and 8 months of recovery. Measured endpoints included mutations in the HRPT gene of alveolar epithelial cells, changes in bronchi alveolar lavage fluid markers, expression of mRNA for chemokines as well as histopathology. Whereas subchronic inhalation of 1.1 mg/m^3 carbon black resulted in no detectable lung effects; especially no inflammatory responses, increased mutation frequencies in the presence of significant pulmonary inflammation and epithelial cell hyperplasia were observed immediately after the exposure period in the mid and high dose groups. Subsequent studies (Driscoll et al, 1997) revealed a relationship between the severity of pulmonary inflammation and ex vivo induced mutations by co-incubating lung lavage inflammatory cells (macrophages, neutrophils, lymphocytes) from carbon black exposed rats with rat lung epithelial cells. Increases of the HRPT mutation rate were observed when the percentage of neutrophils in the lavage fluid increase above 50%. Since this mutagenic response was inhibited in the presence of catalase as antioxidant, the authors concluded that oxidative stress in form of the generation and release of ROS and/or depletion of antioxidants may significantly contribute to this response. These findings support the discussed mechanism that inflammatory cell-derived reactive oxidants and increased cell proliferation play a key role in the pathogenesis of rat lung tumours in response to PSP and are consistent with the existence of a threshold (Oberdörster 1996, Greim et al, 2001). Since then, various additional studies investigating the mechanisms for mutagenicity induction following particulate exposures have demonstrated that reactive oxygen/nitrogen species generated during particle-induced pulmonary inflammation can oxidatively attack DNA resulting in structural alterations of the DNA and ultimately in fixed mutations (Weitzman and Gordon 1990, Risom et al, 2005, Singh et al, 2009).

Several studies in rats have supported the observation that persistent inflammation of the lung is driving particle induced genotoxicity in vivo Beside HPRT mutations in alveolar epithelial cells (Driscoll et al, 1997), also alternative genotoxicity markers like 8-hydroxy-2`-deoxyguanosine (8-OH-dG) (Gallagher et al, 2003, Seiler et al, 2004), DNA strand breaks (Knaapen et al, 2002) or micronuclei (Albrecht et al, 2004) 8-OHdG represents the best investigated oxidative and premutagenic DNA lesion, and consequently is also considered a marker of oxidative stress. Oxidative stress as generally defined by Sies (1991) is "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage". Inflammatory cells, like neutrophils, eosinophils and macrophages, possess a NADPH oxidase, which is induced during cell activation to produce superoxide anions. The influx and subsequent activation of such inflammatory cells into the lung therefore may lead to an oxidative burst which overwhelms the pulmonary antioxidative defences, resulting in oxidative DNA damage. Hence, genetic damage as well as proliferative effects to target cells (i.e. type II epithelial cells, Clara cells) can be induced through the production and release of oxidants.

This promutagenic environment provides favourable conditions for producing both, fibrotic cores and clones of mutated cells that eventually culminate in malignant lung disease if antioxidative defences, DNA-repair and selective apoptosis fail as protective mechanisms.

Based on the above findings, the tumourigenic effect of PSP in rats seems to be closely linked to the induction of persistent inflammation evoking genotoxic events and mutations as prerequisites of tumour formation. The mechanistic understanding by which such secondary genotoxic mechanisms occur following exposure to high concentrations of PSPs is well described in a NIOSH report on the health hazards of TiO_2 (NIOSH, 2011) as well as in appendix R7-1 to Chapter R7a of the ECHA guidance (ECHA 2012). In contrast to rats, no other animal species, including mice and hamsters have developed lung tumours following chronic exposure to PSP. Interestingly, following intra-tracheal instillation of comparable high doses of quartz to rats and hamsters, both species exhibited elevated levels of neutrophils with changes significantly higher in rats compared to hamsters. Rats also showed greater expression of several pro-inflammatory mediators and lower levels of anti-inflammatory mediators (Carter and Driscoll, 2001). Although genotoxic events measured as 8-oxoguanine were observed in both species, increased cell proliferation was only noted in rats but not in hamsters (Seiler et al, 2001). The authors concluded that the differences in pro- and anti-inflammatory mediators may not only contribute to the higher inflammatory reactions seen in rats, but do also indicate less potential for scavenging the mutagenic effects in rats. Moreover, the increased cell proliferation which was only detectable in rats but not in hamsters, may contribute to the observed species differences in sensitivity to tumorigenic effects of PSP.

Ziemann et al (2011) have investigated the in vivo induction of genotoxic effects of fine versus ultrafine particles in the lungs of rats following repeated intra-tracheal instillation of crystalline silica (DQ12, 1300 nm), amorphous silica (Aerosil® 150, 14 nm) and carbon black (Printex® 90, 14 nm) for 3 months. After immunohistochemical quantification, the results of the measured genotoxicity biomarkers poly-ADP-Ribose (PAR), 8-hydroxy-2`-deoxyguanosine (8-OH-dG), 8-oxoguanine DNA (OGG1) and gamma-H2AX were indicative of distinct genotoxic stress, the occurrence of DNA double strand breaks and oxidative DNA damage in lung epithelial cells. Comparisons of these results with existing instillation data from a 3 month BAL study and life time carcinogenicity assay with the same particles, revealed also a good correlation between the biomarker results with the tumour potency data and the histopathologically scored pulmonary inflammation. The authors concluded that the findings after subchronic instillation of these particles are consistent with the picture of an overload phenomenon in the rat lung in that chronic inflammation in the lungs is leading to a persistent exposure of lung epithelial cells to released ROS, oxidative stress and subsequent oxidative DNA-damage including cell death (cell proliferation), double strand breaks, mutations and finally tumour development in the rat lung.

More recently, Gebel (2012) investigated differences as to the pulmonary carcinogenic potency of nanomaterials versus fine dusts. Based on the assumption that inflammatory responses are driving the development of pulmonary tumours in the rat and especially because the particle, whether nanosized or microsized, is considered the toxicological principle as scientifically accepted common mode of action, a meta-analysis of existing rat inhalation carcinogenicity studies was performed. Taking mass concentration as dose metric, relative potency factors based on cumulative particle mass following chronic exposure indicate a 4- to 5-fold higher carcinogenic potency of nanosized particles compared to microsized particles. However, these analyses have not taken into account that tumour induction is age dependent and that particle-induced pulmonary tumours in the rat appear late, generally later than 24 month after start of the exposure

(Mauderly et al, 1987). Taking into account that the median study duration for nanosized particles was 4 months longer than for micromaterials, Gebel (2012) therefore made adjustments for exposure duration as well as for total study duration to consider the age-dependency in tumour formation. Based on his results, the relative carcinogenic potency differences between nano and micro materials is small and indicate only a 2- to 2.5 fold higher potency compared to the 4- to 5-fold difference previously assumed (Gebel 2012).

In conclusion, recent findings have shown that chronic exposure to high concentrations of PSP, whether in nanosize or microsize, will increase the lung burden until a steady state between deposition and clearance is reached. Above this threshold, the mucociliary and alveolar macrophage mediated clearance mechanisms of the lung become overloaded, leading to accumulation of the particles and finally to sustained pulmonary inflammatory responses. Below this lung overload threshold, particles will be removed from the lung at normal clearance rates without any appreciable adverse response. During lung overload conditions, inflammation dependent increase of oxidative stress becomes dominant resulting in secondary genotoxic events. Compared to other species, the inflammatory and subsequent pathological responses are much more pronounced in rats which indicate the special sensitivity and specificity of the rat concerning lung overload driven lung responses. Additionally, from all available data it is concluded that inflammatory processes are responsible for the pathogenic lung responses and that mechanistically no difference between nano- or microsized particles exist. Scientifically accepted is that as common mode of action the particle nature as such can be regarded the causative principle. A critical analysis of data obtained in rats after exposure to high PSP concentrations - whether micro- or nanosized with regard to their relevance for human health risk assessments is therefore crucial.

4.5 Particle translocation

Although phagocytosis by macrophages is the dominant method of trapping and processing particles in the lung tissue, some particles may enter the interstitium by inefficient alveolar macrophages phagocytosis. Some studies indicate that particles pass directly from lung parenchyma into blood vessels (and lymphatics vessels) without being carried by mobile cells.

Early literature hypothesised that very small particles can migrate to perivascular and peribronchial positions passing directly from the lung tissue (Gross and Westrick 1954). Electron microscopic studies (Lauweryns et al, 1974) showed that carbon particles (25 nm diameter) are cleared from the lungs of rabbits via the lymphatics system, while ferritin molecules (10 nm diameter) are absorbed by blood capillaries and lymphatics. After their intratracheal administration and within 30 minutes, both types of particles had translocated to the interstitium and the lymph vessels. Studies in vitro by Pratten & Lloyd, 1986 reported that macrophages show a 70-fold slower rate of uptake for 30-nm particles compared to bigger >100 nm particles. The quantitative role of blood capillaries and lymphatics in alveolar clearance was evaluated (Meyer et al, 1969), and estimated that particle absorption via lymphatics tissues is about a 37-fold that of blood.

PSPs, ultrafine particles (UFPs) show a tendency to translocate outside the lung compared to larger (> 100 nm) particles, and their clearance and retention is through interstitialisation rather than the usual alveolar macrophage mediate clearance (Kreyling et al, 2000). Uptake by epithelial cells can be implied from studies

in rats exposed to UF iridium (15 nm and 80 nm), where some particles escaped AM clearance by penetration through the lung epithelium into systemic circulation reaching extrapulmonary organs. Although the translocated fraction was very low in these organs (< 0.01), the 15 nm fraction was an order or magnitude larger compared to that of the 80 nm material indicating an inverse relationship between size and translocation potential (Kreyling et al, 2002).

Site of retention is also species dependent; in experiments conducted with diesel exhaust following chronic exposure (Nikula el al. 1997), rats tend to retain greater portions of UFP in the lumen of alveolar ducts and alveoli compared to cynomolgus monkeys and humans, who retain more material in the interstitium (Section 5.3.).

Translocation of inhaled UFP from lung deposition sites is of interest because of the hypothesis that translocated particles may pose adverse health effects to extra pulmonary organs. Because poorly soluble particles vary in size, composition and form, it is challenging to define a generic approach to study translocation of PSP to extra pulmonary sites after their deposition in the lung.

There are however some specific studies available which help illustrate the phenomenon of particle translocation from lung tissues to extra pulmonary organs described in the following:

4.5.1 UFP show a rapid translocation from the site of deposition

Oberdörster and colleagues (Oberdörster et al, 2002a) estimated that on average, two-thirds of the lung deposits are cleared between an initial 6-hour exposure and the subsequent 0.5 hour post-exposure measurement. Since no significant lung clearance was measured at the other post-exposure periods, it was implied that very rapid clearance of UF carbon particles from the lower respiratory tract occurred in the initial stages of exposure. This finding was considered consistent with previous observations (Oberdörster et al, 2000), where UF particles depositing in the lower respiratory tract escape AM clearance and have a rapid translocation to epithelial, interstitial and endothelial sites. Once uptake from interstitial sites in the respiratory tract has taken place, further translocation of UF particles to lymphatic channels (or directly via the endothelium) and then to the blood circulation could take place.

4.5.2 Translocation is determined by solubility and size

A biokinetic study was designed (Kreyling et al, 2002), in order to study particle translocation from the lung to secondary organs using radiolabelled iridium (^{192}Ir) in soluble and insoluble forms with the following administration techniques:

a.) soluble $^{192}Ir^{3+}$ administered to the rat lung via instillation;
b.) suspension of UF ^{192}Ir by intratracheal instillation;
c.) suspension of UF ^{192}Ir administered by gavage;
d.) suspension of UF ^{192}Ir via systemic injection.

Administered UF ^{192}Ir via systemic circulation, gavage, or to the lungs have very low solubility and no uptake from the GI tract; particles passed the gut and were excreted via the faeces. The administered soluble ^{192}Ir indicated that, when found in soluble form (administered to the lung), it is excreted mostly through the urine with negligible organ uptake, suggesting that insoluble form of particles is required for extra pulmonary translocation.

Smaller particles (15-nm vs. 80-nm) showed greater uptake to the interstitial spaces. A very small fraction of ^{192}Ir particles deposited in the lower respiratory tract translocated from the lungs into liver, spleen, heart, brain and the carcass. Only the liver showed a time-dependent pattern. Due to the fact that, systemic injection of 192Ir resulted in fractions found in similar organs, the plausible pathway suggested was transport from lung epithelium into the pulmonary vasculature and then into systemic circulation. Translocation was larger for 15-nm particles, which showed higher interstitial uptake than larger particles, by a factor of 5-10. The rate of systemic translocation of insoluble UF ^{192}Ir was rather small but shown to be size dependent, suggesting an inverse particle-size-dependent transport phenomenon into blood circulation.

4.5.3 GI tract may contribute to particle burden in organs

In addition to the interstitial clearance pathway from the alveolar region, clearance via the GI tract should be considered. Deposits of UFP in the tracheobronchial region cleared through the mucociliary escalator as well as particles deposited in the in the head region may contribute to particle translocation through the blood circulation via GI tract clearance. This second pathway was proposed based on the fact that the amount of UF ^{13}C found in lung and liver at 24 h post-exposure was on average 63% greater than the amount predicted to be deposited in the whole respiratory tract, implying that additional to inhalation, uptake from the GI tract via the above mentioned mucociliary and head regions (with possible external contamination via oral uptake from the fur due to the fact that the study was done by whole-body inhalation), should be considered as adding to the lung-liver translocation process (Oberdörster et al, 2002). Hence, the translocated UFP that was measured in the liver should be regarded as blood-borne coming from two sources, lung-interstitial and GI-tract clearance. This is in contrast to other studies with UF radioactive metal particles which do not show significant translocation from the GI tract to other organs neither via the blood circulation, nor by these types of particles deposited in the lung to other extra-pulmonary organs (Kreyling et al, 2002; Kanapilly et al, 1980). These discrepancies were explained by differences in exposure conditions (nose-only by Kanapilly and intratracheal inhalation by Kreyling), chemical nature of UFP, rising questions on organ translocation differences between metals and carbon due to cellular-component interactions (e.g. protein binding). Although the translocated fraction in the Kreyling 2002, study was small (< 1%) and had no GI tract uptake, it did show a correlation to particle size and is probably also dependent of the intratracheal method of exposure.

Early studies by Ferin and Feldstein (1978) showed that rats exposed to TiO_2, had fewer particles in the lymph node after inhalation than after intratracheal instillation, which gives an indication about differences in results because of exposure techniques.

The previous observations are supported by a 3 week inhalation study in Wistar rats exposed to fine and nano TIO_2 particles with recovery times of 3, 28 and 90 days. The results of this study using relative

deposition index indicate that apart from particles found in AM and to some extent in type-1 pneoumocytes; capillary cells show limited particle accumulation either in the nano or fine-particle treated groups. This indicates that following exposure by inhalation translocation of fine and nano TiO_2 particles from the lung to blood stream was minimal (Eydner et al, 2012).

4.5.4 Source of UFP may influence translocation

In both studies to investigate size, solubility and clearance from site of deposition (Oberdörster et al, 2002a; Kreyling et al, 2002), UFP particles were generated by spark discharge. Morfeld et al (2012) have questioned whether only primary nanoparticles or small aggregates/agglomerates generated for research purposes have the ability to translocate; and in the context of real life exposures (formation of microsize aggregates/agglomerates) what is the actual sub distribution of nanoparticles in the lung. For this question, the disintegration of the inhaled microsize aggregates/agglomerates into nanosized primary material was given special attention.

Nanosized TiO_2 was tested in an inhalation study which also corrected for transmission electron microscopy (TEM) slicing bias; since particles are perceived as two dimensional surrogates of a three dimensional structure. A model was developed to estimate the expected numbers of particle diameters below 100 nm due to the TEM slicing bias. Comparisons of observed to expected values did not provide evidence in favour of the presence of nanoparticles in the rat lungs after correcting for TEM slicing bias. This indicated that nanostructured TiO_2 aggregates/agglomerates do not disintegrate into smaller structures when exposed to fluids similar to the lung surfactant.

In the context of translocation, this suggests that contrary to spark generated UFP, nanostructured TiO_2 generated via a different process show a different translocation behaviour. Manufactured nanostructured materials are often present as agglomerates due to van der Waals forces. Dispersing manufactured nanomaterials in a test atmosphere results in agglomerates up to several micrometres with only a minimal fraction present as primary particles. Morfeld et al (2012) indicated that the amount available for translocation is extremely low (below detection limit and about 0.0025% of the total deposition).

4.5.5 Target organs for translocation

Studies observed that the liver had a prominent role in extra pulmonary organ translocation, although other organs like the spleen and pulmonary lymph nodes were also target organs for translocated particles. This may take place through interstitial pathway clearance via blood circulation. When uptake in other organs was assessed, only the liver showed a time-dependence pattern. The fractions found in the pulmonary lymph nodes, although low showed an inverse relationship to particle size (Oberdörster et al, 2002; Kreyling et al, 2002; van Ravenzwaay, 2009).

Uptake into the lymph node is different from other secondary organ uptake as it has been suggested that penetration of particles into lung lymph nodes appears to be highly dependent on the accumulated dose and

inflammatory response. Two types of TiO_2 (a 20 nm ultrafine and a 250 nm fine TiO_2) at similar concentrations were compared with regard to their inflammogenic potential and their impact on clearance behaviour. The ultrafine sample exerted much more inflammation accompanied by a significantly larger fraction migrating to the interstitial space and the lung lymph nodes. (Section 2 on surface area, Oberdörster et al, 1994). In the study by Morfeld et al, 2012; TiO_2 (20-25 nm) was only detected in the lung and the mediastinal lymph nodes, which was considered a consequence of an inflammatory response and not of size characteristics of the test material. The inflammation potential was higher for quartz than for TiO_2 (either in pigmentary or nano-sized form), which was also reflected in the amount found in the mediastinal lymph nodes (van Ravenzwaay 2009).

Taken together, the translocation of PSPs from the deposition site into the lung lymph nodes appears to be a function of inflammation rather than of particle size per se, suggesting that lung lymph nodes should probably not be considered as secondary organs of translocation as they serve as lung draining lymph nodes and thus a clearance mechanism.

4.5.6 Summary

During translocation processes, particles may escape macrophage phagocytosis and enter the interstitium. Controlled exposure to defined particles, have allowed direct measurement of translocation processes in extra pulmonary organs. Uptake into the interstitium has an inverse particle-size dependency but is also species dependent. Rats tend to accumulate more particles in the lumen of alveolar ducts and alveoli; humans retain more material in the interstitium. Once in the interstitium, tiny fractions may translocate to extra pulmonary organs. The plausible pathway suggested is transport from lung epithelium into the pulmonary vasculature and lymphatics then into systemic circulation and so reaching other organs. The liver appears to be the preferred target organ of most of the translocated particles. Translocation to the lymph node is rather a process of clearance related to inflammation than of particle size per se. One prerequisite for extra pulmonary site and rate translocation is the form of particle administration technique and poor solubility, indicative that chemical composition and physical structure of the test material may be an important determinant influencing systemic translocation of particles. It has been suggested that the origin of the particles may play an important role in translocation. Test particles (nano and/or microsized) generated under laboratory conditions show a different translocation behaviour compared to industrially-generated (nano)particles. Nanoparticles appear to form agglomerates which, once deposited in the lung, do not appear to disintegrate into smaller nano-sized particles or translocate outside the lung draining lymph nodes.

5. SPECIES DIFFERENCES AND MECHANISMS OF LUNG TUMOUR FORMATION IN RATS

In experimental toxicity studies, the rat model is known to be particularly sensitive to the development of lung pathological responses under conditions of particle overload. Lung tumours have been reported only in rats, but not mice or hamsters, exposed to low toxicity, low solubility particulates following chronic exposures to high particle concentrations. This disparity in lung responses among the rodent species has clearly been demonstrated following 90-day and chronic (two year) inhalation studies in rats, mice and hamsters to similar test substances and identical concentrations of the same test substances including pigment-grade and ultrafine titanium dioxide (TiO_2) particles and carbon black particulates. It is interesting to note that although particle overload was documented in the mouse lung, it was only in the rat that a sequence of pulmonary pathogenic events was initiated which progressed to fibro-proliferative disease, evidence by septal fibrosis, hyperplasia and eventually lung tumours. The results of experimental studies clearly demonstrate that under virtually similar or identical exposure conditions, pathological changes are documented in rats, but are not observed in other rodent species (mice or hamsters). Furthermore, it has also been well demonstrated that particle deposition, retention and inflammatory patterns are different in the lungs of rats when compared to particle effects investigated in the lungs of non-human primates. It is also noteworthy that detailed epidemiological studies in TiO_2 and carbon black workers provide unequivocal evidence of no causal link between particle exposures and lung cancers or other non-neoplastic lung diseases. This Section details the relevant toxicological database demonstrating the interspecies differences in lung response to particle overload, thus clearly demonstrating that the rat model presents a particularly sensitive pulmonary and, moreover, a unique lung neoplastic response under conditions of particle overload.

5.1 Subchronic inhalation studies

With regard to nomenclature issues, studies on three different test substance particle-types are discussed in the following sections. Pigment-grade TiO_2 particles, are generally considered in the mean, primary particle size range > 100 nm. Pigment-grade or fine-sized TiO_2 particulates are generally in the size range of 250 – 400 nm. In addition, the crystal structure of the pigment-grade particle types were predominantly of the "rutile" type and generally have a measured surface area of 5-6 m^2/g. In contrast, the "ultrafine TiO_2 test substances used in the sub chronic (Bermudez et al, 2004) and Heinrich et al, (1995) had a crystal structure composition of 80% anatase and 20% rutile, with a measured surface area of ~ 53 m^2/g, and a primary particle size (TEM) of 25 nm. It is important to note that the aerosolised distribution of ultrafine and pigment-grade TiO_2 particles included primarily agglomerated particulates. Carbon black particles are generally found in the <100 nm range. In the Elder et al (2005) study, the primary particle size of the high surface area carbon black test substances was approximately 16 nm, corresponding to a specific surface area of 300 m^2/g. The primary particle size and specific surface area of the "lower" surface area carbon black test particle was approximately 70 nm and 37 m^2/g, respectively.

5.1.1 Pigment-grade TiO_2

Subchronic, 90-day inhalation exposures of rats, mice, and hamsters to either pigmentary or ultrafine TiO_2 particles at concentrations likely to induce particle overload resulted in a more severe and persistent pulmonary inflammatory response in rats, when compared with either similarly-exposed mice or hamsters. Rats were unique among these three rodent species in the development of progressive fibroproliferative lesions and alveolar epithelial metaplasia (Bermudez et al, 2002 and 2004). Female rats, mice or hamsters were exposed to 10, 50 or 250 mg/m^3 concentrations of pigmentary (rutile type) TiO_2 particles for 6 hours/day, 5 days/week for 13 weeks followed by 4, 13, 26 or 52 weeks of post exposure (46 weeks for hamsters)(Bermudez et al, 2002). Lung and associated lymph node loads of TiO_2 increased in a concentration-related manner. It is important to note that retained lung burdens were greatest in mice following exposure, with rats and hamsters demonstrating similar lung burdens immediately following 90-day exposures. Particle retention data indicated that particle overload in the lungs was reached in both rats and mice at the 50 and 250 mg/m3 concentrations. Inflammation was observed in all three species at the two highest concentrations. This inflammation persisted in rats and mice throughout the post exposure recovery period at the highest exposure concentration. In hamsters, inflammatory responses were eventually resolved due to the more rapid clearance of particles from the lung. In rats exposed to the highest concentration (250 mg/m^3), pulmonary lesions consisted of epithelial proliferative changes manifested by increased alveolar epithelial cell labelling indices, as evidenced by the results of cell proliferation studies. Associated with these proliferative changes in the rat were enhanced interstitial accumulations of particles along with alveolar septal fibrosis. Although rats exposed to 50 mg/m^3 developed minimal alveolar cell hypertrophy, accumulation of particle-laden macrophages, and inflammation, no alveolar septal fibrosis or relevant cell turnover at alveolar sites were observed at this lower exposure concentration. Similar changes to those seen in rats were not observed in either mice or hamsters. The study clearly demonstrated the uniqueness of the rat pulmonary response to particle overload concentrations.

