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Carcinogenicity and Mutagenicity of Nanoparticles – Assessment of Current Knowledge as Basis for Regulation

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Carcinogenicity and Mutagenicity of Nanoparticles – Assessment of Current Knowledge as Basis for Regulation

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Abstract

Carcinogenicity studies with several types of respirable particles and fibres indicate a carcinogenic potential