5.1.2 Ultrafine TiO_2 particles

In a study with ultrafine TiO_2 (80% anatase: 20% rutile; average primary particle size = 21 nm), female rats, mice or hamsters were exposed to aerosol concentrations of 0.5, 2.0 or 10 mg/m^3 TiO_2 for 6 hours/day, 5 days/week for 13 weeks followed by 4, 13, 26 or 52 weeks of post exposure (49 weeks for hamsters)(Bermudez et al, 2004). Retained lung burdens increased in a concentration-related manner in all three species. Mice and rats had similar lung burdens at the end of exposures but hamsters were significantly lower. Retardation of particle clearance in rats and mice at the highest exposure concentration (10 mg/m^3) was a strong signal that pulmonary particle overload had been achieved. Pulmonary lesions of the lungs in rats included foci of alveolar epithelial proliferation of metaplastic epithelial cells (alveolar bronchiolisation) concomitant with focal areas of heavy, particle-laden macrophages. In rats, these alterations were manifested by increased alveolar epithelial cell labelling indices, as evidenced by increases in cell proliferation indices. Associated with these overload-related developments were areas of interstitial particle accumulation and alveolar septal fibrosis. These pulmonary lesions measured and observed in rat lungs were progressive, i.e., became more pronounced with time. Mice developed a less severe inflammatory response without the progressive epithelial and fibroproliferativefibro proliferative changes.

These data are consistent with the results of a companion study using inhaled pigmentary (fine mode) TiO_2 (Bermudez et al, 2002) and demonstrate that the pulmonary responses of rats exposed to ultrafine particulate concentrations likely to induce pulmonary overload are different from the effects measured in similarly exposed mice and hamsters. These differences can be explained both by pulmonary responses and by particle dosimetry differences between these rodent species.

5.1.3 Carbon black particles

Inhalation exposures to high concentrations of carbon black (CB) particles have produced lung tumours in rats, but not mice or hamsters, presumably due to secondary genotoxic mechanisms involving persistent lung inflammation and injury. Accordingly, for this study it was postulated that the lung inflammation and injury induced by sub chronic, 90-day inhalation exposures of CB would be pronounced in rats than in mice and hamsters. Particle retention kinetics, inflammation, and histopathology were examined in female rats, mice and hamsters exposed for 13 weeks to high surface area CB (HSCb) at doses chosen to span a no-observable adverse effects level (NOAEL) to particle overload (0, 1, 7, 50 mg/m³). Rats were also exposed to low surface area CB (50 mg/m³, nominal; LSCb). Retention and effects measurements were performed immediately after exposure and 3 and 11 months post-exposure. Equivalent or similar mass burdens were achieved in rats exposed to high-dose HSCb and LSCb, whereas surface area burdens were equivalent for mid-dose HSCb and LSCb. Prolonged retention was found in rats exposed to mid- and high-dose HSCb and to LSCb, but LSCb was cleared faster than HSCb. Retention was also prolonged in mice exposed to mid- and high-dose HSCb and to LSCb, and in hamsters exposed to high-dose HSCb. The results demonstrated that pulmonary inflammation and histopathological effects were more severe and prolonged in rats when compared to mice or hamsters, and both lung effects were similar in rats exposed to mid-dose exposures to the two forms of carbon black, i.e., HSCb and LSCb. Similar to the results of both TiO_2 sub chronic inhalation studies, the findings demonstrated that hamsters have the most efficient clearance mechanisms and least severe responses of the three species. The results from rats also show that particle surface area is an important determinant of target tissue dose. The authors concluded that a subchronic NOAEL of 1 mg/m³ respirable HSCb (Printex 90) was determined for female rats (Elder et al, 2005).

5.2 Chronic inhalation studies

Chronic inhalation exposure of rats to very high doses of particles can result in inflammation, fibrosis, and some lung tumours. The tumorigenic response observed in the rat appears to be both species specific and restricted to doses where there is an overload of the lung clearance mechanisms.

5.2.1 Pigment-grade TiO_2

In chronic exposure studies, lung tumours were produced in rats under conditions of particle overload. In a 2-year inhalation study in male and female rats, exposures to titanium dioxide particles (rutile type) at

concentrations of 10, 50, or 250 mg/m^3 produced lung tumours only at the highest concentration (Lee et al, 1985). With one exception, the tumours produced were ultimately characterised as primarily benign pulmonary keratin cysts (Warheit and Frame, 2006). In a study reported by Muhle et al (1991), TiO_2 was used as a negative control dust in a two-year inhalation study with toner particles. Male and female rats were exposed (6 hr/day, 5 days/week) to 5 mg/m^3 TiO_2 (rutile form) of 1.1 mm MMAD with a respirable fraction of 78%. There were no significant increases in lung tumours vs. control rats exposed for up to 24 months by whole body inhalation to TiO_2 in this study.

5.2.2 Ultra-fine grade TiO_2

Heinrich et al (1995) exposed female rats by whole body inhalation to ultrafine TiO_2 (80% anatase: 20% rutile) at an average concentration of 10 mg/m^3 for 24 months followed by 6 months without exposure. The particle size of the TiO_2 used ranged from 15 to 40 nm with a MMAD of 0.8 mm (agglomerates of ultrafine particles). Statistically significant increases in tumours vs. controls were observed in rats from this study (benign keratinising cystic squamous-cell tumours, adenocarcinomas, squamous-cell carcinomas, and adenomas). Exposure of female mice to ultrafine TiO_2 under the same conditions as for rats resulted in a significantly decreased lifespan at an inhalation concentration of approximately 10 mg/m^3 (Heinrich et al, 1995). However, tumour rates were not statistically increased over prevalence in controls.

Groups of female Wistar rats were exposed by inhalation 17 hr/day, 5 days/week to 6 mg/m^3 Printex CB particles primary particle size = 15 nm, MMAD = 1.1 mm). One group was exposed for 43 weeks and placed in post-exposure recovery for an additional 86 weeks in air. The other group was exposed for 86 weeks and placed in post-exposure recovery for an additional 43 weeks. The results demonstrated that no tumours were observed in control rats. In the 43-week exposure group, the lung tumour rate was reported to be 18%. In the 86-week exposure group had a lung tumour rate of 8%.

5.2.3 Carbon black

As another component of the study with uf TiO_2, female Wistar rats were exposed to high-purity furnace carbon black (particle size -= 14 nm, specific surface area = 227 m^2/g), MMAD = 0.64 mm). The extractable organic mass of the furnace black was 0.04%; the content of benzo[a]pyrene was 0.6 pg/mg and that of 1-nitropyrene was < 0.5 ng/mg particle mass. Rats were exposed in whole-body exposure chambers for 18 h/d, 5 days/wk to 7.4 mg/m^3 CB for 4 months followed by 12.2 mg/m^3 for 20 months. After exposure, the rats were kept in clean air for another 6 months. The incidence of benign and malignant lung tumours was increased in the treated groups after 30 months.

5.3 Other mammalian species responses to inhaled particulates

The pulmonary responses of rats are extremely marked when compared to other large mammalian species such as non-human primates and humans

In studies reported by Nikula et al (1997, 2001), it has been proposed that the intrapulmonary particle retention patterns and tissue reactions in rats may not be predictive of pulmonary retention patterns and tissue responses in either primates or humans. Male monkeys and rats were exposed for 7 hours/day, 5 days/week for 24 months to diesel exhaust (2 mg/m^3), coal dust (2 mg/m^3), or diesel exhaust and coal dust combined (1 mg/m^3 each) and were subsequently examined histopathologically (Nikula et al, 1997). In all exposed groups, monkeys retained a similar amount or more particulate material in the lungs than did rats. Rats retained a greater proportion of the particulate material in the alveolar ducts and alveoli, whereas monkeys retained a greater proportion of particulate material in the interstitium. Rats, but not monkeys, had significant alveolar epithelial hyperplastic, inflammatory, and septal fibrotic responses to the retained particles. Similar to the findings in monkeys, up to 91% of the retained particulate material in the lungs of coal miners was located in the lung interstitium (Nikula et al, 2001). It was suggested by the authors that these differences in particulate tissue distribution in rats and humans might bring different lung cells into contact with retained particulates or particle-containing macrophages. This may account for the differences in species responses to inhaled particulates.

Studies also show that the rat's response to retained particles is more serious and adverse than for other species. Nikula et al (1997) compared the anatomical patterns of particle retention and the lung tissue responses between rats and cynomolgus monkeys following chronic exposure to diesel exhaust and coal dust. Lung sections from the monkeys and rats exposed for 24 months to filtered ambient air, diesel exhaust (2 mg soot/m^3), coal dust (2 mg respirable particulate material/m^3) or diesel exhaust and coal dust combined (1 mg soot and 1 mg respirable coal dust/m^3) were examined histopathologically. The relative volume density of particulate material and the volume percentage of the total particulate material in defined pulmonary compartments were determined morphometrically to assess the relative amount and the anatomic distribution of retained particulate material. With one exception (diesel exhaust), relatively more particulate material was retained in the monkey lungs relative to the rat lungs, indicating that the rats clear the dust at a faster rate relative to the monkeys. There was no significant difference between diesel exhaust-exposed monkeys and rats in the relative amount of retained particulate materials. In addition, rats retained a greater portion of the particulate material in lumens of alveolar ducts and alveoli than monkeys; and monkeys retained a greater portion of the particulate material in the interstitium than rats. Rats, but not monkeys, had significant alveolar epithelial hyperplastic, inflammatory and septal fibrotic responses to the retained particles. The authors concluded: "These results suggest that intrapulmonary particle retention patterns and tissue reactions in rats may not be predictive of retention patterns and tissue responses in primates exposed to poorly soluble particles at concentrations representing high occupational exposures. The pulmonary responses of the rats were severe compared to the primate, where the insult to the lungs was handled without adverse consequences."

In a subsequent study, Nikula et al (2001) evaluated the influence of exposure concentration or dose on the distribution of particulate material within the lungs of rats and humans. In this study the investigators used morphometric methods to assess the influence of exposure concentration on particle retention by evaluating histologic lung sections from rats and humans. The rats had been exposed for 24 months to diesel

exhaust at 0.35, 3.5, or 7.0 mg soot/m³. The human subject groups included 1) nonsmokers who did not work as miners; 2) nonsmoking coal miners who worked under the current standard of 2 mg dust/m³ for 10-20 years; and 3) nonsmoking coal miners who worked under the former standard of <10 mg dust/m³ for 33 to 50 years. The distribution of retained particles within the lung compartments was markedly different between species. In all three groups of rats, 82 to 85% of the retained particulate material was located in the alveolar and alveolar duct lumens, primarily in macrophages. In humans, 57, 68, and 91% of the retained particulate material, respectively, was located in the interstitium of the lung in the three aforementioned study groups. The authors concluded: "These results show that chronically inhaled diesel soot is retained predominantly in the airspaces of rats over a wide range of exposures, whereas in humans, chronically inhaled particulate material is retained primarily in the interstitium. In humans, the percentage of particles in the interstitium is increased with increasing dose (exposure concentration, years of exposure, and/or lung burden). This difference in distribution may bring different lung cells into contact with the retained particles or particle-containing macrophages in rats and humans and, therefore, may account for differences in species response to inhaled particles."

The two studies by Nikula et al (1997, 2001) provide important insights, as they clearly delineate significant species differences in pulmonary responses to inhaled particulates between rats and primates, including humans. The studies by Nikula and colleagues comparing lung responses to inhaled particles in rats versus larger mammals demonstrate the following: 1) the disposition and dosimetry differ between rats and either monkeys or humans (rat = alveolar; monkey/human = interstitial sites); and 2) rats produce significantly augmented and sustained pulmonary inflammogenic, epithelial and fibro-proliferative responses when compared to either monkeys or humans. The available data also suggest that rats are significantly more sensitive in the development of adverse lung responses to inhaled particle exposures when compared to: 1) other rodent species; and 2) larger mammals such as monkeys and humans.

In a review paper, Nikula (2000) compared histopathological results from chronic bioassay studies with particulates in rats vs. nonhuman primates. This review summarised data from studies with exposure for 2 years or more using 5 poorly soluble nonfibrous materials - diesel exhaust, carbon black, titanium dioxide, talc, and coal dust. The following studies were reviewed: Chronic inhalation studies of rats and monkeys identically exposed to diesel exhaust (Lewis et al, 1989), coal dust (Lewis et al, 1989), petroleum coke dust (Klonne et al, 1987), raw or processed shale dusts (MacFarland et al, 1982), bertrandite (Wagner et al, 1969), and beryl ore (Wagner et al, 1969). The comparisons of rat and monkey responses to chronic inhalation of poorly soluble particles led to some important insights.

Lewis and colleagues (1989) exposed rats and monkeys to filtered air or to diesel exhaust or coal dust at 2 mg respirable particulate material/m³, 7 h/day, 5 days/wk for 24 months. Diesel exhaust- and coal dust-exposed rats and monkeys retained particulate material in their lungs. The anatomic distribution of the retained material differed between rats and monkeys (Nikula et al, 1997; Lewis et al, 1989). Rats retained particulate material predominantly within alveolar macrophages located in the lumens of alveoli and alveolar ducts. Monkeys retained more of the particulate material within macrophages in the interstitium and pleura than in the lumens of alveoli and alveolar ducts. Responses to the exposures and retained material also differed between rats and monkeys. Rats, but not monkeys, had significant alveolar epithelial hyperplastic and inflammatory responses to the retained particulate material (Nikula et al, 1997; Lewis et al, 1989).

Klonne and colleagues (1987) exposed Sprague-Dawley rats and cynomolgus monkeys to 0, 10.2, or 30.7 mg/m³ micronized delayed process petroleum coke for 6 h/day, 5 days/wk (weekly exposure rate up to 921 mg/m³) for 24 months. The rats exhibited retention of particulate material in macrophages, chronic inflammation, focal fibrosis alveolar-bronchiolar metaplasia, sclerosis, squamous metaplasia of alveolar epithelium, and keratin cysts. Two of 48 males and 10 or 44 (23%) females in the high concentration group had keratin cysts. Several rats had multiple cysts. In monkeys, the pulmonary histopathology was limited to accumulations of macrophages containing particulate material.

MacFarland and co-workers (1982) exposed F344 rats and cynomolgous monkeys to 0, 10, or 30 mg/m³ respirable raw or processed shale dust for 6 h/day, 5 days/wk (weekly exposure rate up to 900 mg/hour/m3) for 24 months. All of the rats developed proliferative bronchiolitis and alveolitis (i.e., inflammation with epithelial hyperplasia) and most developed chronic inflammation with nonprogressive fibrosis, cholesterol clefts, and micro-granulomas. The monkeys accumulated more pigment-laden macrophages in the bronchiolar and alveolar walls than in the alveolar lumens. The majority of monkeys had no reaction to the accumulated material. A few monkeys had occasional foci of sub acute inflammation.

Wagner and colleagues (1969) exposed Sprague-Dawley and Green-acres Controlled Flora (strain not otherwise identified) rats, Syrian golden hamsters, and squirrel monkeys to bertrandite or beryl ore dusts. The exposures were to 0 or to 15 mg/m³ of the ores for 6 h/day, 5 days/week (weekly exposure rate of 450 mg mg/hour/m3) for 17 months (rats and hamsters for 23 months (monkeys). Alveolar epithelial hyperplasia and chronic inflammation with granulomas were present in rats exposed to both materials. By 12 months, 5 of 11 rats had foci of squamous metaplasia or lesions referred to as tiny epidermoid tumours. By 17 months, 7 and 9 of 19 rats had adenomas and adenocarcinomas, respectively and 4 of 19 had epidermoid tumours. The ore-exposed hamsters had alveolar epithelial hyperplasia, bronchiolar-alveolar metaplasia, and a few granulomatous lesions. No lesions other than accumulations of particle-containing macrophages and mononuclear cells around respiratory bronchioles and blood vessels were present in monkeys.

The results of studies wherein rats and monkeys were exposed to identical aerosolised particle test substances indicate that although both rats and monkeys retain particulate material in their lungs when chronically exposed to high concentrations of dusts or soot, the retention pattern within anatomical compartments is different. Moreover, the pulmonary tissue response to the particulate material is different. Specifically rats retain a greater proportion of the material in intralumenal alveolar macrophages, and they respond with a greater degree of epithelial hyperplasia and active inflammation when compared to the response of monkeys.

5.4 Understanding the mechanisms of the unique neoplastic rat lung responses to particle overload

Warheit et al (1997) performed a study that provides a mechanistic explanation for the responses observed in the pivotal rat oncogenicity study (Lee et al, 1985). The study demonstrated that the lungs of particle-overload exposed rats are characterised by impaired pulmonary clearance, sustained pulmonary inflammation, cellular hypertrophy and hyperplasia; and that these effects, following continuous exposure at 250 mg/m³ (for two years), likely could result in the development of overload-related pulmonary tumours.

The study with pigment-grade TiO_2 in rats used exposure concentrations similar to those in the Lee et al, study (1985) to detail the characteristics of 'lung overload' in this species, along with an assessment of the rat's ability to recover from this challenge. Male rats were exposed to TiO_2 particles 6 hours, 5 days a week for 4 weeks at concentrations of 5, 50, and 250 mg/m³ and evaluated at selected intervals through 6 months post-exposure. Exposure to high dust concentrations produced pulmonary inflammation, proliferation of pulmonary cells, impairment of particle clearance, deficits in macrophage function, and the appearance of macrophage aggregates at sites of particle deposition. Rats exposed to 250 mg/m³ TiO_2 had lung burdens of 1600 mg/g of fixed lung tissue or 12 mg/lung. TiO_2 particles produced sustained pulmonary inflammatory responses in animals exposed to 250 mg/m³, corresponding to substantial numbers of neutrophils recruited to alveolar regions. Rats exposed to 50 mg/m³ TiO_2 had small, sustained inflammatory responses. Rats exposed to 250 mg/m³ demonstrated diminished lung clearance after 1 week through 1 month post-exposure. Mono-exponential clearance modelling indicated that TiO_2 particles were cleared with half-times of approximately 68, 110, and 330 days for the 5, 50, and 250 mg/m³ test groups, respectively. Lymph node burdens of rats exposed to 250 mg/m³ TiO_2 demonstrated TiO_2 particles had translocated to tracheobronchial lymph nodes. In vitro phagocytosis studies demonstrated that alveolar macrophages exposed to 250 mg/m³ TiO_2 were impaired in their phagocytic responses. At high concentrations (50 to 250 mg/m³) of TiO_2, cellular hypertrophy and hyperplasia were evident at alveolar wall and duct bifurcations that were adjacent to the macrophage.

Despite a comparable behaviour of rats and humans to accumulate PSP within similar lung compartments, morphometric analysis by Nikula et al (2011) have shown that the relative amounts of intraluminal and interstitial particle load differ markedly between rats and humans with particles being found predominantly in the interstitium in man and intraluminarly in rats. Additionally, quantitative effect response analysis to PSP exposures revealed differences at cellular levels between rats and humans. Especially the occurrence of acute intra-alveolar inflammatory responses, alveolar epithelial hyperplasia and alveolar lipoproteinosis were all significantly more pronounced in rats compared to humans exposed to the same particles (Green et al, 2007). According to the authors, these differences may also account for the species differences seen in the long-term responses to high PSP exposures.

In evaluating mechanistic differences in the response of rats vs. mice or hamsters to inhaled carbon black particles, Carter and coworkers (2006) assessed the levels of several key pro- and anti-inflammatory mediators in the lungs of exposed animals. These investigators postulated that the unique response of the rat may relate to an inability to generate sufficient anti-inflammatory mediators in the face of continuing pulmonary particulate overload exposures. Thus, the aim of the study was to study pro-inflammatory and anti-inflammatory mechanisms underlying species specificity in carbon black-induced lung inflammation. Accordingly, rats, mice, and hamsters were exposed to carbon black particulates at 3 concentrations for 13 weeks as described in the Elder et al, 2005 study, at 3 concentrations (1, 7 and 50 mg/m^3) for 13 weeks. Bronchoalveolar lavage along with reactive oxygen and nitrogen endpoints, and cytokine levels were measured. Ex vivo mutational analysis of inflammatory cells was measured by co-incubating with lung epithelial cells. In addition, lung tissue was evaluated for gene expression of various anti-inflammatory mediators. The investigators reported a dose- and time-course related effect with all the parameters.

Rats demonstrated greater propensity for generating a pro-inflammatory response, whereas mice and hamsters demonstrated an increased anti-inflammatory response. These incipient findings suggest a

potential mechanism for delineating the differences in rat responses to particle overload-induced lung inflammation when compared to mice or hamster responses.

5.5 Conclusions

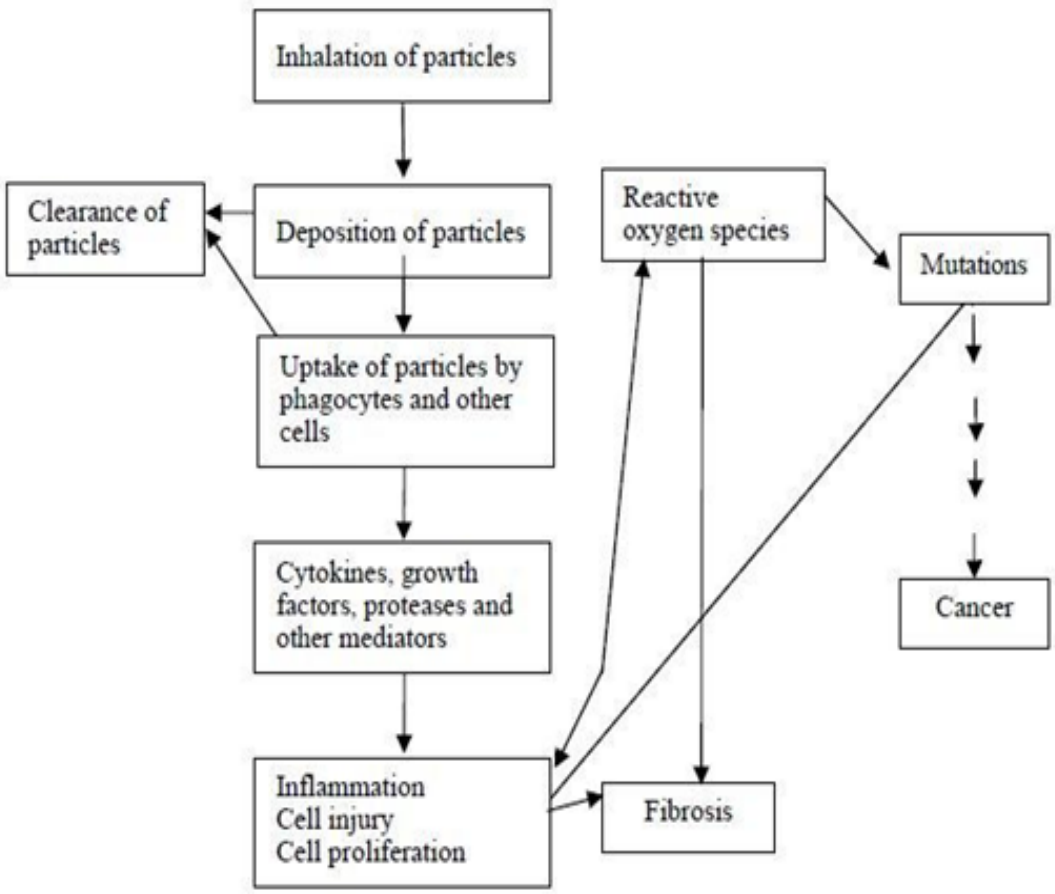
The development of pulmonary tumours in rats after particle exposures to poorly soluble, low toxicity dusts occurs only under the conditions of particle overload in the lungs. This pulmonary pathological response in rats to chronic particulate exposures ending in the development of pulmonary tumours at high concentrations is unique, as other rodents, such as mice and hamsters; and other mammals such as humans or non-human primates do not develop lung tumours under similar conditions of particle lung overload from PSPs. The evidence to support this position has been addressed in some of the studies listed above. To summarise the results of those studies: 1) chronic exposures to PSPs at high concentrations leads to develop of lung tumours in rats, but not mice; 2) rats, mice and hamsters have been exposed to identical test substances at identical concentrations of pigment-grade TiO_2, ultrafine TiO_2 as well as carbon black particles (at particle overload concentrations). Rats, but not mice developed a pathological sequelae of sustained lung inflammation and cytotoxicity, followed by increased cell turnover and fibroproliferative effects (e.g., hyperplasia, septal fibrosis, etc.) ultimately leading to metaplasia and secondary genotoxicity. All of the interspecies studies point to an identical conclusion regarding the unique characteristics of a pathophysiological process operating in the rat, which ultimately leads to the formation of primarily benign lung tumours. The development of pulmonary tumours at particle overload exposures is triggered by the inability of the rat to effectively clear particles from the respiratory tract concomitant with the sustained development of inflammation and cytotoxic effects. Several lines of evidence suggest that the mechanistic differences could be related to the propensity of rats to undergo specific pathological sequelae in response to particle overload conditions. This is initiated by a sustained lung inflammatory and cytotoxicity response, followed sequentially by the development of enhanced cell proliferative activity and corresponding fibro proliferative effects, including septal fibrosis, hyperplasia, and secondary genotoxicity, ultimately leading to the development of lung tumours. Contributing to this pathological effect could be the unique response of rat lung cells to particle overload concentrations – generating "abnormally high" proinflammatory response, concomitant with a deficiency in anti-inflammatory cytokine responses. This would appear to be a plausible "mechanistic recipe" for enhancing the sensitivity or overriding the protective capacity of the rat lung response to particle overload in rats.

In support of these above conclusions, it is instructive to explore the discussion on mechanistic considerations that took place at the IARC Monograph meeting on titanium dioxide and carbon black (IARC 2010). In these deliberations, they postulated on the pathways of biochemical and patho-physiological events which took place during and following lung overload from PSPs in the rat and other experimental species to see if this could be considered a generic mode of action (MoA) that was relevant to the prediction of a potential lung cancer risk to humans. Although much of the examples used drew on studies involving carbon black, TiO_2 and talc, the general pathophysiological features of lung overload as a MoA for the induction of lung tumours in rats are considered to be broadly applicable to all PSPs of low intrinsic toxicity.

Firstly, their general description of the cascade of events that begins with the particle deposition of particles within critical target cells or tissues within the lung that leads to lung tumours in the rats involves: “sustained

inflammation, production of reactive oxygen species, depletion of antioxidants and/or impairment of other defence mechanisms, cell proliferation and gene mutations." They considered that this can be considered to be a generic MoA for the rats and the steps can be compared to other species, including humans.

Figure 4. Conceptual framework of carcinogenesis induced by poorly soluble particles in rats a(IARC, 2010)



[a] Sequence of events and MoAs leading to formation of lung tumours in rats after high exposure to PSPs

They note that the dose metric that best describes the dose-response relationship for PSPs with lung tumour induction in the rats can be surface area, particle and size (Driscoll et al, 1996; Pott & Roller, 2005; Morfeld et al, 2006). Interestingly, they remark that the degree of sustained inflammation experienced by rodents (most notably rats) at high lung burdens has not been observed in humans. However, humans may experience sustained inflammation in certain disease states such as late-stage, interstitial pulmonary fibrosis (Daniels & Jett. 2005)

Notably they draw attention to the fact that: "The precise role of chronic inflammation in the development of cancer is uncertain, but there is considerable evidence that chronic inflammation may have a multifaceted role in this process. Activated cells in the lung are known to release various reactive intermediates, most notably those derived from oxygen. Sustained excess of oxidant activity is known to deplete antioxidant defences gradually. These defence mechanisms in the lungs of of humans and rats clearly differ, in that humans are overall relatively deficient in some of them (Slade et al, 1985)." This latter is an important point as it emphasises the potential for differences in pathological response between the two species. In addition,

the IARC Working Group agreed with the now generally accepted position that: "The mechanism that involves inflammation and oxidative stress which lead to tumour formation is considered to be a secondary genotoxic mechanism, in contrast to a primary genotoxic mechanism in which the agent interacts directly with DNA (Knaapen et al, 2004)."

Probably, the most illuminating part of the IARC Working Group discussion relates to their deliberations on interspecies extrapolation in which they make use of the cascade of events, illustrated above in Fig 4.2, and try to evaluate these events seen in rats from particle deposition to lung tumour formation to these events in other experimental species and humans. They note that: "An important question that needs to be addressed is the extent to which the steps outlined in Fig. 4.2 Fig. 5.5 for rat lung cancer are also operative in other animal species including humans."

Their considerations in this regard lead them to make to following statement.

"Rats and mice, in contrast to hamsters, exhibit sustained inflammation associated with particle lung burden, but lung tumours induced by poorly soluble particles have only been observed in rats. It has been shown that rats are uniquely susceptible to poorly soluble particle-induced lung cancer relative to mice and hamsters. While some of the steps indicated in Fig. 4.2 have been demonstrated in humans exposed to poorly soluble particles, it is not known to what extent humans are susceptible to particle-induced lung cancers associated with titanium dioxide, carbon black or talc."

Clearly, the IARC Working Group have drawn a weight-of-evidence distinction between the steps from particle induction to lung tumour induction in rats and the situation in humans, where the totality of these necessary MoA steps have not been demonstrated. Although these conclusions were directed towards the experimental and human findings for titanium dioxide, carbon black and talc (the three particulate substances that the IARC Monograph meeting was evaluating), it is considered that the same considerations would equally apply to all respirable PSPs.

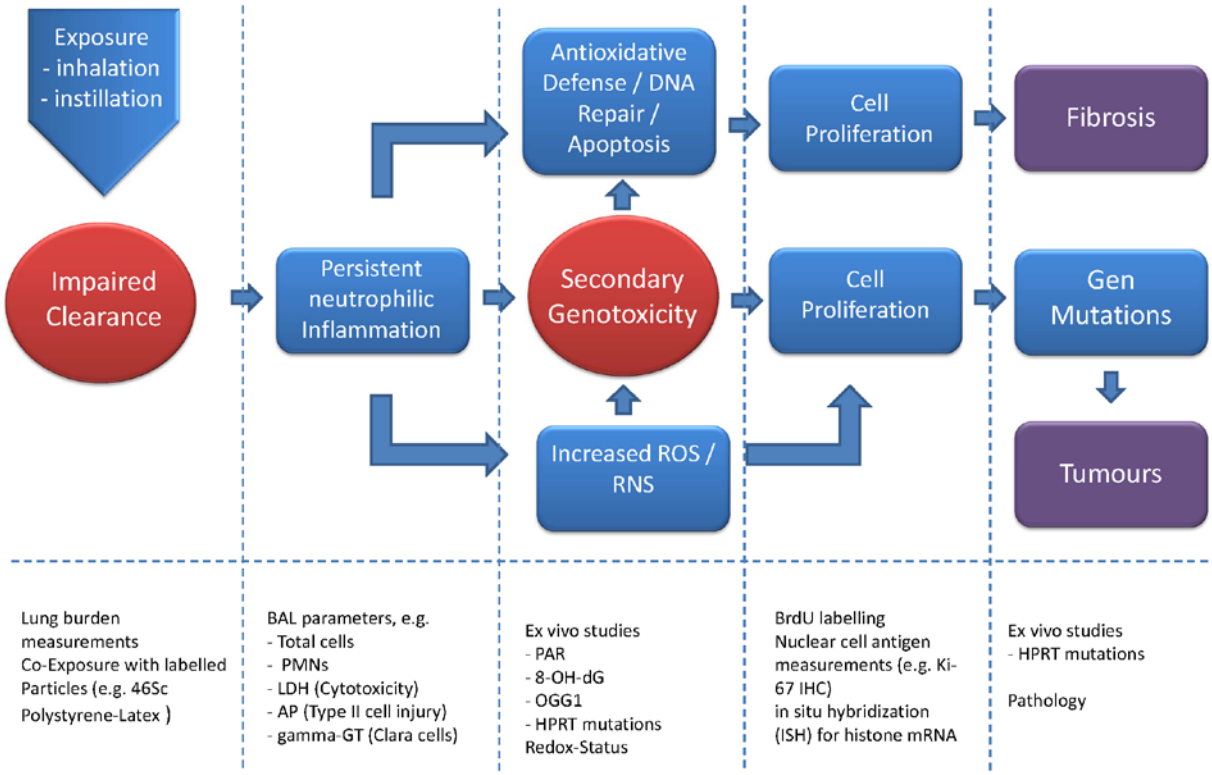
5.6 Adverse outcome pathway

An `Adverse Outcome Pathway`(AOP) describes the sequential progression of events evolving in an organism from the first contact of a toxicant at the molecular level, via a subset of following key effects or biological responses to a final adverse outcome at the individual or population level (OECD 2013). Although AOPs can be outlined as a linear cascade of consecutive events, where one common molecular initiating effect is the prerequisite for all subsequent steps, the `adverse outcome` may vary significantly. In this respect AOPs take into account that different molecular initiating events can cause the same adverse outcome as well as that many different `mode-of actions` (MoA) share common key molecular initiating events. Even though the adverse outcome observed in vivo is the result of a sequential cascade of biological events, each step in this pathway may itself be influenced by other pathways ongoing and/or dominating within the biological system of interest. Thus it is the intrinsic chain of causally linked biological events that determines the AOP and in cases where species specific toxicodynamics can influence various possible MoAs different phenotypic outcomes may be triggered. Species related differences associated with specific biological functions expressed by particular cell types, such as the inhibition or generation of reactive oxygen species in

various organs and/or tissues, may therefore be responsible that the same substance can cause different pathological outcomes based on a common initial molecular event.

The development of an AOP depends largely on the identification of the `molecular initiating event`, the relevant intermediate events and the final adverse outcome. Based on above outlined concept it becomes clear that each AOP will have only one `molecular initiating event` and one `adverse outcome` as apical endpoint. With regard to "lung overload" effects following chronic inhalation to PSP of low acute toxicity, obvious species specific differences exist in the `adverse outcome`. Rats are considered to be particularly sensitive towards PSP induced lung toxicity compared to other species. Although an accumulation of particles in the deep lung is a common finding in all investigated species, significant differences in the phenotypic `adverse outcome` between rats and all other mammalian species exist. Lung tumours have been reported exclusively in rats, but not in mice, hamsters, non-human primates or humans. It is well established that lung `overload` contributes to the observed (species independent) pathogenesis of non-neoplastic lung responses, with the significant impairment of pulmonary particle clearance as `initial event` relevant for AOP considerations. Since the induction of persistent neutrophilic pulmonary inflammation, apoptosis, generation of ROS/RNS and increased cell proliferation are also recurring descriptors of events occurring during "overload" triggered lung pathology, they may be regarded "intermediate events" in the sense of an AOP. However, with regard to the final biological effect tumours have to be considered the `adverse outcome` only in rats whereas non-neoplastic changes, e.g. fibrosis, seem to be the `adverse outcome` in other species. Key events in the context of an AOP, which are relevant for the diversion of the so far common mechanistic sequence of effects leading to different outcomes may be the consequence of differently equipped cell systems, e.g. in the diversity of toxification/detoxification systems, like anti-oxidants impacting the degree of resulting `oxidative stress`, apoptosis as well as DNA repair capacity. A simplified depiction of an AOP linking a common initial event with different phenotypical outcomes is shown in Figure 5.

Figure 5: Conceptual AOP of `lung overload` related key events and possible investigative methods



5.6.1 (AOP) rat

The development of pulmonary tumours in rats after PSP exposures occurs only under the conditions of sustained particle overload in the lungs. This pulmonary pathological response in rats is unique, as other rodent species (i.e., mice/hamsters) and larger mammals such as humans or non-human primates do not develop lung tumours under similar conditions of particle lung overload. The evidence to support this conclusion has been addressed in some of the studies listed above: 1) chronic particle overload PSP exposures produces lung tumours in rats, but not in mice; 2) in subchronic inhalation studies with ultrafine TiO_2 and carbon black particles rats, mice and hamsters have been exposed to identical test substances at the same concentrations. Rats, but not mice or hamsters developed atemporal pathological sequelae in lung responses to particle overload conditions. This is initiated by sustained lung inflammation and cytotoxicity responses, followed sequentially by accelerated cell proliferative and fibroproliferative effects (e.g., hyperplasia, septal fibrosis, secondary genotoxicity, etc.); ultimately leading to lung tumour development. Contributing to this pathological scenario conceivably could be the generation "abnormally high" pro-inflammatory responses, concomitant with a deficiency in anti-inflammatory cytokine responses, leading to a perpetual imbalance of adverse effects in the rat lung. This would appear to be a potentially important mechanism for either increasing the sensitivity and/or overriding the protective capacity of the rat lung response to particle overload. Table 2 summarises the difference in the pulmonary responses to particle overload exposures in rats vs. mice, hamsters and primates/humans.

5.6.2 (AOP) mouse and hamster

There are three informative sub-chronic inhalation studies from which to compare the Adverse Outcome Pathways of the rat, mouse and hamster. In all three studies, females from each of these species were exposed to the same dose-related concentrations of identical particulates for 13 weeks duration, and evaluated at the same post exposure time points by the same criteria.

In the first study with pigment-grade TiO_2 particles, groups of female mice, rats and hamsters were exposed to 10, 50 or 250 mg/m3 TiO_2 particles for 13 weeks and evaluated at several post-exposure time points up to 52 weeks (Bermudez et al, 2002). A variety of pulmonary endpoints were investigated, including inflammatory parameters, cytotoxicity indices, lung cell proliferation labelling kinetics and histopathological alterations. The authors noted that retained lung burdens following exposures were greatest in mice. Using particle retention data, it was determined that lung particle overload was achieved in both rats and mice at the exposure levels of 50 and 250 mg/m^3. Lung inflammation and cytotoxic effects were noted initially in all three species at 50 and 250 mg/m^3. The ranking order of severity of effects among the species was the following: rats > mice > hamsters.

In mice and rats, the BALF inflammatory responses remained elevated compared to controls throughout the entire post exposure recovery period in animals exposed to 250 mg/m^3. In comparison, inflammation in hamsters was short-lived, likely due to the more rapid clearance of particles from the lung concomitant with the more protective, anti-inflammatory cytokine responses in this species. Pulmonary lesions were most severe in rats, where progressive epithelial- and fibroproliferative changes were observed in the particle overload 250 mg/m^3 group. These epithelial proliferative changes were characterised in rats as increased alveolar epithelial cell proliferation. Associated with these foci of epithelial proliferation were interstitial particle accumulation and alveolar septal fibrosis. It was interesting to note that although exposed female mice demonstrated evidence of particle overload and pulmonary inflammation, unlike the exposed rats, high-dose TiO_2-exposed mice were devoid of the fibro-proliferative and fibrotic tissue responses measured and observed in exposed rats. Moreover, when compared to identically exposed rats and mice, female hamsters had the weakest pulmonary inflammatory responses to the titanium dioxide dust burden concomitant with the fastest clearance times.

The second study of note consisted of an interspecies 13-week subchronic inhalation study; with female rats, mice and hamsters exposed to 3 concentrations of ultrafine TiO_2 particles (Bermudez et al, 2004). Female rats, mice and hamsters were exposed to aerosol concentrations of 0.5, 2.0 or 10 mg/m3 of uf-TiO_2 particles for 13 weeks and assessed over several post-exposure time points up to 1 year. Similar to the study effects measured with pigment-grade TiO_2 particles, mice and rats had similar retained lung burdens at the end of the exposures, whereas hamsters had retained lung burdens that were significantly reduced. Lung burdens in all three species decreased with time after cessation of exposures. Moreover, at the end of the recovery period (i.e., ~ 1 year post-exposure), the percentages of particle burden remaining in the lungs of the 10 mg/m^3 group were 57, 45 and 3% for rat, mouse, hamster, respectively. The retardation of particle clearance from the lungs in mice and rats of the 10 mg/m^3 groups were indicators that particle overload had been achieved in these animals, but not in hamsters. BAL fluid biomarkers demonstrated that lung inflammation and cytotoxicity were apparent in rats and mice exposed to 10 mg/m^3 uf-TiO_2. The neutrophilic inflammatory responses in rats, but not mice, declined in a time-dependent manner correlating with a reduction in lung burdens; however, the fraction of recovered PMNs at 52 weeks post-exposure was equivalent in the two

species. There were no significant changes in cellular responses, or with markers indicating toxicity in hamsters; reflecting the capacity of these animals to rapidly clear particles from the lung. Similar to the results reported in the interspecies, pigment-grade TiO_2 study, progressive epithelial and fibro proliferative changes were observed in rats exposed to 10 mg/m^3 TiO_2 particles. Lung lesions were characterised by foci of alveolar epithelial proliferation of metaplastic epithelial cells which were located adjacent to heavily particle-laden macrophages; along with augmented alveolar epithelial cell proliferation indices. Associated with these foci of epithelial proliferation were interstitial particle accumulation and progressive alveolar septal fibrotic responses. It is noteworthy that epithelial, metaplastic and fibroproliferative changes were not noted in the lungs of either mice or hamsters.

To summarise the results of the study findings, there were significant species differences in the pulmonary responses to inhaled uf-TiO_2 particles. Rats developed a more severe inflammatory and fibro proliferative response compared to mice. Clearance of particles from the lung was markedly impaired in mice and rats exposed to 10 mg/m^3 uf-TiO_2, whereas clearance in hamsters did not appear to be affected at any of the administered doses.

From a qualitative standpoint, these data are consistent and virtually identical with the results of the subchronic inhalation study with inhaled pigmentary TiO_2 and demonstrate that the pulmonary responses of rats exposed to ultrafine particulate concentration likely to induce pulmonary overload are different from similarly exposed mice and hamsters. These differences can be explained both by pulmonary responses and by particle dosimetry differences among these rodent species.

The third study was again an interspecies, 13-week sub chronic inhalation study; with female rats, mice and hamsters exposed to three concentrations of carbon black (CB) particles (Elder et al, 2005). In this study, the authors postulated that the lung inflammation and injury induced by subchronic inhalation of CB are more pronounced in rats than in mice and hamsters. Particle retention kinetics, inflammation, and histopathology were assessed in female rats, mice, and hamsters exposed for 13 weeks to high surface area CB (HSCb) at doses chosen to span a no observable adverse effects level (NOAEL) to particle overload (0, 1, 7, 50 mg/m^3). Pulmonary retention and lung effect measurements were conducted immediately after exposure as well as 3 and 11 months post-exposure; retention was also evaluated after 5 weeks of exposure. Significant decreases in body weight during exposure occurred only in hamsters exposed to high-dose HSCb. Lung weights were increased in high-dose CB-exposed animals, but this persisted only in rats and mice up to the end of the study period. Prolonged retention was measured in rats exposed to mid- and high-dose HSCb. Retention was also prolonged in mice exposed to mid- and high-dose HSCb, and in hamsters exposed to high-dose HSCb. Lung inflammation and histopathological effects were more severe and prolonged in rats when compared to mice and hamsters, and both indices were similar in rats exposed to mid-dose HSCb. The results show that hamsters have the most efficient clearance mechanisms and least severe responses of the three species.

An additional component of the CB study was published one year later, wherein the investigators assessed several pro and anti-inflammatory mediators to detect underlying mechanistic differences, which could account for the disparity in lung inflammatory responses to inhaled particulates among the rodent species (Carter et al, 2006). Here, the investigators looked at comparative dose-related responses of several key pro- and anti-inflammatory mediators in the lungs of rats, mice, and hamsters after subchronic inhalation of CB. As discussed above, rats, mice, and hamsters were exposed to air, 1, 7, or 50 mg/m^3 of CB for 13 weeks and subjected to bronchoalveolar lavage 1 day, 3 months, and 11 months post- exposure. Some of the endpoints

included recovered cell number and differential type, reactive oxygen and nitrogen species, and cytokine levels. Ex vivo mutational analysis of inflammatory cells was performed by co-incubating with lung epithelial cells. In addition, lung tissues were processed and evaluated for gene expression of selected anti-inflammatory mediators. The results demonstrated that a dose- and time-related effect existed with all the parameters. Rats demonstrated greater capacity for generating pro-inflammatory responses. In contrast, mice and hamsters demonstrated an increased proclivity for generating anti-inflammatory responses. The authors concluded that the cytokine differences in generating pro- and anti-inflammatory responses correlated with in vivo lung tissue responses and could play a significant role contributing to the apparent species differences in inflammation and tumourigenesis.

In summary, the results of these three above similarly designed sub chronic studies with particulates are remarkably similar in their findings. In all three studies, the rat model appeared to be the most sensitive to the development of lung inflammation, cytotoxicity, fibro-proliferative effects, and septal fibrotic response to high concentrations of particles that reached overload status. In contrast, under identical exposure circumstances, mice developed particle overload status with reduced or absent lung clearance effects, concomitant with strong evidence of robust lung inflammatory responses. However, despite reaching particle overload and lung inflammatory effects, the pulmonary tissue responses in the mouse were fundamentally different from the rat. Indeed there was no evidence of enhanced cell proliferative changes, fibro-proliferative effects or septal fibrosis – all of which are likely to lead to secondary genotoxic effects and the development of lung tumours following high-dose chronic inhalation of low solubility particulates. These extensive investigative findings separate the mouse and hamster responses from the pulmonary overload responses observed and measured in time course studies with rats. In addition, it should be noted that the hamster tends to have an accelerated particle clearance mechanisms, concomitant with reduced lung inflammatory responses to high dose particle exposures. Moreover, the reported findings by Carter et al, 2006, suggest that one potential mechanism for the differences in adverse lung responses in mouse and hamster vs. rats could be related to the enhanced generation of anti-inflammatory cytokines in mice and hamsters and the augmented generation of pro-inflammatory cytokines in the rats. This would likely lead to an imbalance in the rat response to overload particle exposures and could foster the development of the mechanistic pathological sequelae/events which have been postulated to occur in the rat lung response to high-dose particulates.

5.7 Relevance of `Lung Overload` for humans

As noted in the ILSI Workshop Consensus Report in 2000, high-dose, long-term exposures to poorly soluble particulates (PSPs), which produce lung tumours in rats, do not induce neoplastic pulmonary effects in similarly exposed mice and hamsters. Moreover, the abundance of available clinical and epidemiological data in occupationally-exposed workers is consistently negative for lung cancer as well as non-neoplastic lung diseases. Levy (1995) concluded that the findings of rat-specific lung neoplastic responses to chronic PSP exposures are unique to that species. Therefore, it was noted that the findings in rats are not useful endpoints for human risk evaluations of poorly soluble particulate exposures. In contrast to the experience with rats, epidemiological findings in coal mine workers, a -well studied occupationally- exposed group of workers with routine "particle overload" in their lungs, clearly demonstrate a lack of lung cancer risk when correlated with exposures. In addition, results from several extensive human epidemiology studies in

titanium dioxide or carbon black exposed workers clearly have demonstrated that long-term occupational exposures to these particle-types do not cause lung cancer or non-cancerous diseases of the respiratory tract.

In attempting to elucidate differential mechanisms between rats and humans when investigating pulmonary responses to high dust concentrations, the studies reported by Nikula et al, 1997 serve to be particularly instructive. By studying lung morphological and morphometric responses of rats and humans under similar exposure conditions, the researchers demonstrated fundamental differences between the two species when assessing particle retention patterns and lung tissue and cellular reactions following high dose dust exposures. Interpretation of the findings appears almost counterintuitive – that is, under a wide range of chronic exposure conditions, inhaled diesel soot is retained predominantly in the alveolar regions of rats. In contrast to the dosimetry in rats, long-term inhaled particulates are retained primarily in the interstitial compartments in humans. This finding implies a much greater rate of particle translocation from deposition sites through pulmonary epithelium. The measured differences of particle retention and distribution patterns in rats vs. humans, may account for the following findings: 1) longer particle clearance rates in humans vs. rats; 2) enhanced sensitivity in sustained rat lung inflammatory and cytotoxicity responses; and 3) sustained respiratory tract pathological tissue responses in rats corresponding to a unique pathological pattern commencing with inflammation, fibro proliferative disease, cell proliferation, genotoxicity and lung tumour development. In contrast, none of these parameters have been documented in PSP-exposed workers (Table 2).

Table 2: Interspecies lung responses[a] following long-term or chronic inhalation exposure to PSPs

Species			
Rat	**Mouse**	**Hamster**	**Primate/Human**
Likelihood for developing particle overload (slow lung clearance)			
+++	+++	+	Not determined
Alveolar macrophage participation			
Active (accumulation in alveolar ducts)	Active (accumulation in alveolar ducts)	Extensive (rapid clearance)	Not as extensive (translocation to interstitial sites)
Pulmonary (neutrophilic) inflammation			
+++	+++	+	+
Epithelial and interstitial cell proliferation			
+++	+	(+)	(+)
Septal fibrosis			
+++	+	(+)	(+)
Anatomical location of retained particulates			
Primarily alveolar (some increased translocation at overload)	Primarily alveolar (some translocation at overload)	Rapid clearance	Primarily interstitial
Lung tumours following chronic exposure			
Yes	No	No	No

[a] Severity low +, moderate ++, high +++, or questionable (+)

5.8 Biomathematical modelling of respirable dust in human lungs

Based the particle burdens in the lung and hilar lymph nodes measured in US coal miners, Kuempel et al, developed biomathematical model of particle clearance and retention (Kuempel et al, 2001a). They found that the particle clearance and retention as observed in the lungs and lymph nodes of coal miners was poorly described by the one-compartment dosimetry model which is applicable to rats, e.g. (Muhle et al, 1990). Kuempel et al developed a 3-compartment model consisting of the deposition of inhaled particles in the alveolar region, competing processes of either clearance from the alveolar region or translocation to the lung interstitial region, and very slow irreversible sequestration of interstitialised material in the lung-associated lymph nodes. Kuempel et al (2001a) also compared the required lung dust burden in rats and humans which would lead to a decline of the alveolar-macrophage-mediated clearance rate. Based on the differences in macrophage volume and number, the minimal (onset of clearance rate decrease) and maximal (levelling off of the clearance rate decrease) coal dust burdens were estimated and these were found to be approximately 33% higher in humans than in rats (Kuempel et al, 2001a). The decrease of the macrophage-mediated lung clearance as observed in rodent studies was shown not to be a major determinant of the lung burdens in the US miners. The half-time of respirable particle retention in coal miners' lungs was shown to be 5 to 15 years, compared to less than 2 years in persons without dusty jobs. The increased retention half-time in coal miners might be consistent with a substantial portion of the dust being sequestered at all lung dust burdens, due to the longer residence time in the alveolar region. Only in the "overloaded" rat, i.e. at significantly slower alveolar-macrophage-mediated clearance rates, does the lung burden reach concentrations as high as those observed in some humans with occupational dust exposure, such as coal miners. Kuempel et al, (2001b) explored the biomathematical model further in a second publication looking at the model's variability and uncertainty which confirmed the earlier findings, i.e. the best-fitting exposure-dose model to the US miners data had substantial interstitialisation/sequestration of particles and no dose-dependent decline in alveolar clearance. Tran and Buchanan (2000) confirmed the model proposed by Kuempel et al, using a data set from UK coal miners. In addition, they observed a different behaviour of the quartz and non-quartz fraction of coal dust, the first showing a higher tendency to remain in the lung and lymph nodes. More recently, the 3-compartment model has been shown to also best predict the exposure to radioactive cobalt or plutonium (Gregoratto et al, 2010a,b). The model structure was recently adopted by the International Commission on Radiological Protection (ICRP) to describe the long-term clearance and retention of inhaled particles in the alveolar-interstitial region of the human respiratory tract and an additional statistical analysis of the model confirmed once more the conclusions made earlier by Kuempel et al (Sweeney et al, 2013).

In conclusion, albeit that the model has only been validated using a limited number of dusts, it provides rather strong indications that the overload concept as being described for rats is of little relevance for humans which have been chronically exposed to high levels of dusts. The relevance of effects which are observed in rats in conditions at which the alveolar-macrophage-mediated lung clearance has been seriously affected might be limited because such conditions do not appear to occur in humans according to the model developed by Kuempel et al, On the other hand, the interstitialisation/sequestration of particles from the alveolar spaces, possibly followed by translocation to the draining lymph nodes appears to be of higher relevance in humans, even at lower level exposures.

6. INFLUENCE OF METHODOLOGY USED

There are different in vivo and in vitro methods that are used to study pulmonary toxicity of various chemicals including PSP. In vivo, test substances are generally administered mainly by inhalation of aerosolised material or by direct intratracheal instillation of suspended material into the lungs of rodents. Other methods e.g. "pharyngeal aspiration" or "endotracheal respiration" have been applied by a few laboratories for mechanistic examinations, are not widespread yet. In vitro, there are quite a few cell lines or multi-culture models available. In the following Section the major methods and their pros and contras are summarised.

6.1 Inhalation

Inhalation tests were developed, updated and refined during the past few decades to examine potential hazard of inhalable substances. In an inhalation study, atmospheres containing aerosols are generated in inhalation chambers, animals are exposed either nose-only or whole body to an atmosphere. The concentration of the test item in the atmospheres is to be determined appropriately. During the whole exposure period, the atmospheric concentrations have to be kept as constant as possible. For aerosols, particle size distributions have to be determined by cascade impactors or other equivalent devices.

This type of application provides the natural route of entry into the host and has gained significance in the regulatory context. OECD test guidelines (No. 403, 412 and 413) for inhalation exposure was adopted first time in 1982 and revised in 2009. A comprehensive guidance document (GD 39) was also released by OECD in 2009. Beside the OECD test guidelines there were guidelines released by European Commission Regulations (EC No 440/2008 in 30 May 2008) and US Environmental Protection Agency (OPPTS 870.1300 and 870.3465). The technical requirements described in these guidelines are comparable. Based on the determined no observed adverse effect concentration, considering certain inter- and intra-species differences, occupational limit concentrations can be derived.

6.2 Instillation

Although inhalation technique is more physiological, direct intratracheal instillation has often been performed to assess toxicity in the lung. During intratracheal instillation, animals are narcotised. For rodents, short-acting anaesthetics, which suppress reflexes for a minimal period of time and allow the animal to recover quickly and regain normal respiration, are preferred. Halothane, metaphane, enflurane and isoflurane are inhaled anaesthetics frequently used. The deeply narcotised animal is positioned on an angled restraining stand, its throat illuminated by a light source. The test substance is suspended in an appropriate vehicle and instilled into small laboratory rodents usually by inserting a catheter or needle trans-orally into the tracheal lumen (Brain et al, 1976; Saffiotti et al, 1968, 1972).

6.3 Pros and cons of instillation and inhalation

Both inhalation and instillation are in vivo methods that have their strengths and weaknesses. Being aware of them may help the researcher who has to choose the appropriate method in a scientific and economic way. Therefore, this Section focuses on the differences between the two methods, which are discussed first in the text and then presented in a short form in Table 3 followed by some salient examples.

In a number of ways inhalation exposure is preferable to intratracheal instillation exposure. First, a key advantage of inhalation exposure is that it is the physiological route of exposure to airborne particulates. Second, inhalation considers any effect on the upper respiratory tract, while instillation bypasses upper respiratory tract (e.g. nasal cavity, larynx) and its defence mechanisms. Third, inhalation exposure leads to a more even distribution throughout the lung lobes, while instillation produced highly uneven patterns of lung retention (Brain 1976, Pritchard et al, 1985, Leong et al, 1986, Dorris and Valberg 1992). Fourth, inhalation exposure provides a continuous low deposition and retention rate of test material, while intratracheal instillation delivers an abrupt, concentrated (bolus) dose (Pritchard et al, 1985). This later may overwhelm and overload specific respiratory defence mechanisms and cause more severe lesions (Driscoll et al 1990). Finally, the instillation procedure and liquid per se may produce tissue inflammation not typical of inhalation (Kazmierowski et al, 1977, Driscoll et al, 1990).

However, inhalation exposure requires extensive technical experience and exposure chamber facilities and is expensive. The amount of material that is deposited in the lungs is dependent upon numerous variables, including the aerodynamic diameter and concentration of aerosol particles, the minute ventilation, the breathing pattern, and the disease state of the animals (Brain 1979). These uncertainties may lead to inter-laboratory differences. Another shortcoming is that most particulates have to be micronised by grinding to generate respirable sizes which involves strong mechanical forces and high temperatures and may change particle characteristics.

Instillation technique does not require elaborate exposure chamber and aerosol generation and characterisation equipment. As the equipment required is simple, the technique is inexpensive compared to inhalation exposure. As reproducibility of delivery is highly dependent upon the experience of the individual performing the procedure, appropriate training is essential. Provide that well-trained technicians perform the procedure, the amount of substance delivered is easy to quantify and it is less variable among animals. Thus, intratracheal instillation has been used in many studies as an alternative to inhalation. When substances are very toxic, radioactive or available only in small quantities, instillation may however the only way to examine pulmonary toxicity of substances because it requires significantly less test material and enables safe handling of the test article.

Table 3 summarises the above considerations as follows.

Table 3: Pros and cons of inhalation and instillation techniques

Aspect	Inhalation	Instillation
Technical requirements	High	Low. Appropriate training is essential
Test substance	Large quantity	Quantity as for usual oral toxicity studies
	Handling of highly toxic or radioactive substances prohibited	Handling of highly toxic or radioactive substances possible
Cost	High	Low
Dose	Defined by test atmosphere concentration as mg/m^3, real deposition in the respiratory tract is influenced by, inter alia,. MMAD, particle density and respiration physiology of test animal	Exactly defined as instillated dose (mg/kgbw or mg/animal)
Deposition	Continuous low deposition and retention rate of test material. Overload condition achieved after a certain exposure period	Abrupt, concentrated dose. Overload condition already achieved after one to a few instillations
Effects	Less severe. Consider whole respiratory tract	More severe. Bypass upper respiratory tract
Regulatory applicability	Occupational equivalent value can be derived	Appropriate for mechanistic examinations, screening studies to compare toxic potency

For poorly soluble particles, instillation of quartz and titanium dioxide caused more severe and early effect than inhalation due to the bolus dose (Driscoll et al 1990). However, the type of responses was similar irrespective of the exposure technique. A study conducted by Henderson et al (1995) with the same materials at lower doses/concentrations indicated similarity of the two exposure methods. Interestingly, after inhalation exposure to quartz micro granuloma was observed on pleura surface besides bronchial-associated lymphoid tissue, whereas those formed after instillation was not on pleura surface. This finding indicates that instillation may fail to reveal affected tissue in inhalation study.

A major concern regarding the use of intratracheal instillation is that the introduction of a large bolus dose of the test substance into the lung in a short period of time will overwhelm the normal lung response and defence systems to the extent that it may produce responses that are pathophysiological artefacts that would not be seen if the same dose was delivered over a longer period of time via an inhalation exposure. This can thus produce serious problems for the interpretation of both hazard identification and risk assessment.

Based on their chemical composition as well as their density, surface, mean particle size and behaviour in water Pott and Roller selected 19 different mostly bio persistent dust samples for intratracheal instillation experiments with 2050 female rats in 43 dose (mostly 2 per dust) and 3 control groups (Pott and Roller, 2002). A total of 5 to 20 instillations (once per week) and a broad dose spectrum between 1 and 6 mg per rat and instillation in most cases summed up to a cumulative dose of 30-120 mg dust per rat lung. For quartz, diesel soot, hydrophobic TiO_2 (P805) and Ruß, carbon black (Printex 90), smaller doses were used, however with only the latter one fulfilling the criteria for granular biopersistent dust. Unfortunately, with Printex 90 a dosing errors occurred in the lowest dose group (5x1, 5 mg group) by instillation of additional 3 mg dose of ultrafine titanium dioxide. The selection of the highest and the lowest dose level was based on the lifetime particle lung retention of coal miners' and women in cities in the last decades, respectively. Each exposure

experiment comprised a total of 130 weeks after which the rat lungs were examined for tumour incidence and tumour type (Pott and Roller 2002)

Valberg et al, (2009) critically reviewed the outcome of the Pott and Roller study and came to the conclusion that the tumour incidence reflected a saturated inflammatory response due to a rat specific lung overload and is not useful for human risk assessment. The dose selection was based on the assumption that the rat is a miniature human, however, differences in particle translocation (Section 5.7) do not allow for this simple dose transformation. With only providing the tumour data the studies do not provide any new information but just show what has been expected to happen with a biopersistent dust in the rat at lung under overload conditions capable of inducing an inflammatory response.

Overall, when testing toxicological impact of occupationally-relevant dusts in animal models, both inhalation of airborne material and intratracheal instillation of fluid-suspended dust into airways may be employed. Although intratracheal instillation is non-physiological and less desirable than inhalation of airborne dust, it may be helpful if certain conditions are met. Keeping in mind that instillation has distinct differences in the distribution, clearance and retention of particles; the doses should not exceed a specific quantity in order to prevent results being dominated by non-specific artefacts. While inhalation studies produce data that can be used for risk assessment, instillation studies are more appropriate when the toxic potency of several materials should be compared.

6.4 In vitro methods

The successful development of in vitro assays with cultured lung cells and aerosols have been critical for fostering predictive screening tests during the early phases of product development and mitigate some acute inhalation toxicity testing efforts. The potential advantages of these tests result from simpler, faster, and less expensive acute studies when compared to their in vivo counterparts. Perhaps more importantly, accurate and reliable development of in vitro testing systems will result in the reduction of animal use (3Rs) for screening studies of potential pulmonary test materials. Currently however, the accuracy of predictive results from in vitro screening studies for estimating in vivo pulmonary effects of respirable particles is unreliable, although there are a number of initiatives underway to develop such reliable testing schemes

Although some progress has been made in the development of alternative (in vitro) methods to estimate in vivo toxicity endpoints of substances, reliable and predictive methodologies are still lacking. Most approaches thus far have revealed a lack of convergence when comparing particle induced effects when comparing in vitro vs. in vivo results on identical or similar particle-types (Seagrave et al, 2005, Sayes et al, 2007). These studies have utilised inflammatory and cytotoxic endpoints and reported little correlation among the biomarkers (e.g., inflammation/cytokines and LDH indices (Sayes et al, 2007).

The lung is a complex organ system comprised of many different interdependent cell-types (i.e., types I and II alveolar epithelial cells, macrophages, interstitial cells, vascular cells, etc.), thus the pulmonary microenvironment and the complex interactions in the lung which occur following inhalation and subsequent deposition of particles is difficult to simulate using in vitro techniques. Numerous studies have reported toxicity results with single cell-types. However, these are rather simplistic in scope and ignore the complex interactions in the lung milieu that occur following exposure to aerosols of particles, bacteria, etc.

Ng and Warheit (2013) have attempted to develop a complex in vitro system which can, in part, simulate particle phagocytosis, an important aspect in the simulation of lung defence responses to particles, using lung epithelial – macrophage cellular co-cultures. The development of such in vitro techniques may serve to expedite the transition from the current animal-based testing system to one that is based primarily on human cell lines and in vitro assays. Such reproducible, accurate, and validated cell-based in vitro assays for assessing pulmonary hazards will have important benefits in screening more compounds in a faster, more reliable, and less expensive manner. In earlier comparative reports, Warheit et al (2007, 2009a,b) published the results of three studies demonstrating that in vitro pulmonary toxicity studies with particulates in a submerged culture system did not correlate with the measured in vivo effects when assessing identical particulate-types. Therefore, it would be useful to develop and implement a more efficacious/predictive cell culture/exposure system, in order to bridge the major technical gap on simulating in vitro exposures with relevance to the in vivo lung micro-environment. During the past year, some progress has been made in transitioning (and optimising) from a submerged in vitro cell culture system to a more physiologically relevant, in vitro air-liquid interface (ALI) system, using Transwell® Permeable Support devices (microporous membranes), for providing co-cultures (of rat lung epithelial L2 cells and NR8383 alveolar macrophages) applicable to potential in vitro cell exposures and toxicity assays. Certain cell growth conditions and parameters, and the applicability of several toxicity (XTT, LDH, and cytokine release) assays to the ALI co-culture cell model, are being confirmed and verified. Since an advanced aerosol exposure system is critical for exposing the co-cultures on ALI, Warheit reports that they are now testing a new device, the Nano-Aerosol Chamber for In vitro Toxicology (NACIVT); while developing methodologies to generate and document aerosol exposure concentrations at verifiable dose metrics. The output of this system will be to measure a variety of cellular toxicity end-points (of the co-cultures), namely, cytotoxicity indices, as well as generation of several inflammatory cytokines in a dose-dependent, time-course experimental protocol. The efficacy and validation of this approach remains to be determined. The proposed studies are designed to provide greater sophistication and relevance to the in vitro ALI cell exposure system by assessing the performance of the NACIVT to provide informative and reproducible results. Collectively, these efforts could expedite and facilitate the inevitable transition (as promulgated in the National Academy of Sciences Toxicology in the 21st Century report) from the current animal-based testing system to one that is based, in part, on more sophisticated in vitro toxicity and screening tests.

To summarise this Section, validation of the predictive value of in vitro screening assays for gauging in vivo pulmonary toxicity of particulates will be essential. In addition, study designs will require a focus on developing test with relevant particle dosimetry and realistic doses, and time course studies to gauge the sustainability of any measured endpoints. The cell-types used in the in vitro study designs should simulate respiratory tract point-of-entry routes of exposures. These integrated components are important prerequisites for developing in vivo predictability responses.

7. HUMAN DATA, INCLUDING EPIDEMIOLOGY

7.1 Existing data inclusive epidemiology

7.1.1 Various dusts

Past exposures to dust in coal miners have resulted in the development of pneumoconiosis, with good correlation between dust exposure (concentration), duration and coal workers pneumoconiosis (Attfield and Morring, 1992).

Additionally, King et al (1956) showed a relationship between the severity/grading of diagnosed pneumoconiosis and lung dust burden, i.e. high lung burdens correlated in general with more severe disease. This study found, on average, 35 g of dust in colliers' lungs.

Stöber et al (1967) reported similarly high dust burdens in the lungs of miners together with extended clearance half-lives. However, overloading of human lungs and reduced clearance rates are not associated with the excessive inflammation and subsequent development of pulmonary tumours which appears to be the typical response of the rat lung to such conditions. This indicates that there are distinct differences in the tissue responses of rat and human lungs to high burdens of low toxicity particulates.

In this context, Nikula et al (1997) compared the anatomical patterns of particle retention and the lung tissue responses between rats and cynomolgus monkeys following chronic exposure to diesel exhaust and coal dust. There was no significant difference between diesel exhaust-exposed monkeys and rats in the relative amount of retained particulate materials but a very important difference was that rats retained a greater portion of the particulate material in the lumina of alveolar ducts and alveoli than monkeys; and monkeys retained a greater portion of the particulate material in the interstitium than rats. Rats, but not monkeys, had significant alveolar epithelial hyperplastic, inflammatory and septal fibrotic responses to the retained particles. The authors concluded that the results suggest that intrapulmonary particle retention patterns and tissue reactions in rats may not be predictive of retention patterns and tissue responses in primates exposed to poorly soluble particles at concentrations representing high occupational exposures. Additionally, they comment that the pulmonary responses of the rats were severe compared to the primate, where the insult to the lungs was handled without adverse consequences (Nikula et al, 1997).

Nikula et al (2001) concluded: ‘these results show that chronically inhaled diesel soot is retained predominantly in the airspaces of rats over a wide range of exposures, whereas in humans, chronically inhaled particulate material is retained primarily in the interstitium. In humans, the percentage of particles in the interstitium is increased with increasing dose (exposure concentration, years of exposure and/or lung burden). This difference in distribution may bring different lung cells into contact with the retained particles or particle-containing macrophages in rats and humans and, therefore, may account for differences in species response to inhaled particles'. These two publications (Nikula et al, 1997, 2001) demonstrate significant species differences in lung responses to inhaled particulates between rats and primates, including humans. They provide some evidence to suggest that not only does the rat differ from other experimental species with respect to the pulmonary response to high doses of low toxicity particulates, but that this

difference extends also to primates and humans and there is no reason to consider that TiO_2 would be any different (Nikula et al, 2001).

The epidemiology studies investigated whether there was a link between increased incidence of lung cancer and exposure to TiO_2 dust. In all the studies the overall conclusion was the same: 'The results of the studies do not suggest a carcinogenic effect of TiO_2 dust on the human lung' (Hext et al, 2005). This negative finding for lung cancer has been confirmed in other, more recent studies (Ellis et al, 2010, 2013; IARC 2010)

The largely agent-specific lung parenchymal dust diseases (in particular, the pneumoconiosis) were responsible for most of the lung disease mortality and morbidity attributable to occupational exposures in the first half of the century. By the later decades of the century their place had been ceded to disease of the airways, acute and chronic, part of a mid-century epidemic in which tobacco usage was a major environmental cause. Airways disease was broadly defined and included asthma, chronic bronchitis, emphysema, and chronic obstructive pulmonary disease (Becklake, 1998).

The results support four basic mechanisms in the etiology of Coal Workers Pneumoconiosis (CWP) and silicosis:

a) direct cytotoxicity of coal dust or silica, resulting in lung cell damage, release of lipases and proteases, and eventual lung scarring;
b) activation of oxidant production by pulmonary phagocytes, which overwhelms the antioxidant defences and leads to lipid peroxidation, protein nitrosation, cell injury, and lung scarring;
c) activation of mediator release from alveolar macrophages and epithelial cells, which leads to recruitment of poly-morphonuclear leukocytes and macrophages, resulting in the production of pro-inflammatory cytokines and reactive species and in further lung injury and scarring;
d) secretion of growth factors from alveolar macrophages and epithelial cells, stimulating fibroblast proliferation and eventual scarring.

Results of in vitro and animal studies provide a basis for proposing these mechanisms for the initiation and progression of pneumoconiosis. Data obtained from exposed workers lend support to these mechanisms (Castranova and Vallyathan, 2000).

In an epidemiological study in Germany, mortality and cancer morbidity were investigated over the period 1980-2002 (Morfield et al, 2007). In this study, a cohort of coal miners from Saarland (#4579) was analysed regarding mortality and cancer morbidity over the period 1980-2002. Data on causes of death and cancer history was collected form national registers, allocation in dust exposure groups (high or low) was executed by expert judgement and retrospective modelling. The male population of Saarland was used as a control group (Morfield et al, 2007).

With an average work history of 30.4 years in the coal mines, and an average cumulated exposure - between 1400 and 1900 mg/m3 quartz dust times the number of shifts and 16000 to 22000 mg/m3 non-quartz dust times the number of shifts (Morfield et al, 2007).

The health status could be assessed for 99.9% of the cohort population: 1181 deaths (SMR = 0.87; 0.95 interval 0.82-0 92). Cause of death was established in 99.5% of the cohort population: 399 cancer deaths (SMR 0.88; 0.80-0.97) of which 143 lung cancer cases (SMR 0.89; 0.75-1.05). 752 Primary cancer cases were

documented (SIR = 0.86; 0.80-0.92), of which 158 lung cancers (SIR =0.92; 0.78-1.08). Lung cancer risks varied with the history of coal miners' pneumoconiosis; the SMR and SIR ratio is for coal miners approx. 2.5. Exposure modelling did not show any relation with dust exposure. Despite long and high dust exposures no adverse effects of dust exposure could be related with cancer mortality or morbidity. In the analysis effects such as survivor bias, intermediate confounding and dependent censoring were not seen, although the limited number of people in the cohort does limit the complex model analysis. The data of the study are consistent with the scenario that pneumoconiosis acts as a biomarker for lung cancer and not as an exposure marker. A similar association was described for fibrosis (Morfield et al, 2007).

The mortality experience over 22–24 years of 8,899 working coal miners initially medically examined in 1969–1971 at 31 U.S. coal mines was evaluated (Attfield und Kuempel, 2008).

A cohort life-table analysis was undertaken on underlying causes of death, and proportional hazards models were fitted to both underlying, and underlying and contributing causes of death. Elevated mortality from non-violent causes, non-malignant respiratory disease (NMRD), and accidents was observed, but lung cancer and stomach cancer mortality were not elevated. Smoking, pneumoconiosis, coal rank region, and cumulative coal mine dust exposure were all predictors of mortality from nonviolent causes and NMRD. Mortality from nonviolent causes and NMRD was related to dust exposure within the complete cohort and also for the never smoker subgroup. Dust exposure relative risks for mortality were similar for pneumoconiosis, NMRD, and chronic airways obstruction.

In conclusion, the findings from this study show elevations in non-violent cause and NMRD mortality overall and in association with dust exposure, after allowance for age, smoking, and coal rank region. Little definitive evidence was found, however, for any increase in deaths from lung cancer or stomach cancer. A large healthy worker effect appeared to be present, and may have had the effect of attenuating the exposure-response relationships. Mortality was increased with severity of pneumoconiosis as ascertained at start of follow-up. Regional effects, probably associated with coal rank, were very obvious. The results in this study provide additional evidence that exposure to coal mine dust leads to lung diseases other than pneumoconiosis. In particular, the analysis of underlying, and of underlying plus contributing, mortality from chronic airways obstruction shows not only that obstructive airways disease is elevated in coal miners, but also that (1) the risk increases with increasing dust exposure, and (2) manifestation of the disease can occur independently of pneumoconiosis (Attfield und Kuempel, 2008). An interesting review on why there appears to be no increase in lung cancer in coal miners is given by Stayner and Graber (2011)

As an overall conclusion, these more recent studies on the mortality in coalminers seems to support the findings of earlier studies regarding the lack of lung cancer risk, in spite of the presence of inflammatory effects and particle lung overload in this large and well investigated group of workers. This further emphasises the uniqueness of the lung tumours seen in rat lungs under conditions of lung overload caused by exposure to PSPs and their lack of reliability as a predictor to risk to humans from these materials.

7.1.2 Toner

In a retrospective mortality study among employees who were occupationally exposed to toner, it was concluded that the results were consistent with the general mortality patterns among healthy working

populations. There was no evidence that toner exposure increases the risk of all-cause mortality or cause-specific mortality for the 23 categories of death analysed (Abraham et al, 2010).

This cohort study examined the effects of occupational exposure to toner, a particulate material with widespread use in today's society, on mortality. The study included 33,671 employees of a xerographic company employed between 1960 and 1982 as manufacturing workers or customer service engineers. Vital status was tracked through 1999. Standardised mortality ratios (SMRs) were calculated using the US population for comparison. Results: All-cause SMRs for toner-exposed populations were 0.65 and 0.84 for white men and women, respectively, and 0.37 and 0.74 for non-white men and women, respectively. SMRs for all cancers, lung cancer, respiratory disease, and cardiovascular disease in toner-exposed men were lower than 1.0 (Abraham et al, 2010).

This report, using data updated for vital statistics through December 31, 1999, extends the average follow-up time to 26 years and provides strong support that toner exposure does not increase mortality. The exposure to total dust concentrations from the CSE's ranged from below the limit of detection to 0.34 mg/m3 (average value: 0.33 mg/m^3). Field measurements resulted in total dust concentrations of 0.09 to 0.94 mg/m3, with an average of 0.28 mg/m^3. One higher value (3.0 mg/m^3) was considered suspect and was rejected. The average TWA concentration for total dust measured during worst-case testing was only slightly higher (0.38 mg/m^3).

In general, there was a pattern of lower mortality in the Xerox population than expected compared with US mortality rates, consistent with the "healthy worker effect." The SMRs for all causes were 0.65 and 0.91 for the white male exposed and control populations, respectively; 0.84 and 0.92 for the white females exposed and control populations, respectively. There was a similar indication of the healthy worker effect among non-white men and women. In addition, the SMRs for all cancers, lung cancer, respiratory disease, and cardiovascular disease in white and non-White men were all lower than 1.0 in the toner-exposed population, with the confidence limits not including 1.0. These SMRs suggest that exposure to toner in an occupational setting does not cause an increase in mortality from cancer, respiratory disease, or cardiovascular disease (Abraham et al, 2010).

7.1.3 Carbon black

In a report based on an evaluation performed in February 2006, International Agency for Research on Cancer classified carbon black as Group 2B - *possibly carcinogenic to humans* (IARC, 2010). Subsequently, analysis of data from 1147 workers in five factories in the United Kingdom suggested that carbon black might have a late stage effect in respiratory carcinogenesis. Nevertheless, there was heterogeneity across the five plants with a significant association at only two of the five plants. This finding was not replicated in a set of comparable analyses in a German cohort of 1528 carbon black workers; and in this same cohort, there was no indication of increased lung cancer risk. Lung cancer risk was also not elevated in cohorts of German rubber workers and of US black carbon workers. In two case-control studies in Montreal, carbon black was not associated with increased lung cancer risk. In the Xerox workers, who had exposure to carbon black in toner particles, the researchers also did not find an increased risk of lung cancer. It is also important to note that carbon black in commercially available toners is non-respirable and is bound within the toner particle. In

conclusion, this analysis evaluated 3374 deaths occurring over 832,064 person-years of follow-up time (average follow-up time 26 years). The results of this analysis are consistent with the general mortality patterns among healthy working populations. No evidence was found that toner exposure increases the risk of all cause or cause-specific mortality for 23 categories of cause of death (Abraham et al, 2010).

Interestingly, the US ACGIH derived a TLV (3 mg/m^3, inhalable particulate matter, TWA) for carbon black based on bronchitis as primary effect. The most important health outcomes from elevated exposure to carbon black appear to be respiratory symptoms; decreased lung function; possible changed in the lung as indicated by changes on chest X-ray; and in the rat carcinogenesis (ACGIH, 2011). The carcinogenic effects, which were seen in rat studies under overload conditions, are considered to have questionable predictive power for human lung cancer (Mauderly, 1997).

Carbon blacks are characterised by the size distribution of the primary particles and the degree of their aggregation and agglomeration. Human exposure is primarily to carbon black particles in aggregate and agglomerate forms. Average aggregate particle diameters in several commercially produced carbon blacks range from 50 to 600 nm and the more loosely associated agglomerates can reach up to many micrometres in diameter. The majority of carbon blacks currently manufactured have small quantities (< 1%) of organic compounds, including polycyclic aromatic hydrocarbons, adsorbed onto their surface (IARC, 2010).

About 90% of carbon black is used in rubber products, predominantly in tires. Carbon black is also used as a pigment in inks, paints and coatings and in plastics (IARC, 2010).

Exposures to carbon black vary markedly between and within production facilities and over time. The highest levels of exposure are experienced by packers and site cleaners. Some studies prior to 1970 found that extremely high levels of carbon black exposure could have occurred in the carbon black manufacturing industry. Exposure studies in this industry in the USA and western Europe after the late 1970s found personal inhalable dust exposures to be less than 5 mg/m^3 (geometric mean). By the mid to late 1990s the inhalable dust levels were below 2 mg/m3 (geometric mean); the respirable dust levels were below 0.5 mg/m^3. No data were available that would allow the characterisation or quantification of exposure to ultrafine primary particles (IARC, 2010).

The study of workers in five carbon black production facilities in the United Kingdom involved a large group with a long follow-up. When compared with national mortality rates, there was a clear excess of mortality from lung cancer. Although smoking histories were not known, there was no corresponding excess of other diseases known to be associated with smoking. The excess risk was manifest in two of the five factories. Exposure was assessed using last job from worker records and a job–exposure matrix based on expert judgment and measurements from two of the five plants. When adjusted for age and divided into four subgroups based on cumulative exposure levels, relative risk for lung cancer did not increase monotonically with increasing exposure, although the two highest exposure categories showed higher relative risks than the two lowest categories. There was no excess risk for cancer at any other site (IARC, 2010).

A cohort study was conducted among blue-collar workers in a long-standing, large German carbon black production plant. When mortality was compared with regional rates, there was an approximate doubling of risk for lung cancer. Exposure was assessed using full work history records from the plant and expert judgments. Further, company medical records provided some information on tobacco smoking for most of the workers. Compared with the lowest exposure group, after adjusting for smoking, and using several

indices of exposure, there was no indication that workers with higher exposure to carbon black had higher risks. However, the precision of these subgroup risk estimates was low. There were no excess risks for most other cancer sites, including oesophagus, stomach and urinary bladder, although the numbers were small (IARC, 2010).

Another group of investigators analysed the same German cohort of carbon black workers, but used rather different methods. They confirmed that there was no exposure–response relationship within the cohort between estimated exposure to carbon black and lung cancer. After accounting for regional variations in cancer and different methods of adjustment for tobacco smoking and other exposures, the overall risk for lung cancer was slightly elevated, although the Working Group was not persuaded that all the adjustments were warranted (IARC, 2010).

The US study included a large cohort of workers from 18 plants with good ascertainment of cohort members and effective mortality follow-up over a long period of time. There was no indication of excess risk for cancer at any of the reported cancer sites. There was no indication that long-service workers had higher risks than short-service workers. For most types of cancer, including lung cancer, the numbers of deaths observed did not exceed the numbers expected on the basis of national rates. No results were provided according to levels of exposure to carbon black, and the analyses did not take into account tobacco smoking habits (IARC, 2010).

Summary evaluation

Overall, seven studies were considered to be informative for lung cancer, of which three were among carbon black production workers. The IARC Working Group considered the studies of carbon black production workers in the United Kingdom, Germany and the USA to be the most informative for assessing cancer risk. The two studies from the United Kingdom and Germany indicated excess risk compared with external references. Confounding by smoking could not be excluded, although it was unlikely to have explained the entire excess risk. However, in both cohorts, internal analyses by level of exposure to carbon black gave equivocal but mainly null results. The study of carbon black workers in the USA suggested no excess mortality, but did not assess risk by level of exposure. In studies that assessed risks for lung cancer among user industries, the most informative study of German rubber workers showed some indication of excess risk that disappeared when asbestos and talc were adjusted for in the analysis. Of the remaining studies, two others showed non-significant excesses (US formaldehyde cohort and the Canadian community-based case–control study) and one showed no excess risk for lung cancer linked to the handling of carbon black (Italian dockworkers). For cancers of the urinary bladder, kidney, stomach and oesophagus, isolated results indicate excess risks, but these are not sufficient to support an evaluation of human carcinogenicity. There is no evidence of an effect of carbon black for other cancer sites (IARC, 2010).

The deposition pattern of carbon black particles depends on the particle size (aerodynamic or thermodynamic) and on the anatomical and physiological characteristics of the host. The deposition fraction of carbon black influences the dose to a given region of the respiratory tract. Several studies describe the retention of carbon black in the respiratory tract of exposed workers, as well as the health effects of these exposures. For example, lung tissues from workers in carbon black factories contain deposits of carbon black. Lung diseases or conditions may influence the deposition and retention of particles such as carbon black. For instance, asthmatics had a higher total deposition of ultrafine carbon particles in the respiratory tract

compared with healthy individuals. The amount of carbon particles deposited can also increase with increasing minute ventilation, for instance in individuals taking exercise or during heavy physical labour. High retained mass lung burdens and decreased lung clearance have been observed in coal miners (IARC, 2010).

Non-cancer respiratory effects in carbon black workers that have been reported include cough, sputum production, bronchitis, chest radiographic opacities (e.g. pneumoconiosis) and decrements in lung function (IARC, 2010).

There are many studies on the deposition and retention kinetics of inhaled carbon particles following intratracheal instillation or inhalation in rodents. In general, all rodent species investigated show evidence of rapid clearance of inhaled carbon particles when exposure concentrations did not result in impaired clearance resulting in accumulation of particles in the lung (i.e. lung overload). The experimental studies of ultrafine particles of carbon black have shown that rodents experience dose-dependent impairment of alveolar macrophage-mediated clearance, which occurs at lower mass doses of ultrafine particles than with larger particles. Overloading has been observed in rats, mice and hamsters exposed to carbon black. Hamsters appear to exhibit the most efficient clearance of carbon black particles compared with rats and mice. Adverse lung responses to inhaled carbon black (pulmonary inflammation and epithelial injury) increase significantly with increasing retained lung dose of carbon black particles. Fine and ultrafine carbon black particles can translocate beyond the lungs to other organs (IARC, 2010).

A number of toxic effects of carbon black have been reported in various rodent species. The toxic effects reported are dose-dependent and include inflammation, lung epithelial cell injury and lung lesions that are more severe and prolonged in rats than in mice and hamsters. Exposure to carbon black particles modulates the immune system. In vitro studies show evidence that carbon black particles can generate reactive oxygen species in cell-free systems, increase the production of tumour necrosis factor-α and activate serum factors such as complement (IARC, 2010).

7.1.4 Carbon black evaluation IARC (2010)

Carbon black evaluation IARC (2010):

There is *inadequate evidence* in humans for the carcinogenicity of carbon black.
There is *sufficient evidence* in experimental animals for the carcinogenicity of carbon black.
Carbon black is *possibly carcinogenic* to humans (Group 2B).

Rationale IARC (2010): In making this evaluation the Working Group considered the human and animal evidence as well as the evidence on potential mechanisms through which carbon black may cause cancer in humans (IARC, 2010).

The human epidemiological evidence was inconsistent. Two of the three studies of carbon black production workers observed excess risk for lung cancer and other studies provided mixed evidence for an increased risk for lung and other cancers. The few studies that assessed exposure–response for lung cancer, including the two that observed excess risks compared with the general population, provided weak or inconclusive evidence of a dose–response. Overall, these results led the Working Group to conclude that there was

inadequate evidence from epidemiological studies to assess whether carbon black causes cancer in humans (IARC, 2010).

Three studies of female rats that inhaled carbon black and three additional studies of female rats exposed intratracheally found significant increases in the incidence of malignant lung tumours, providing *sufficient evidence* that carbon black can cause cancer in animals. Solvent extracts of carbon black were used in one study of rats in which skin tumours were observed after dermal application and several studies of mice in which sarcomas were seen following subcutaneous injection, providing *sufficient evidence* that carbon black extracts can cause cancer in animals (IARC, 2010).

The Working Group considered a large body of mechanistic information. For lung cancer in rats, it was concluded that a sequence of events that starts with impaired clearance and accumulation of particles in the lung, causing inflammation, cell injury and production of reactive oxygen species that eventually lead to mutations, was well supported by experimental evidence, although some data also supported alternative pathways. High retained mass lung burdens and decreased lung clearance have been observed in coal miners, which led the Working Group to conclude that animal cancer data obtained under conditions of impaired lung clearance are relevant to humans. There was a minority opinion in the Working Group that would support the classification of carbon black in Group 2A, and invoked the analogy with quartz particles, which are carcinogenic in the lung of rats and humans. However, based on current evidence, the Working Group considered that the degree to which all elements of the above-mentioned mechanism may operate in humans is not clear and, thus, the mechanistic information did not alter the overall evaluation of Group 2B (IARC, 2010).

Numerous epidemiology studies have failed to adequately demonstrate an increased risk of lung cancer due to occupational exposure to carbon black. Carbon black is not carcinogenic to mice (oral, skin or inhalation), hamsters (inhalation or intratracheal), guinea pigs (inhalation), rabbits (skin or inhalation), primates (skin or inhalation) or rats (oral). Only studies conducted by inhalation and intratracheal administration in rats have shown significant increases in benign and malignant lung tumours and lesions described as benign cystic keratinising squamous-cell (KSC) tumours. Carbon black -induced lung tumour formation, including KSC lesions, occurs only in rats. An expert panel reviewing KSC lesions (induced in rats by TiO_2 or p-aramid) concluded that KSC lesions are not seen in humans. Lung tumours in humans are primarily located in the bronchial airways, whereas in the rat they occur in the parenchyma and are alveolar in origin. This species-specific response (tumour formation and KSC lesions) by the rat to carbon black, not seen in any other laboratory species and which has not been reported in humans, strongly suggests that the results of the rat inhalation bioassay should not be considered directly relevant when assessing human risk. Therefore, carbon black should not be classified as carcinogenic to humans based on the rodent bioassay data (Rausch et al, 2004).

7.1.5 Titanium dioxide

The human pulmonary studies of titanium dioxide are largely limited to case reports of one or more highly exposed individuals that detail the location of large amounts of titanium dioxide in the tissues. Interpretation of these studies is complicated by co-exposures to other compounds (e.g. cigarette smoke and silica) and a

lack of information regarding the estimated delivered pulmonary doses. Therefore, clearance kinetics following acute and chronic exposure to titanium dioxide is poorly characterised in humans relative to animals (IARC, 2010).

Three epidemiological cohort studies and one population-based case–control study from North America and Western Europe were available for evaluation of titanium oxide exposure (IARC, 2010).

The largest of the cohort studies was among white male production workers in the titanium dioxide industry in six European countries. The study indicated a slightly increased risk for lung cancer compared with the general population. However, there was no evidence of an exposure–response relationship within the cohort. No increase in the mortality rates for kidney cancer was found when the cohort was compared with the general population, but there was a suggestion of an exposure–response relationship in internal analyses. The other cohort studies, both of which were conducted in the USA, did not report an increased risk for lung cancer or cancer at any other site; no results for kidney cancer were reported, presumably because there were few cases (IARC, 2010).

One population-based case–control study conducted in Montréal did not indicate an increased risk for lung or kidney cancer (IARC, 2010).

In summary, the studies do not suggest an association between occupational exposure to titanium dioxide as it occurred in recent decades in Western Europe and North America and risk for cancer (IARC, 2010).

All the studies had methodological limitations; misclassification of exposure could not be ruled out. None of the studies was designed to assess the impact of particle size (fine or ultrafine) or the potential effect of the coating compounds on the risk for lung cancer (IARC, 2010).

Respiratory effects that have been observed among groups of titanium dioxide exposed workers include a decline in lung function, pleural disease with plaques and pleural thickening, and mild fibrotic changes. However, the workers in these studies were also exposed to asbestos and/or silica (IARC, 2010).

No data were available on the genotoxic effects in titanium dioxide-exposed humans. Many data on deposition, retention and clearance of titanium dioxide in experimental animals are available for the inhalation route. Titanium dioxide inhalation studies showed differences—both for normalised pulmonary burden (deposited mass per dry lung, mass per body weight) and clearance kinetics—among rodent species including rats of different size, age and strain. Clearance of titanium dioxide is also affected by pre-exposure to gaseous pollutants or co-exposure to cytotoxic aerosols. Differences in dose rate or clearance kinetics and the appearance of focal areas of high particle burden have been implicated in the higher toxic and inflammatory lung responses to intratracheally instilled versus inhaled titanium dioxide particles. Experimental studies with titanium dioxide have demonstrated that rodents experience dose-dependent impairment of alveolar macrophage-mediated clearance. Ultrafine primary particles of titanium dioxide are cleared more slowly than their fine counterparts (IARC, 2010).

Titanium dioxide causes varying degrees of inflammation and associated pulmonary effects including lung epithelial cell injury, cholesterol granulomas and fibrosis. Rodents experience stronger pulmonary effects after exposure to ultrafine titanium dioxide particles compared with fine particles on a mass basis. These

differences are related to lung burden in terms of particle surface area, and are considered to result from impaired phagocytosis and sequestration of ultrafine particles into the interstitium.

Fine titanium dioxide particles show minimal cytotoxicity and inflammatory/profibrotic mediator release from primary human alveolar macrophages in vitro compared with other particles. Ultrafine titanium dioxide particles inhibit phagocytosis of alveolar macrophages in vitro at mass dose concentrations at which this effect does not occur with fine titanium dioxide (IARC, 2010).

In vitro studies with fine and ultrafine titanium dioxide and purified DNA show induction of DNA damage that is suggestive of the generation of reactive oxygen species by both particle types. This effect is stronger for ultrafine than for fine titanium dioxide, and is markedly enhanced by exposure to simulated sunlight/ultraviolet light (IARC, 2010).

In vivo studies have shown enhanced micronucleus formation in bone marrow and peripheral blood lymphocytes of intraperitoneally instilled mice. Increased Hprt mutations were seen in lung epithelial cells isolated from titanium dioxide-instilled rats. In another study, no enhanced oxidative DNA damage was observed in lung tissues of rats that were intratracheally instilled with titanium dioxide (IARC, 2010).

Most in-vitro genotoxicity studies with titanium dioxide gave negative results (IARC, 2010).

A cohort of 3,607 workers employed in three DuPont titanium dioxide production facilities was followed from 1935 through 2006 (Ellis et al, 2010, 2013). The cohort included workers employed at least 6 months (183 days) on or after January 1, 1935 and prior to January 1, 2006 at any of the three DuPont TiO_2 facilities. In addition, the worker had to have a job history, which resulted in exposure to TiO_2 or $TiCl_4$ based on the exposure assessment. If exposure to TiO_2 or TiCl4 was unknown for more than 5 years or greater than 25% of the employment period, the worker was excluded from the study. For the Edgemoor plant, study eligibility was determined by considering work history prior to 1935, but entry into the study could not be earlier than January 1, 1935. Among the 8,359 workers identified as ever working at the three plants, 3,607 met the criteria for inclusion in the study. Workers with unknown gender or ethnicity were included in the study.

The exposure assessment was performed by an industrial hygienist experienced in historical occupational exposure assessment. Working with knowledgeable industrial hygienists at each site, we performed a walkthrough of each plant to gain knowledge of potential exposures throughout the processing operations and conducted interviews with long-term employees knowledgeable in plant operations to gain insight into changes in the plant operations over time. Detailed job descriptions which covered various time periods of plant operations as well as hard copy and electronic chemical monitoring data for a wide variety of materials were collected from each plant. The 3,488 industrial hygiene-monitoring records for TiO_2 and $TiCl_4$ collected from 1974 through 2002 were used in the exposure assessment.

Because of the focus on respiratory disease and the presence of asbestos in the plants, potential for asbestos was also determined for each worker as an ever/never exposure.

For TiO_2 and $TiCl_4$, the cumulative occupational exposure for each material was calculated for each job increment as the product of the number of calendar days in the job and the exposure level value specific for the job. The exposure level value was set as the midpoint of the range defined for each level of exposure. Annual cumulative occupational exposures were determined for each year of employment.

SMRs for all five outcomes of interest were less than 1 when compared to the US population. When stratified by plant, only the Edgemoor plant had any elevated SMRs for all malignant neoplasms, lung cancer, and all heart disease. None of these results was statistically significant. For the other two plants, all SMRs were statistically significantly reduced except the SMR for non-malignant respiratory diseases. In contrast to the results from the comparison to the US population, all the SMRs were elevated when the whole cohort was compared to other DuPont workers. Three outcomes had statistically significantly increased mortality: all causes, all malignant neoplasms, and lung cancer. The plant-specific comparison to the regional DuPont employee population produced significantly increased SMRs for four of the five outcomes among workers at the Edgemoor plant. For NJV and DL combined, all SMRs were less than 1.

When TiO_2 exposure was not lagged, there was no evidence of an increase in the RR with increasing TiO_2 exposure for any outcome. Although the RR was greater than 1 at every level for all five outcomes compared to the referent, the CIs were wide and overlapped across all levels. Only three of the 20 RR estimates were statistically significantly elevated. Two of the three occurred for all causes (exposure groups 15–35 and 80+ mg/m^{3}year) and the third for heart disease (exposure group 15–35 mg/m^3 year). There was no evidence of increasing risk with increasing exposure for any of the five outcomes. When exposure was lagged 10 years, results were similar with the RR estimates similar or slightly higher for each exposure level. For all causes, the RR estimates were statistically significantly increased for every exposure level except 35–80 mg/m^3 year.

For heart disease, the RR estimates were statistically significantly increased at two exposure levels (5–15 and 15–35 mg/m^3 year) and the lower bound was 1 for 80+ mg/m^3 year. The only outcome with an indication of a positive relationship between risk and exposure was non-malignant respiratory disease with exposure lagged 10 years, but all the CIs overlapped and included unity.

For $TiCl_4$ with no exposure lag, the trend with exposure was the same as TiO_2, namely no positive association between exposure and disease outcome with CIs overlapping across exposure levels for each of the five outcomes. Looking at results for specific outcomes, lung cancer was the only outcome with increased RR estimates at all exposure levels. For all cancers similar results were seen except that one level had a reduced RR estimate (exposure group 1–5 mg/m^3 year). For all causes, the RR estimates were less than 1 at all exposure levels. For non-malignant respiratory and heart diseases, three of the four RR estimates were less than 1. When the analysis was repeated using a 10-year lag, the overall results were the similar but the RR estimates tended to be higher.

Combined and plant-specific cohort mortality was compared with the overall US population and other DuPont employees. The relationships between selected causes of death and annual cumulative exposures to titanium dioxide and chloride were investigated using Poisson regression methods to examine trends with increasing exposure.

Among the 833 deaths, no causes of deaths were statistically significantly elevated either overall or plant-specific when compared to the US population. Compared to DuPont workers, statistically significantly elevated SMRs for all causes, all cancers, and lung cancers were found driven by the workers at the oldest plant. Comparing increasing exposure groups to the lowest group, disease risk did not increase with exposure.

There was no indication of a positive association between occupational exposure and death from all causes, all cancers, lung cancers, non-malignant respiratory disease, or all heart disease.

The results of this study were similar to those in previous studies of these DuPont workers. The 3,607 workers in the current study overlap with the 5,054 workers at these three plants from our earlier study (Ellis et al, 2010). While the previous study criteria only required work in the process area at any of these three DuPont plants for 6 months, the current study was more restrictive. Although these restrictions reduced the size of the study cohort substantially, they were applied in an effort to reduce the uncertainty in the exposure estimates. Retaining the excluded workers and assigning their unknown exposure years to the exposure control group would likely have resulted in bias towards the null, which may have masked any increased risk from exposure.

The restrictions were also made to facilitate comparison to results from the two other cohort studies of TiO_2 workers (Fryzek et al, 2003; Boffetta et al, 2004). Similar to these two studies, the current study was restricted to workers with employment of at least 6 months, a job that had potential for exposure to TiO_2, and not more than 25% or 5 years missing job history. Although a large percentage of the workers in our previous study were excluded from this study, the workers in the current study had similar characteristics to our earlier study with respect to gender, ethnicity, birth year, age at entry, length of follow up, and plant of employment. The results of the exposure analysis were also similar to those for the previous studies of Chen and Fayerweather (1988) and Fayerweather et al (1992), which found no association between lung cancer and TiO_2 or $TiCl_4$ regardless of whether TiO_2 or TiCl4 exposure assessment was based on a time-weighted average, exposure duration or cumulative exposure index.

The statistically significant SMRs seen in the study cohort when the referent was other DuPont workers did not result from the truncation of the analysis to 1955. When we repeated the SMR analysis using the US population as the referent truncating the rates at 1955, the results were similar to the analysis using US rates beginning at 1935. The increased SMRs using a DuPont referent were reported in the previous study of the Edgemoor by Chen and Fayerweather (1988). Similar results have been seen for studies of other DuPont worker cohorts when US and DuPont rates were used as referents in the same study (Leonard et al, 2007, 2008). This difference in the SMRs using a general population versus an employed population as the referent demonstrates the bias associated with the healthy worker effect (Monson, 1986).

The increased SMRs consistently observed for the Edgemoor workers using the DuPont referent rates may result from the way these rates were assembled. Although the DuPont death registry began in 1957, only deaths for pensioned and active workers were included prior to 1979. With the inception of the

NDI in 1979, deaths for non-pensioned former workers were added. Since our SMR study began with DuPont reference rates for the 1955–1959 time period and includes deaths for all workers regardless of pension status, using the DuPont referent rates would underestimate the number of expected deaths for our study cohort which would increase the estimates of the SMR.

The limitations include lack of smoking history information when the outcomes of interest include lung cancer and non-malignant respiratory disease, reconstruction of work histories resulting from different practices in recording and retaining work history both within and between plants, lack of information on ethnicity, and reliance on work history and cause of death data from Chen and Fayerweather (1988) without complete documentation on how the data for the study were assembled.

Strengths include the completeness of vital status ascertainment and collection of cause of death information as well as the availability of multiple sources of information to capture the data needed to complete this study.

With no exposure data available prior to 1975, the likelihood of misclassification of exposure was increased. This lack of data prior to the 1970s is common in occupational epidemiologic studies since monitoring was limited prior to the imposition of regulatory guidelines in that era.

However, the hazards assessment was done by an industrial hygienist experienced in historical occupational exposure reconstruction with input from knowledgeable individuals at each plant. There is no reason to believe that any systematic bias occurred since the exposure assignment for each worker was done without knowledge of the worker's vital status or cause of death. As a result, the effect of any exposure misclassification would be to bias the results toward finding no effect.

In conclusion, the results of this study are consistent with those of other studies of TiO_2 workers. There is no indication of a positive association between occupational exposure to TiO_2 or $TiCl_4$ and death from all causes, all cancers, lung cancer, non-malignant respiratory disease or all heart disease.

7.1.6 Titanium dioxide IARC evaluation (2010)

Titanium dioxide IARC evaluation (2010)

There is *inadequate evidence* in humans for the carcinogenicity of titanium dioxide.

There is *sufficient evidence* in experimental animals for the carcinogenicity of titanium dioxide.

Titanium dioxide is *possibly carcinogenic* to humans (Group 2B).

Rationale: In making this evaluation the Working Group considered the human and animal evidence as well as the evidence regarding potential mechanisms through which titanium dioxide might cause cancer in humans.

The Working Group found little evidence of an increased risk for cancer among humans based on epidemiological data, although relatively few studies were available (IARC, 2010).

The single most informative study was a multi-country study of titanium dioxide production workers that found a slightly increased risk for lung cancer compared with the general population and a suggestive dose–response, but no overall excess risk for kidney cancer. The two other cohort studies reported no increased risks and evidence from the case–control study did not indicate an increased risk for either lung or kidney cancer (IARC, 2010).

Overall, these results led the Working Group to conclude that there was *inadequate evidence* from epidemiological studies to assess whether titanium dioxide causes cancer in humans (IARC, 2010).

In two studies of rats that inhaled titanium dioxide, one observed an excess incidence of lung tumours in both sexes and another in females only. Studies of rats exposed intratracheally found increases in the incidence of lung tumours. No increases were observed among mice and hamsters exposed intratracheally. Other studies that used different routes of administration did not observe excesses in tumour incidence. On the basis of the results of an increased incidence of lung tumours in rats, the Working Group concluded that there was *sufficient evidence* that titanium dioxide is carcinogenic in experimental animals (IARC, 2010).

The Working Group considered the body of evidence regarding the pathways and mechanisms by which titanium dioxide or other poorly soluble particles may cause cancer. Following the same line of reasoning as that for the other particles reviewed in this volume, the Working Group considered that the available mechanistic evidence for titanium dioxide was not strong enough to warrant a classification other than Group 2B (IARC, 2010).

Two main pathological processes, atherosclerosis and thrombosis, lead to acute coronary syndromes such as unstable angina and myocardial infarction (Peters, 2006).

Systemic inflammation induced by ambient air pollution is one of the potential mechanisms linking particle deposition in the lung to myocardial infarction. Initial evidence came from an air pollution episode recorded in the mid-1980s when elevated plasma viscosity was observed. Ambient particulate matter has been associated with systemic responses including increases in C-reactive protein (CRP) and fibrinogen in healthy individuals in cross-sectional studies in longitudinal studies, changes in ambient particulate air pollution were associated with changes in the CRP level (Peters, 2006).

There is a strong link between inflammation and coronary heart disease, since factors involved in inflammation and infection seem to play a pro-atherogenic role and inflammation has been identified as a potent risk factor for the acute coronary syndromes (Peters, 2006).

Acute phase proteins, like CRP or fibrinogen, have been identified as biomarkers for inflammatory processes and are important determinants of plaque rupture (Peters, 2006).

Systemic inflammation induced by ambient air pollution is one of the potential mechanisms linking particle deposition in the lung to myocardial infarction. In longitudinal studies, changes in ambient particulate air pollution were associated with changes in the CRP level (Peters, 2006).

The publication of Zeka et al (2006) shows an association between high concentrations of traffic-related particles and increases in inflammatory and thrombotic markers.

Particle number concentrations in ambient air are dominated by the so-called ultrafine particles, particles smaller than 100 nm (Peters, 2006).

An association is observed between particle number concentrations and fibrinogen levels with a lag of 48 hours, and these effects were still evident if particle number concentrations were elevated for at least a week. These data may indicate an acute phase response in association with the high surface area of ultrafine particles and the related oxidative stress exhibited. However, there is also room for the speculation that translocation of ultrafine particles into the blood may be responsible for these observed associations leading to endothelial cell action and subsequent shift to a pro-thrombotic state, here indicated by elevated fibrinogen concentrations (Peters, 2006).

The study is important as it indicates that locally emitted particles with high surface areas and small enough to enter the circulation may be responsible for systemic pro-inflammatory and pro-thrombotic effects. In addition, it suggests that the inflammatory pathways requiring macrophage activation due to fine particle exposures may be only partially responsible for the cardiovascular disease exacerbation observed. Thereby, the data highlight that potentially different particle properties impact on the vascular physiology differently, but the underlying mechanisms are only poorly understood to date (Peters, 2006).

It seems very reasonable that aged aerosol, which is dominated most probably by internally mixed, partially soluble particles, may have more local effects in the lung, while relatively insoluble ultrafine particles with high surface areas may directly interact systemically with vascular functions (Peters, 2006).

7.2 Conclusion

Epidemiology studies show that under the worst-case exposure scenarios (which occurs in production, and exposure in the past was much worse, in comparison with today), the lung dust burdens of miners exceeded those observed in rodent studies in which overloading of lung clearance has been observed (Morrow 1992). However, numerous epidemiology studies have failed to adequately demonstrate an increased risk of lung cancer due to occupational exposure coal dust.

Carbon black is not carcinogenic to mice (oral, skin or inhalation), hamsters (inhalation or intratracheal), guinea pigs (inhalation), rabbits (skin or inhalation), primates (skin or inhalation) or rats (oral). Only studies in rats conducted by inhalation and intratracheal administration have shown significant increases in benign and malignant lung tumours. This species-specific response by the rat to carbon black, not seen in any other laboratory species and which has not been reported in humans, strongly suggests that the results of the rat inhalation bioassay should not be considered directly relevant when assessing human risk. Therefore, carbon black should not be classified as carcinogenic to humans based on the rodent bioassay data (Rausch et al, 2004). So overload in rodent tests cannot be used as a predictive parameter for adverse human health effects, such as lung cancer.

The IARC evaluations of carbon black and titanium oxide (*possibly carcinogenic to humans,* group 2B; based on *inadequate evidence* for humans and *sufficient evidence* for animals; IARC, 2010) do not distinguish between the different species response to lung burden by rodents and humans as suggested by Rausch et al (2004), and seems to be over conservative in the precautionary approach that lung cancer may occur, although epidemiology and various animal tests do not support this causal relation.

Chronic particle exposure may cause adverse health effects other than lung cancer in humans, such as systemic inflammation and pneumoconiosis. However, further analyses of these data including toxicokinetic modelling to evaluate hypotheses about relationships between lifetime exposures, retained lung dust, overloading of lung clearance and disease development are recommended.

8. REGULATORY CONSIDERATIONS

To identify the potential hazards associated with human exposure to chemicals in general at the work place, through the environment or during the use of a chemical-based product, chemical classification and labelling schemes have been formulated to help reduce potential risks. The objectives of such systems are to identify in a systematic way the hazards of chemicals ('classification'), to draw attention of the workers or users to those hazards ('labelling') and to enable them to take action to protect themselves as appropriate or use the chemicals safely (Pratt, 2002). Hazard classifications are entirely based on the inherent properties of the chemical in question. They do not provide information on the level of human risk, e.g., cancer risk that will be associated with a given chemical exposure.

Various chemical hazard classification systems exist. Although closely correlated from a scientific point of view, one has to distinguish between risk management systems such as the United Nations globally harmonised system of classification and labelling of chemicals (UN GHS) which are legally required to be followed by chemical manufacturers according to the respective national implementation laws of the responsible jurisdiction and, hazard classification schemes, such as those developed by the International Agency for Research on Cancer (IARC) or the German MAK-Commission for the setting of exposure limits ('MAK'). The latter two schemes are advisory and do not have direct regulatory consequences, but informs the regulator making classification and labelling decisions or the setting of occupational exposure limits.

The following chapter provides an overview of key chemical classification schemes focusing on those classifications that may be triggered by the tumorigenic or non- tumorigenic effects seen in long(er) term rodent inhalation studies with PSPs. It also highlights issues associated with the application of existing classification criteria and guideline values and identifies where further clarifications are deemed necessary.

8.1 Carcinogen classifications of relevance to inhalation exposure to PSP

8.1.1 United Nation's Globally Harmonized System (UN GHS) and its European implementation law EU regulation (EC) 1272/2008 (`CLP`)

An individual substance may be further distinguished:

- Category 1A: Known to have carcinogenic potential for humans;
- Category 1B: Presumed to have carcinogenic potential for humans;
- Category 2: Suspected human carcinogens

The placing of a substance in Category 1 is done on the basis of epidemiological (Category 1A) and/or animal data (Category 1B. Placement of substances in Category 2 is done on the basis of evidence from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1. Carcinogen classification under UN GHS or EU CLP is a one-step, criterion-based process that involves evaluation of the strength of evidence and consideration of all other relevant information to place a

chemical with human cancer potential into the respective hazard category. The UN GHS as well as the EU CLP carcinogenicity classification schemes uses the terms ‘sufficient' and ‘limited' as defined by the International Agency for Research on Cancer (IARC).

Both, the UN GHS and the EU CLP system provide only little guidance on how to consider the tumourigenic effects of PSP in experimental animals upon inhalation under the conditions of lung overload in terms of carcinogenicity classification. Beyond the determination of the strength of evidence for carcinogenicity, the guidance documents indicate a number of generic factors that should be considered when determining the carcinogenicity hazard of a substance in humans. The full list of factors that influence this determination is very lengthy, but some factors that are of particular relevance when evaluating the tumorigenic effects of PSP in experimental animals upon inhalation, should be noted here:

Progression of lesion to malignancy;
The possibility of a confounding effect of excessive toxicity at test doses;
Mode of action and its relevance for humans, such as (secondary) mutagenicity, cytotoxicity with growth stimulation, mitogenesis, immunosuppression.

Beyond this generic identification of potential influencing factors to be considered in the classification decision, both regulations refer to the need to follow the IPCS "Conceptual Framework for Evaluating a Mode of Action for Chemical carcinogens' as part of a weight of evidence analysis.

It is of note and direct relevance to the inhalation of PSP, however, that the UN GHS states that ‘if a mode of action of tumour development is conclusively determined not to be operative in humans, the carcinogenic evidence for that tumour may be discounted following expert review and weight of evidence analysis.' Also, in a subsequent Chapter on confounding effects of excessive toxicity or localised effects, it states that tumours occurring only at excessive doses associated with severe toxicity or occurring only at sites of contact at excessive doses may have doubtful potential for carcinogenicity. In this context the EU CLP goes one step further as it states in Chapter on ‘Specific Target Organ Toxicity – Repeated Exposure' that ‘lung overload'-induced effects in the rat require specific consideration as they may be species-specific with no relevance to humans.

8.1.2 International Agency for Research on Cancer (IARC)

The International Agency for Research on Cancer (IARC) categorises chemicals with regard to carcinogenicity into the following groups: Group 1 (‘agent is carcinogenic to humans'), Group 2A (‘agent is probably carcinogenic to humans'), Group 2B (‘agent is possibly carcinogenic to humans'), Group 3 (‘agent is not classifiable as to its carcinogenicity to humans') and Group 4 (‘agent is probably not carcinogenic to humans').

While IARC does not specifically consider the mechanism of lung overload in rats as a species-specific effect which is not relevant to humans, it recognised the issue of excess incidences of lung cancer in rats after chronic inhalation of overloading doses of PSP in context of its evaluation of the carcinogenicity of carbon black, titanium dioxide and talc. It highlighted the need of assessing the scientific evidence allowing for comparison of exposure, dose-response and mode of action among the different species. IARC further

proposed a conceptual framework of carcinogenesis induced in the lungs of rats after high exposure to PSPs. (IARC, 2010).

8.1.3 German MAK-Commission

Contrary to the chemical carcinogen classification schemes of the UN GHS, EU CLP or IARC, the German Committee for the determination of occupational exposure limits ('MAK-Commission') considers the mode of action of tumour development in its classification scheme. It distinguishes the following 5 groups:

Group 1: Substances that cause cancer in humans;
Group 2: Substances that are considered to be carcinogenic in humans;
Group 3: Substances that cause concern of being carcinogenic to humans but which cannot be conclusively evaluated due to lack of data;
Group 4: Substances with carcinogenic potential for which a non-genotoxic mode of action is of prime importance; no significant contribution to human cancer risk is expected at exposures at MAK and BAT values;
Group 5: Substances with carcinogenic and genotoxic effects; the potency of these substances is considered to be so low that, provided MAK and BAT limit values are respected, no significant contribution to human cancer risk is to be expected.

With categories 4 and 5 the MAK commission identifies those substances which have been shown to cause carcinogenicity for which a safety threshold can be established.

In 2011, the German MAK-Commission reviewed the epidemiological and animal data available for the inhalation toxicity of granular persistent dusts (GBS), the German analogue to PSP, and kept its existing occupational exposure limit for GBS at 4 mg/m^3 for the inhalable fraction and lowered the existing limit of 1.5 mg/m^3 to 0.3 mg/m^3 for the respirable fraction.

In addition to lowering the existing MAK value for the respirable fraction of GBSs, the MAK-Commission classified GBS as a Category 4 carcinogen. Under the MAK carcinogenicity classification scheme, Category 4 carcinogens are substances with carcinogenic potential for which a non-genotoxic mode of action is of prime importance and no significant contribution human cancer risk is expected at exposures below the MAK (or BAT) value. Such classification is generally applied to substances for which the mode of action is well understood and the tumorigenic responses are related for example to increases in cellular proliferation, inhibition of apoptosis or disturbances in cellular differentiation.

The MAK-Commission justified the classification of GBS as Category 4 carcinogens on the basis of existing evidence that inhalation exposure to high levels of GBS leads to inflammation in the bronchial and alveolar region and associated release of radical oxygen species leading to tumour formation in the rat. It was concluded that the effects seen in the rat are also applicable to humans.

8.2 Other non-tumorigenic classifications of relevance to PSP inhalation exposure

8.2.1 United Nation's Globally Harmonized System (`UN GHS`) and its European implementation law EU Regulation (EC) 1272/2008 (`CLP`)

Under both classification systems, the UN GHS and the EU CLP, non-tumourigenic longer term effects associated with inhalation exposure to PSP are addressed under the so-called 'STOT-RE' classification. Definitions, criteria and guidance to classify substances that produce specific target organ toxicity arising from repeated exposure ('STOT-RE'), including inhalation exposure to particles are provided. Final classification depends upon the availability of reliable evidence that a repeated exposure to the substance has produced a consistent and identifiable toxic effect in humans or, that toxic effect which have been observed in experimental animals are relevant for human health. In both schemes, two STOT-RE hazard categories exist: Category 1 for substances that have produced significant toxicity in humans or experimental animals following repeated exposures or Category 2 for substances that on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure.

The following guidance values assist with classification based on the results obtained from 90-day dust inhalation studies in experimental animals:

Table 4: STOT-RE values (mg/m^3) for grouping inhalation studies [a]

Species	Category 1	Category 2
Rat	≤ 20	≤ 200

[a] In rats exposed by inhalation (6 h/d) to dust/mist/fume for 90 days

The above values are not intended as strict demarcation values but should be used as guidance as part of a weight of evidence approach. If a specific toxicity profile is seen in animal studies below the guidance values and the nature of the effect is such that it appears only in a single species that is particularly susceptible to this effect, then a classification may not be warranted. Likewise, a specific toxicity profile may be seen in animal studies occurring above the guideline values, but there is supplementary information from other sources (*e.g.*, human case experience) which support a conclusion that in view of the weight of evidence, classification would be the prudent action to take.

While the UN GHS does not specifically recognise the phenomenon of "lung overload" in rats for the inhalation toxicity of poorly soluble particles of low toxicity ('PSP') within its classification schemes, it is considered as a rat specific mechanism which may not be relevant to humans.

Adverse effects considered supporting STOT-RE classification

The UN GHS as well as the EU CLP regulation, here specifically the ECHA guidance document to EU CLP, describes the evidence required to associate repeated exposure to a substance with a consistent and identifiable toxic effect triggering a STOT-RE classification. It is recognised that evidence from human

experience/incidents is usually restricted to reports of adverse health consequences, often with uncertainty about exposure conditions, and may not provide the scientific detail that can be obtained from well-conducted studies in experimental animals.

Evidence from appropriate studies in experimental animals can provide much more detailed information such as clinical observations, haematology, clinical chemistry, macroscopic and microscopic pathological examination. This can often reveal hazards that may not be life-threatening but could indicate functional impairment. Consequently all available evidence, and relevance to human health, must be taken into consideration in the classification process. Examples of relevant toxic effects in humans and/or animals that are of particular relevance to inhaled PSP are provided below:

- Morbidity or death resulting from repeated or long-term exposure. Morbidity or death may result from repeated exposure, even to relatively low doses/concentrations, due to bioaccumulation of the substance or its metabolites, or due to an overwhelming of the de-toxification process by repeated exposure;
- Significant organ damage that may be noted at necropsy and/or subsequently seen or confirmed at microscopic examination;
- Multifocal or diffuse necrosis, fibrosis or granuloma formulation in vital organs with regenerative capacity;
- Evidence of appreciable cell death (including cell degeneration and reduced cell number) in vital organs incapable of regeneration.
- However, the UN GHS also recognises that a number of effects may be seen that would not justify classification as STOT-RE. The ones of relevance to the rat PSP inhalation studies, are
- Adaptive responses that are not considered toxicologically relevant;
- Substance-induced species-specific mechanisms of toxicity that have been demonstrated with reasonable certainty to not be relevant for human health.

While the criteria and classification guidance values as well as the described effects considered to support or not to support a STOT-RE classification are the same as those presented in the UN GHS, ECHA has developed a substantial guidance document assisting in deciding whether inhalation exposure to particles warrants a STOT-RE hazard classification.

As stated before, of particular relevance for the classification of particles causing effects by inhalation is the specific recognition of the phenomenon of ‘lung overload' in Section 3.9 as a mechanism not relevant to humans Here, the ECHA guidance for the EU CLP Regulation states that ‘The relevance of lung overload in animals to humans is currently not clear and is subject to continued scientific debate.' (ECHA, 2012b). While this statement is inconclusive with regard to the dealing with effects observed under the conditions of lung overload from a classification standpoint, it at least recognises the issue suggesting the need for applying weight of evidence and expert judgment in the final classification decision.

Lastly, it should be noted that numerous sub-chronic or chronic experimental rat inhalation studies with PSPs such as titanium dioxide, coal dust or talc revealed that at typical particle exposure concentrations of 1 to 30 mg/m^3, the conditions of lung overload were achieved and pulmonary inflammatory responses, altered morphology, epithelial hyperplasia and finally chronic disease including fibrosis observed (ILSI, 2000). Applying strictly above UN GHS/EU CLP guideline values to those data without taking into account dose-response differences between rat and humans, also with regard to particle size distribution differences

would basically lead to a STOT-RE classification for virtually any poorly soluble particle. Hence, similarly to the need of adjusting the NOAELs from animal experiments by dosimetric modelling (e.g., MPPD) for setting health based exposure limits for PSP (Section 8.3), it is recommended to consider at the minimum the UN GHS/EU CLP guideline values for STOT classifications as human equivalent concentrations (HECs).

8.3 Proposed approaches to derive health based exposure limits for PSPs

8.3.1 Generic versus substance specific approaches

Based on regulatory provisions, like REACH, a high level of protection of humans from exposure to harmful chemicals which might compromise their health must be ensured. For this it has to be demonstrated that hazards and resulting risks are properly controlled to exposures which are considered to be safe. Within REACH, such ‘human exposure limit values' (Derived No Effect Levels; DNELs) are generally based on dose descriptors like NOAELs or benchmark doses for critical effects observed in animal studies which have to be modified to human exposure situations by applying so called assessment factors to account for species differences and uncertainties.

With respect to ‘poorly soluble particles of low toxicity' (PSP), it should be noted that per definition, they are devoid of any known specific toxicity and thus localised ‘lung overload' driven pulmonary toxicity is considered the only relevant or ‘critical' effect for risk assessment purposes. This also means that for risk extrapolation merely local effects and only the inhalation route of exposure have to be considered. From this it follows, that using the described conceptual 'adverse outcome pathway' for PSP, the avoidance of lung inflammatory responses may be used as criteria for setting a safe exposure limit.

Based hereupon, there are various approaches to establish safe ‘human exposure limit values' for PSP that can be envisaged. In this respect, beside the derivation of `substance specific no effect levels' like the ‘DNELs' under REACH, also the use of `binding no effect levels` and/or ‘indicative no effect levels' like ‘general dust limit values' may be appropriate.

Indicative / Binding OELs

EU Indicative OEL values (IOELVs) are only set for substances for which an effect threshold can be identified and such values are claimed to be purely health based. Binding OEL values (BOELVs) are set according to more pragmatic principles including a toxicological evaluation as well as issues of feasibility. As the name implies, the IOELVs are not mandatory and the member states may implement them at higher, equal, or lower numerical values in their national legislations. In contrast, the BOELVs must be implemented at the same or a lower level (i.e., providing the same or a higher safety margin).

According to the `Scientific Committee on Occupational Exposure Limits`(SCOEL), uncertainty is handled by the application of uncertainty factors, henceforth called assessment factors (AFs) in concordance with the nomenclature in the REACH guidance document (ECHA, 2008). The SCOEL guidance gives no numerical recommendations for AFs but lists a number of aspects of uncertainty that might need consideration.

Aspects which should be taken into account are inter alia nature and severity of critical effect (local or systemic), nature of PoD, dose-response considerations and known species differences (SCOEL 2009).

General dust limit values

In Europe, general dust limit values are set for so called ‘inert dusts', ‘nuisance dust' or ‘particulates not otherwise regulated' (PNOR) either by regulatory bodies including EU member states or independent scientific bodies like the German MAK Commission (MAK values).

The German workplace regulation specifies an occupational exposure limit (OEL) for so called `granular bio-persistent dusts`(GBS) of 4 mg/m^3 for the inhalable fraction and 1.5 mg/m^3 for the respirable fraction. However, the MAK Commission recently has lowered the MAK value of the respirable fraction (for dusts of density 1) from 1.5 mg/m^3 to 0.3 mg/m^3 (MAK 2012a).

For workplace exposure, official occupational exposure limits (OELs) for PSP (or GBS) may already exist. Under certain circumstances OELs and/or the underlying information used for setting the OELs can be used to derive a respective DNEL. With regard to setting DNELs specifically for dusts, the REACH technical guidance document states that "for exposure to dust, it should be considered whether a derived DNEL for inhalation may have to be lowered. However, when using existing OELs for the inhalable and/or respirable fraction of dusts, the following should be considered according to ECHA:

- For non-soluble inert dusts and if the derived DNEL for inhalation is above these dust limits, the general dust limits should apply for exposure scenarios with exposure to dust.
- For significantly soluble dusts, if the derived DNEL for inhalation is above (these dust limits), the general dust limit might apply. Where it is not to be used, the rationale for any deviation from the general dust limits should be justified.

In principle, this approach is supported, with some minor specification, by the Task Force members. For PSP, as defined in this report, general dust limits may be used as long-term DNEL for workers, unless there is some substance specific toxicity data suggesting different level(s) of toxicity. For PSPs, where substance-specific inhalation data point to potential health effects at exposure levels below those general OELs, DNELs should be derived on the basis of those substance specific data but taking into account the wealth of data suggesting that the rat is the most sensitive species to effects of PSP as a result of lung overload (Chapter 5).

Generic volumetric overload Limit Value according to Pauluhn

Published evidence suggests that repeated exposure inhalation studies on rats represent the most sensitive bioassays in regard to poorly soluble particles of low inherent toxicity and that the prevention of any overload-like condition will also prevent adverse effects to occur from secondary inflammatory responses or long-term sequelae. Considering this particular high sensitivity of rats to inhaled PSP, the use of additional assessment factors to account for inter- or intra species differences is not considered to be necessary for the establishment of long-term chronic inhalation DNEL based on rat inhalation data. This conclusion matches the deliberations of expert groups convened to address PM-related chronic toxicity and is supported by an ILSI workshop which concluded that no default uncertainty factor is required to account for quantitative

differences in deposition, air flow patterns, clearance rates and protective mechanisms between humans and animals (ILSI 2000).

Recently, a volume based generic concentration of 0.54l PM resp/m^3 was proposed as a defensible OEL based on both, generic theoretical considerations as well as empirical evidence, which allow easily the calculation of respective mass concentrations by multiplication of the volume concentration with the particle agglomerate density (Pauluhn 2010, 2011). However, a proper definition of the relevant type of density is still due (also Section 2.2.1). In addition, Pauluhn also concluded that repeated inhalation studies on rats should be performed using an experimental window of a cumulative volume load of respirable particles in the `no-adverse-effect-range` not exceeding 10 µl/lung to avoid that retention half-times of 1 year would be surpassed. According to Pauluhn (2011), inhalation studies exceeding such a threshold volume may lead to meaningless findings which are difficult to extrapolate to any real-life exposure scenario.

Human equivalen concentration (HEC)

In times of growing interest in computational toxicology, the calculation of a human equivalent concentration (HEC) for inhalable dusts by dosimetric models like MPPD is inviting (Jun Ho Ji 2012, Hartwig 2011, Pauluhn 2011). The general hypothesis behind this concept is that equivalent doses lead to equivalent effects in different species. Hence, a HEC calculated from a NOAEL could in principle be considered as a conservative OEL.

However, the necessary adjustments for deriving such a reference concentration needs careful consideration as these adjustments may influence what is considered the most sensitive organ and/or critical effect, i.e. the most sensitive endpoint may vary for different durations or routes of exposure resulting in different HECs from the same external inhaled concentration. In this respect, the adjustment for systemic effects requires the integration of a more complex set of dose-response considerations compared to adjustments for local portal-of-entry effects between animals and humans. For local non neoplastic effects, there are strong indications that equivalent exposures generally result in similar effects independent whether experimental animals or humans are considered. In these cases comparable and less complex biological feed-back mechanisms between species do allow a more reliable derivation of reference concentrations. Such generalised procedures in deriving HECs do also exist for substances eliciting their responses locally, e.g. particles effects in the respiratory tract. By applying proper dosimetric adjustment factors, respecting species-specific physiology and anatomy, to duration-corrected exposure concentrations (e.g. daily average), such human equivalent concentrations are already used to derive risk based OELs by various regulatory bodies, e.g. within the German Committee on Hazardous Substances Committee (AGS).

Although the MPPD model already considers dosimetric principles like deposition and clearance there are some points that may limit the value of a HEC calculated by the MPPD model (Kalberlah and Schuhmacher-Wolz 2011):

- Different lung tumour types and tumour locations in animals and humans indicate that different target tissues should be considered for a proper dose transformation.
- The actual regional deposition fraction may deviate significantly from the mean values taken for the different lung compartments and used for HEC calculation.

- In humans deposition and retention may be significantly influenced as they are normally not exposed to the identical particle distribution as is in the animal experiment.
- The conservative differences in the clearance mechanisms, expressed by an elimination half life time of 700 days may be already compensated by alternative mechanisms of "detoxification", e.g. translocation into the interstitium.

Whereas the first problems are of general nature and cannot be considered easily in calculations the latter one can be adapted by using different clearance rates than the defaults. Proposals for alternative clearance rates have already been made in literature (Pauluhn 2011, Hartwig, 2011) and were considered by the German MAK commission in their recently derived MAK value of 0.3 mg/m^3 for (unit density) respirable granular biopersistent dusts (`GBS`) based on the HEC concept (MAK 2012a).

Experimental case-by case approach

REACH requires endpoint specific information to identify the so called 'leading health effect' which then has to be used for derivation of the final DNEL (ECHA 2012c). Generally this information is coming from sub chronic (90-day) experimental animal studies. According to the REACH framework, every endpoint specific DNEL has to be calculated, one for each identified adverse health effect and relevant exposure route.

The OEL hazard assessment basically aims at identifying the `critical effect`, i.e., the first adverse effect that appears as dose (or exposure level) increases. The underlying assumption is that if exposure is kept below the critical effect level, neither the critical effect nor other more serious effects will appear. In contrast, in the derivation of DNELs according to the REACH framework, several endpoint-specific DNELs have to be calculated, one for each identified adverse health effect. The lowest of the endpoint-specific DNELs for each relevant exposure route is then chosen as the final DNEL and the corresponding effect being called the `leading effect`. In the case of PSPs, which by definition are devoid of any specific systemic toxicity, only the inhalation route of exposure is relevant to generate meaningful data for assessing related risks. Based hereupon and taking into account `adverse outcome pathway` principles, screening studies with focus on the 'critical health effect', namely lung inflammatory responses, may be useful to generate the necessary data for extrapolation purposes and for ensuring animal welfare principles. A conceptual outline of a respective study design allowing for model specific adjustments for testing is given in Appendix A of this document.

8.3.2 Specific DNEL derivation for PSPs following inhalation exposure

In general, REACH requires the establishment of DNELs also for the inhalation route of exposure following a generic scheme involving, e.g. identification of the mode of action (MoA), determination of the relevant dose descriptor and point of departure (PoD) as well as applying a set of AFs.

Step 1: Gather typical dose descriptors (e.g. NOAEL, LOAEL) and/or other information on potency (dose-response / dose-effect relationship)

Step 2: Decide on mode of action (threshold or non-threshold)

Step 3: Derivation of effect levels (DNEL in case of threshold effects / DMEL in case of non-threshold effects) or the use of a qualitative approach

Step 4: Selection of the leading health effect

In the absence of relevant data from humans (e.g. epidemiological data), animal inhalation studies using rodents as preferred animal species generally represent the basis for the calculation of such DNELs (for more details see Appendix B).

In the case of low soluble particles that are regarded to be of low toxicity and do not have specific systemic toxicity, several aspects relevant for DNEL derivation have to be considered. The default extrapolation factors to be used according to REACH were established for systemic effects, rather than for local or `portal-of-entry` effects. Another difficulty is the discrimination between "critical effects", "leading effects", and "critical DNEL". Quantitative effect data may be derived from comparative response data for the toxic effect itself or for a "point in the chain of events" that is considered critical to the toxic response ("biomarker of effect"). In general OEL settings, the "critical effect" refers to the first "adverse" response (or measure of response) within the MoA that occurs at a dose / concentration below which neither this "critical effect" nor any other effects of health concern will appear (EU Key Documentation, Version 7, Methodology for the Derivation of Occupational Exposure Limits, 2013). The "critical DNEL" for the "leading effect" according to REACH, however, is defined as the lowest DNEL for a given exposure pattern amongst all different endpoint-specific DNELs which have to be calculated (ECHA 2012c).

The extrapolation by simple multiplication of default assessment factors from animal derived NOAELs/LOAELs (respectively NOAECs/LOAECs) as provided in the ECHA Guidance, allows only limited deviation from this approach. However, there is increasing scientific advice to incorporate biologically and mechanistically based data into such extrapolation schemes to take account of critical effects defining "overall thresholds" (Renwick & Lazarus 1998, Dorne 2010). Mechanistic understanding of the key events and processes leading to a distinct toxicological response may be the basis of a more flexible framework of "pathway" related assessment factors.

It is now scientifically accepted that all kind of PSPs (micro-sized and nano-sized) are eliciting localised pulmonary toxicity via processes that causes oxidative stress and are pro-inflammatory in nature, and which are initiating an acute, neutrophil driven inflammatory response. Acknowledging this, all particles of low solubility and no/low inherent toxicity, independent of the particle size, are following the same mode of action. Oxidative stress is evidentially implicated in this process and experimental evidence suggests a relationship between the induction of inflammation and the severity of the oxidative insult (Nel et al, 2006). It should be noted that the mechanisms leading to an oxidative and inflammatory pulmonary status is clearly threshold related as exemplified in a number of studies (Driscoll et al, 1997, Seiler et al, 2001, Gallagher et al, 2003, Rehn et al, 2003, Seiler et al, 2004, Valberg et al, 2009). In this context, a recent APSGB/HESI workshop on how to discriminate adverse effects from normal physiological lung responses concluded that increases in alveolar macrophages/neutrophils (hyperplasia) following exposure to PSP should not be interpreted as "adverse" in the absence of other lung effects like inflammation or fibrotic tissue changes (Kirsten et al, 2012). Jones and Neef (2012) concluded that independent of the duration of the study, lung burdens of approximately 0.5 mg/g lung tissue appeared to be a "transition point" between adaptive and adverse changes in the lung which is remarkably similar to "lung overload" conditions as defined by Morrow (1988). Similar views have been expressed also by Pauluhn (2010, 2011).

DNEL derivation according to REACH defaults

According to REACH guidance, a stepwise approach is proposed for establishing endpoint-specific DNELs which requires the consideration of various key aspects like mode of action, the identification of the leading health effect, impact of species differences in sensitivity and the subsequent extrapolation of animal derived data to humans (also appendix B). Critical in this process are questions concerning the appropriateness and applicability of the assessment factors predefined by the REACH default approach for DNEL derivations.

Mode of Action and Dose Descriptors:

As discussed within this report, there is substantial evidence that poorly soluble particles of low toxicity, whether nanosized or microsized, exert toxicological relevant adverse effects via a threshold mode of action. Plausible mechanism of the observed pulmonary responses, even the tumorigenic action of high exposure concentrations of PSPs like TiO_2 and carbon black in rats, can be interpreted by overloading of lung clearance capabilities accompanied by increased pulmonary inflammation and oxidative stress, cellular proliferation, secondary genotoxic events and (in rats) tumourigenesis. Hence, the derivation of DNELs based on NOAELs/NOAECs for poorly soluble (nano)particles of low toxicity is toxicologically justified.

Leading Health Effect/Point of Departure:

According to REACH, endpoint-specific DNELs for so called leading health effects have to be calculated and the endpoints carcinogenicity, mutagenicity and reproductive toxicity (CMR) are considered to be more severe than other endpoints. However, evidenced by several investigations, it is hypothesised that the reported tumourigenic results from PSP (nano)particles in rats are not a reliable basis for predicting human lung cancer risk (Valberg et al, 2009). This view is supported by experimental animal studies and epidemiology, which have failed so far to provide conclusive evidence of poorly soluble (nano)particle induced systemic toxicity (Gebel 2012). Based on the underlying mode of action, an impairment of lung clearance mechanisms with pulmonary inflammation and oxidative stress can be considered as critical (leading) health effect for which thresholds can be derived. Below this threshold neither the critical nor any other more serious effect will appear. Hence, substance specific NOAELs/LOAELs defining lung inflammation/oxidative stress as pathway defined leading health effects seem to be toxicologically justified dose descriptors.

Considering PSPs and with respect to the related lung overload phenomenon, the appropriate conversion of the respective NOAELs/ OAELs into an adequate `point of departure` (PoD) for DNEL derivation is of special importance. In fact, differences between various animal species and humans with regard to ventilation rates, respiratory volumes, airflow patterns and particle deposition and clearance characteristics are well documented. However, Pauluhn (2010, 2011) compared the larger size and higher number of human alveolar macrophages with that of rats and concluded from the resulting higher human alveolar macrophage volume that humans are six-times more resistant to attaining lung overload conditions compared to rats. Based hereupon and leveraged by earlier conclusions from an ILSI expert group (ILSI 2000) that no default uncertainty factor is required to account for quantitative differences in deposition, air flow patterns, clearance rates and protective mechanisms between humans and animals, NOAELs/LOAELs from rodent inhalation studies can be regarded as appropriate PoDs without additional conversion. However, it should be noted that epidemiology studies show that even under worst case exposure scenarios (exposures during

former occupational conditions, e.g. amongst coal workers) no rat specific pathological lung overload conditions have been seen in humans.

Assessment Factor accounting for interspecies differences:

The interspecies assessment factor takes into account potential species-specific differences in the sensitivity towards adverse health effects and should cover uncertainties based on the default assumption that humans are about 10-fold more sensitive than experimental animals. It is argued that this difference in sensitivity between species mainly relates to a non-existing correlation between body weight with many physiologically functions. Based hereupon, the REACH guidance has split the interspecies factor into allometric scaling ("toxicokinetic") and a default factor for remaining interspecies differences ("toxicodynamic"). The latter, however, is not scientifically based and seems to fit the original interspecies factor of 10.

However, several evaluations are highlighting that rats seem to be far more sensitive to pulmonary particle effects than humans or even other animal species. The estimation of interspecies differences in the retention kinetics demonstrated a 10-fold interspecies difference in lung burdens of rats exposed for 3 months and humans exposed long enough to attain steady state in lung burdens. Interestingly, the alveolar clearance rate of the human is thought to be independent from the particulate matter load for expected exposures, but the clearance rate for a rat depends on the amount of particles in the alveolar region. Hence, rats appear to be more susceptible to ‘‘overload”-related effects due to impaired macrophage-mediated alveolar clearance (Brown et al, 2005). In case of PSP induced lung effects, this contradicts the conservative default position that humans are more sensitive than animals. Taking the higher sensitivity of rats compared to humans as an "intrinsic" safety factor, it seems appropriate and justifiable to reduce the default AF for interspecies differences with regard to pulmonary responses from PSP exposure based on rat data. Comparable considerations have led already to proposals for respective reductions for interspecies variations (Vermeire 1999, Christensen et al, 2011, Pauluhn 2011). Also ECETOC (2003, 2010) suggests an interspecies assessment factor of 1 for local effects following inhalation exposure to dusts.

As PSP driven pulmonary responses are local (point-of-entry) effects and are not depending on metabolic rate or systemic absorption, allometric scaling does not apply. Additionally, a species independent generally low distribution rate of low soluble nano- or microsized particles within the organism is to be expected and thus no significant differences in the toxicokinetics have to be assumed. Additionally, the systemic toxicity of such particles and thus also the toxicodynamic differences, are regarded to be low. In this respect, ILSI (2000) proposed an uncertainty factor of 1 for both, neoplastic and non-neoplastic endpoints as sufficient to account for toxicokinetic and toxicodynamic parameters. Since the factor for remaining uncertainties (default 2.5) is mainly introduced to cover differences in the toxicodynamics, a factor of 1 for remaining species differences is also justified.

Based on the established higher sensitivity of rats compared to humans, an assessment factor of 1 for remaining species differences is also supported by the members of this ECETOC Task Force.

Assessment Factor accounting for intraspecies differences:

Based on REACH, an intraspecies assessment factor should account for the heterogeneity in the sensitivity of the human population. Variations in the individual responses may be due to genetic polymorphisms affecting metabolism, differences in toxikinetics and toxicodynamics, sex, general health status, age but also various

other factors. Although for concentration dependent local irritative port-of-entry effects an interspecies and intraspecies AF of 1 may be considered sufficient, information on intraspecies variations in response to particle induced pulmonary inflammatory responses is relatively scarce. Based hereupon, ECHA has not refined the default intraspecies factors for local effects but is proposing the same as for the extrapolation of systemic effects (5 for workers and 10 for the general population). This is not in line with the recommendations from the ILSI workshop (ILSI 2000), where uncertainty factors of 1 to account for toxicokinetic and toxicodynamic parameters were considered sufficient for both, neoplastic and non-neoplastic endpoints following chronic particulate exposures. Pauluhn (2010) recently proposed an approach for (nano)particulate induced lung overload effects using a mechanistic model in which intraspecies adjustments were also not applied because such local port-of-entry effects exhibits thresholds and are not dependent on metabolism. A comparable approach was followed within the NEDO project from the Japanese National Institute AIST as adverse effects related to pulmonary inflammation are local and only dependent on the surface related alveolar deposition of particles (Nakanishi et al, 2011). ECETOC (2003, 2010) evaluated the intraspecies variability within the human population based on the data sets from Hattis et al (1987, 1999, 2002), Hattis and Silver (1994) and Renwick and Lazarus (1998) and concluded that intraspecies factors of 3 for workers and 5 for the general population seems to be sufficient for irritative local effects from (bio)soluble substances where effects are mainly driven by cytotoxicity. However, for poorly soluble particles of low toxicity the general dust limit should apply (ECETOC 2010).

A synopsis of the available data indicates that the potency of PSP to induce inflammation-related pulmonary responses due to lung overload seems to be solely related to the biokinetics rather than on PSP inherent properties. As insoluble particles of low toxicity generally lack any significant systemic bioavailablity and lung overload related findings are considered localised `portal-of entry` effects independent of any local metabolism, default factors used to extrapolate systemic toxic effects are exaggerated and scientifically not plausible. The ECETOC Task Force members of this Report therefore support the conclusion that for concentration dependent local effects, like inflammation driven portal-of-entry effects under lung overload conditions, an intraspecies AF of 1 is considered sufficient.

Assessment factor for exposure duration extrapolation:

According to REACH Guidance, default assessment factors for the duration of the exposure have to be used. A factor of 3 for extrapolation from subacute to subchronic extrapolation and a factor of 2 for subchronic to chronic exposure should be used. This in turn means, that the same default AF should be used for both systemic effects and for local `port-of-entry` responses in the respiratory tract following inhalation exposure. The view of using the same default AF concurrently was justified by a statistical analysis of technical reports from the US National Toxicology Program (NTP) on subacute, subchronic and chronic inhalation studies with locally acting substances which revealed decreases in effect concentrations by factors of 3.2 (subacute to subchronic), 2.7 (subchronic to chronic) and 6.6 (subacute to chronic) (Kalberlah et al, 2002).

However, a re-analysis of the NTP data sets carried out by ECETOC (2010) was not able to confirm these findings. Additionally, as most of the analysed data sets refer to substances not falling under the definition of PSP, the appropriateness of these default AFs seem to be scientifically not plausible and are considered to be excessively conservative. However, the Task Force members of this report were nevertheless not able to identify sufficient specific examples to analyse the influence of study duration on the NOAELs of PSPs to

derive scientifically defendable AFs and recommends that the AFs should be further investigated and reconsidered as new knowledge becomes available.

8.4 Conclusions on the rat lung overload issue from a regulatory perspective

Various chemical hazard classification systems that aim identifying the potential hazard associated with the inhalation effects of poorly soluble particles of low toxicity (PSP) exist. While some of them, like the United Nations globally harmonised system of classification and labelling of chemicals (UN GHS) or its EU implementation law CLP have direct regulatory consequences, others such as the IARC or the German MAK cancer classification schemes may not have such direct consequences, but inform regulators for classification and labelling decisions or the setting of occupational exposure limits. All these systems have in common that they are hazard and not risk-based. It is noteworthy, however, that the German MAK cancer classification system allows recognition of threshold effects as a pragmatic way to manage risks associated with exposure to PSP and nuisance dusts in general.

The classification systems typically foresee that hazards are established on the basis of animal studies, most often in rats. While this is also possible on the basis of human data, the burden on data quality is high. Good quality human data allowing hazard identification or to eventually question the relevance of hazards identified in animal studies, are rarely available. As has been shown in the previous chapters, the rat is more sensitive than humans, primates or other rodents to the effects of inhaled particles predominantly because of their tendency to impair lung clearance mechanisms leading to an overloading of the rat lungs at high particulate exposure conditions. Such overload conditions induce lung inflammation and histopathological changes which can ultimately lead to fibrosis and in some cases also lung tumours.

The existing regulatory frameworks only provide little considerations of these very rat specific responses to PSP under the conditions of overload. Only the EU CLP Regulations identifies for the hazard of 'specific target organ toxicity following repeated exposure' (STOT-RE) the condition of rat lung overload as a mechanism which may not be relevant to humans. While the EU CLP guidance states that the relevance of lung overload in animals to humans is not clear and subject to continued scientific debate, it is recommended to consider at the minimum the UN GHS/EU CLP guideline values for STOT classifications as human equivalent concentrations (HECs) and not NOAELs stemming from the animal inhalation studies. With regard to the carcinogenicity classification, neither the UN GHS nor the EU CLP Regulation identify or at least recognise the lung overload condition as a rat-specific mechanism of toxicity. Specific recognition and clarification of the issue in a chemical hazard classification context is necessary.

8.5 Recommendation from a regulatory perspective for classification and DNEL derivation

8.5.1 Classification for tumorigenic and non-tumorigenic effects

Based on the information provided in this Report, one can conclude that lung overload in its distinct form with all its consequences is a rat specific phenomenon. Rat inhalation studies with PSPs in which lung overload has been observed have therefore no human relevance with regard to any observed tumourigenic effects and, without appropriately considering the dose-response differences between rat and humans, of little human relevance for non-tumourigenic effects.

From a worker and consumer protection point of view, it is not considered to be meaningful or helpful if, as a general principle, all poorly soluble particles of low toxicity would be classified as STOT-RE and/or inhalation carcinogens on the basis of data that are not relevant to humans. Such situation would undermine the objective of chemical hazard classification schemes. The mechanisms of toxicity of inhaled PSPs are well understood, allowing the establishment of safe exposure levels and identification and use of adequate risk management measures. This knowledge already results in practical application for setting of occupational exposure limits and, under the EU REACH Regulation, the setting of derived no effect levels (‘DNELs'). For hazard classification purposes, it is recommended to clarify the UN GHS/EU CLP guideline values for STOT classifications as human equivalent concentrations (HECs).

8.5.2 REACH DNEL establishment for PSP

There is substantial evidence that poorly soluble particles of low toxicity, whether nano- or microsized, exert toxicologically relevant adverse effects via a threshold mode of action. Hence, the derivation of DNELs based on NOAELs/NOAECs as derived in animal inhalation studies, adjusted for human equivalent concentrations by appropriate dosimetry modelling, is toxicologically justified. Due to the higher sensitivity of the rat compared to humans with regard to lung overload driven effects, an overall assessment factor of 1 for intra- as well as interspecies differences is considered suitable and sufficient. While within the scope of this work it was not possible to scientifically justify a default assessment factor for exposure duration extrapolation, the Task Force recommends further investigations on the need of such AF and reconsideration as new knowledge becomes available.

9. CONCLUSIONS/DATA GAPS

9.1 Current knowledge regarding lung overload

The concept of `lung overload` was first introduced by Morrow in 1988 who defined it as an impairment of alveolar macrophage mediated lung clearance following exposure to high concentrations of low soluble particles of low inherent toxicity (PSP), thereby triggering accumulation of particles in the deep lung, persistent pulmonary inflammation, epithelial cell proliferation as well as non-neoplastic and in the case of rats also to neoplastic lung lesions. In the context of the rat lung response to PSPs a more refined definition had been formulated following an ILSI expert workshop (ILSI 2000) as follows: "For chronic inhalation of PSPs, particle overload is a consequence of exposure that results in a retained lung burden of particles that is greater than the steady-state burden predicted from the deposition rates and clearance kinetics of particles inhaled during exposure.

The present comprehensive review on the available scientific evidence on `lung overload` generally does support the conclusions drawn by the ILSI expert group but allow also additional insights into the mechanistic understanding and obvious species-specific differences important for risk characterisation exercises. In this respect the available data clearly demonstrate that the rat represents a particularly sensitive model with regard to pulmonary non-neoplastic effects and, moreover, a unique model with regard to lung neoplastic responses under conditions of particle overload. In fact, lung tumours following chronic exposure to PSP have been reported exclusively in rats, but not in mice, hamster, non-human primates or humans.

Epidemiological studies to date have not found comparable `lung overload` conditions in workers exposed chronically to PSPs, not even in former coal miners having experienced worst-case exposure conditions. Furthermore, well-conducted epidemiological studies thus far have not been able to detect an association between occupational exposure to PSPs and an increased risk for cancer.

The synopsis of current experimental data allow us to conclude that `lung overload` contributes to a cascade of species independent pathogenic events leading to non-neoplastic lung responses in all animal species investigated so far but to a specific neoplastic pathogenesis only in rats. Although finally resulting in different adverse outcomes, the induction of pulmonary inflammation, generation of reactive oxygen and nitrogen species and increased cell proliferation seems to be common and recurring effects described for all animal species during lung overload conditions. As such they can be considered as `intermediate steps` in the sense of an adverse outcome pathway (AOP).

Lung tumours have to be regarded the final phenotypic `adverse outcome` only in rats whereas in other species non-neoplastic lesions seem to be the respective `adverse outcome`. Key events for this divergence in the largely common mechanistic sequence of the AOP may be seen in a species specific biological diversity of detoxification systems, e.g. anti-oxidant defences reducing `oxidative stress` as well as differences of particle translocation processes. In fact, it could be demonstrated that the rat has greater propensity for generating a pro-inflammatory response, whereas mice and hamsters show an increased anti-inflammatory response which may not only contribute to the higher inflammatory reactions seen in rats, but do also indicate less potential for scavenging secondary mutagenic effects in rats.

The state of knowledge on particle deposition in the respiratory tract of the experimental animals (rats) and humans is sufficiently well developed to be used in dosimetry models for extrapolation of animal data on humans. The regional deposition fraction is one of the key parameters for the calculation of the retained dose from exposure concentrations and the determination of HECs. AS well as the MPPD model providing average values for the deposition fraction, there is experimental evidence (supported by CFD calculations) of significant heterogeneity in lung deposition on the surface within each compartment. This is particularly the case for the trachea-bronchial and extrathoracic region but less of an issue in the alveolar region. Translocation seems also to be species dependent. Whereas in rats most of the particulate matter is located in the lumen of the alveolar ducts and alveoli, the larger part of particles in humans is translocated into the interstitium.

Beside physical translocation of deposited particles, chemical dissolution is one of the basic clearance principles of the respiratory tract. Clearance of biosoluble particles by dissolution can occur in all three major regions of the respiratory tract, i.e. the nasopharyngeal, tracheobronchial and/or alveolar regions. In contrast, physical translocation of inhaled particles of low (bio)solubility is different in these regions and depends on particle characteristics such as size and shape. As the most prevailing clearance mechanism for solid particles in the alveolar region is mediated by alveolar macrophages, (bio)solubility of particles is considered a major determinant in the establishment of `particle lung overload' conditions.

The Task Force also concluded that there is no clear evidence showing that particles below 100 nm exhibit any kind of step-change in their hazard status or for the onset of any novel nano-specific hazard. A higher biological activity of smaller particles is not necessarily to be expected and notwithstanding their smaller size, nanoparticles are no more hazardous than conventional particles. Normal toxicological principles can therefore be applied equally, and conventional particle toxicology data are useful and relevant to the determination of nanoparticle hazard and risk evaluation.

It is now scientifically accepted that all kind of PSPs (micro-sized and nano-sized) are eliciting comparable localised pulmonary toxicity via processes that causes oxidative stress and are pro-inflammatory in nature, and which are initiating an acute, neutrophil driven inflammatory response. In this respect, there is more and more evidence that all particles of low solubility and no/low inherent toxicity, independent of the particle size (i.e. whether in the nano or microsize) are following the same mode of action and basic mechanism. Oxidative stress is evidentially implicated in this process and experimental evidence suggests a relationship between the induction of inflammation and the severity of the oxidative insult. It should be noted that the mechanisms leading to an oxidative and inflammatory pulmonary status is clearly threshold related as exemplified in a number of studies.

As discussed within this Report, there is substantial evidence that poorly soluble particles of low toxicity, whether nanosized or microsized, exert toxicological relevant adverse effects - even the tumourigenic action of high exposure concentrations of PSPs, such as TiO_2 in rats - via a threshold mode of action. Hence, the derivation of DNELs based on NOAELs/NOAECs for poorly soluble (nano)particles of low toxicity is toxicologically justified.

The mechanisms of toxicity of inhaled PSPs are well understood, allowing the establishment of safe exposure levels and identification and use of adequate risk management measures. This knowledge already results in practical application for the setting of occupational exposure limits and, under the EU REACH Regulation, the setting of derived no effect levels (DNELs). DNELs based on NOAELs/NOAECs as derived in animal inhalation

studies and adjusted for human equivalent concentrations by appropriate dosimetry modelling, is recommended. Due to the higher sensitivity of the rat compared to humans with regard to lung overload driven effects and based on comparable biokinetics, an overall assessment factor of 1 for intra- as well as interspecies differences is considered suitable and sufficient. While within the scope of this work it was not possible to scientifically justify a default assessment factor for exposure duration extrapolation, the Task Force recommends further investigations on the need of such AF and reconsideration as new knowledge becomes available.

Since the ILSI 2000 report, there has been a vast amount on in vivo and in vitro work on poorly soluble particles of low toxicity but there have been no compelling studies or a weight of evidence that would allow the Task Force to conclude that the rat lung overload findings is a reliable predictive model, in particular for neoplasia, with regard to hazard or risk assessment for humans who are exposed to poorly soluble particles of low toxicity.

9.2 Existing data gaps & proposed way forward (to be addressed at a workshop)

Several PSP properties have been shown to be determinants of the biological activity (Section 2.2). Therefore a thorough physico-chemical characterisation of PSPs is very important. However, standardised test protocols for a number of important particle characteristics have not yet been developed yet, e.g. surface reactivity.

If it is assumed that volume of the particles retained in the lung is a relevant dose metric, the density becomes an important factor to be considered, since limit values are usually given in terms of mass concentration. Retained volume but not retained mass is related inversely to the density of the particles. Volume and mass are both related inversely to the density of the particles. It is still an open question which type of density (material density, envelope density, true density) has to be selected when the particles are suspended in biological fluids, particularly the macrophage fluid where particles are engulfed shortly after deposition, and how to correctly determine this density (in vitro).

Is it possible to distinguish between the biological effects of particles transferred to the interstitium and those of particles residing on the alveolar surface and interacting with the macrophages? According to the analysis of Kümpel (2001) translocation to the interstitium seems to be much higher in humans than in rats.

There is a need to consider the uncertainties in setting human equivalent mass concentrations that rely on the correctness of the particle density determination. This is particularly the case for powders composed of nanoscaled building blocks. In most cases these powders exist as agglomerates with variable degree of compactness and it is the envelope density of the respirable, airborne agglomerates that counts for the assessment of the aerosol volume based on the measurement of the aerosol mass, both, in inhalation experiments as well as for workplace measurements.

ABBREVIATIONS

ACGIH	American conference of governmental industrial hygienists
AF	Assessment factor
AM	Alveolar macrophage
AOP	Adverse outcome pathway
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BAT	Biological tolerance value for occupational exposure
BET	Brunauer-Emmet-Teller
BOELV	Binding occupational exposure limits value
BrdU	Bromodeoxyuridine
CB	Carbon black
CLP	Classification, labelling and packaging of substances and mixtures
CWP	Coal workers pneumoconiosis
DNA	Deoxyribonucleic acid
DNEL	Derived no effect level
DR	Dose rate
ECHA	European Chemicals Agency
EU	European Union
fD	Fluorescence detection
GHS/CLP	Globally harmonised system of classification and labelling of chemicals
HEC	Human equivalent concentration
HPRT	Hypoxanthine phosphoribosyltranferase

HRTM	Human respiratory tract dosimetry model
I	Index
IARC	International Agency for Research on Cancer
ICRP	International Commission on Radiological Protection
ILSI	International Life Sciences Institute
IOELVs	Indicative occupational exposure limits values
IPCS	International programme on chemical safety
IUPAC	International Union of Pure and Applied Chemistry
KSC	Keratinising squamous-cell
LDH	Lactate dehydrogenase
LOAECs	Lowest observed adverse effect concentration
LOAEL	Lowest observed adverse effect level
MAK	Maximum workplace concentration (Maximale Arbeitsplatzkonzentration)
MMAD	Mass median aerodynamic diameter
MoA	Mode of action
MPPD-model	Multiple path particle dosimetry model
NMRD	Nonmalignant respiratory disease
mRNA	Messenger RNA
MWCNT	Multiwalled carbon nanotube
NADPH	Nicotinamide adenine dinucleotide phosphate
NOAEC	No observed adverse effect concentration
NOAEL	No observed adverse effect level
NTP	National toxicology program
OECD	Organisation for economic co-operation and development

OEL	Occupational exposure limits
OPPTS	(US EPA's) office of prevention, pesticides and toxic substances
PAR	Proximal aveolar region
PMN	Polymorphonuclear leukocyte
PNOR	Particulates not otherwise regulated
PSP	Poorly soluble particle
REACH	Registration, evaluation, authorisation and restriction of chemicals
RMV	Respiratory minute volume
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Relative risk
SAS	Synthetic amorphous silica
SCOEL	Scientific committee on occupational exposure limits
SMRs	Standardised mortality ratios
STOT-RE	Specific target organ toxicant-repeated exposure
Ti	Tolerable intake
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TWA	Time-weighted average
UF	Uncertainty factor
UFP	Ultrafine particles
WG	Water-dispersible granule
ZnO	Zinc oxide

APPENDIX A: TEST METHODOLOGY: BALF STUDY

Screening study to identify effects characteristic for 'lung overload'

Test methodology: BALF Study

Proposal to use early indicators (e.g. biochemical, cytological) as screen for functional/pathological changes in the lung in combination with histopathological evaluation of the respiratory tract and potential lung burden measurements.

Test System: Nose Only

Avoid uptake via other routes (oral due to cleaning of fur, dermal exposure related), especially with regard to "nano".

Test Species: Rat

Most sensitive species (argument of "intrinsic" biological safety factor) for AOP relevant key events in "particle lung overload" / unique concerning lung tumour development as endpoint

Exposure Duration: fixed exposure period + various recovery periods

An exposure period of 5 to 7 days seems sufficient to induce early indicators of inflammation. Different durations of recovery (e.g. 1, 3, and 7 days up to a maximum of 4 weeks post exposure) to inform about reversibility or progressive course of alterations should be considered.

Exposure Dose: 3 different concentrations + control

Exposure doses of 0, 1, 5 and 10 mg/m^3 will allow establishing different lung burdens and dose-response analysis. Modified exposure concentrations to investigate lower or higher exposures may be applicable.

Parameters:

Histolpathology of respiratory tract (e.g. whether inflammation is granulomatous or not)

Lung burden measurements (if applicable)

Biochemical / Cytological Parameters (selection of endpoints)

- Lactate Dehydrogenase (LDH)
- Alkaline Phosphatase (AP)
- γ-Glutamytransferase (γ-GT)

- Total protein

- N-acetylglucosamidase

- ß-Glucuronidase

- Total cell count

- Differential cell count

Additionally, cell proliferation marker as well as marker for genotoxicity (e.g. 8-OH-dG; OGG1) and oxidative stress may be included.

The advantages of such a study design can be seen in its screening character, flexibility, possibility to differentiate between a progressive or reversible course of effects. Depending on the design, dose (lung burden) – response analysis are possible.

Additionally, by including respective (negative, positive) control substances with known toxicity profile, comparative benchmark analysis may be possible.

APPENDIX B: DERIVATION OF A HUMAN NO EFFECT LEVEL (‘DNEL') UNDER REACH'

The European regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) should ensure a high level of protection of human health and the environment. Key element within this regulatory framework are `Derived No Effect Levels` (DNEL), which are representing levels of exposure above which humans should not be exposed. While DNELs are defined as safe exposure levels for threshold effects, such "safe” levels cannot be defined for non-threshold effects, e.g. genotoxic carcinogens. In these cases so called DMELs (derived minimal effect levels) have to be calculated which are considered to minimise potential human health risks. Guidelines on how these DNELs and DMELs are to be derived together with recommended assessment factors for different aspects of uncertainty are specified in Reach Guidance Chapter R.8 (ECHA 2010) and Appendix R8-15 (ECHA 2012).

Based on Appendix R8-15 of ECHA Guidance R.8 (2010), the approach for the generation of DNELs considers the following steps:

Step 1: Gather typical dose descriptors (e.g. NOAEL, LOAEL) and/or other information on potency (dose-response / dose-effect relationship)

Step 2: Decide on mode of action (threshold or non-threshold)

Step 3: Derivation of effect levels (DNEL in case of threshold effects / DMEL in case of non-threshold effects) or the use of a qualitative approach

Step 4: Selection of the leading health effect

Following this tiered approach, the underlying mode of action and identified dose descriptor provides the "point of departure” (POD) as basis for DNEL derivation. Adjustments of the POD may be necessary to address all local and systemic toxic effects, and to consider all relevant combinations of likely routes, durations and frequency of exposures. The DNEL for the specific endpoint is finally derived by dividing the PoD by a series of assessment factors (AFs). Workers as well as consumers are regarded as subpopulations that require specific DNELs. The most obvious difference in DNEL setting between these subpopulations is that the interindividual (intraspecies) variability in workers is considered to be smaller compared to the general population.

In contrast to various other regulatory areas, the ECHA guidance explicitly requires the use of specific adjustments for uncertainties (e.g. intra- and interspecies variation, duration of exposure, nature and severity of effects) based on given default values for the respective uncertainty (Table B1).

Table B1: Default values for assessment factors (ECHA)

Effect		Description
Systemic	**Local**	
Interspecies		
AS [a,b]	–	Correction for differences in metabolic rate per body weight
2.5	1 [c], 2.5 [d]	Remaining differences
Intraspecies		
5	5	Worker
10 [e]	10 [e]	General Population
Exposure duration		
3	3 [f]	Subacute to subchronic
2	2 [f]	Subchronic to chronic
6	6 [f]	Subacute to chronic
Dose-response		
1 [g]	1 [g]	Issues related to reliability of the dose-response, including LOAEL/ NAEL extrapolation and severity of effects
Quality of whole database		
1 [h]	1 [h]	Issues related to completeness and consistency of the available data
1 [i]	1 [i]	Issues related to the reliability of the alternative data

[a] AS = Factor for allometric scaling,

[b] Caution should be taken when the starting point is inhalation or diet study

[c] For effects on skin, eye and GI tract via simple destruction of membranes

[d] For effects on skin, eye and GI tract via local metabolism

[e] Not always covering for very young children; see text for deviations from default

[f] For effects on respiratory tract

[h] See text for deviations from default

[i] Special consideration needed on a case-by-case basis

While these default factors should be used in the absence of substance related data, ECHA notes that chemical-specific AFs can be used if scientifically justified data on the substance and/or for an established chemical category is available. During the adjustment, several AFs are combined by multiplication to obtain an `overall adjustment factor`.

It should be noted, that "simple" multiplication of default assessment factors in many cases will result in overly conservative DNELS. Such formalistic approaches have been already criticised as too overprotective (Fairhurst 1995; Groeneveld et al, 2004; ECETOC 2010) and a recent quantitative comparison of health based European indicative OELs with DNELs revealed that the REACH safety margins were approximately six times higher than those derived by the Scientific Committee for Occupational Exposure Limits (SCOEL), resulting in considerably lower worker-DNELs than the recommended OELs (Schenk and Johanson 2012). Hence, to avoid unrealistic low DNELs, generic AFs have to be critically scrutinised as to their scientific accuracy when discussing DNEL setting for specific endpoints. Especially when dealing with particles (substances) without any known significant specific toxicity, greater flexibility in the use of AFs to not overstating real risks seems to be scientifically justified.

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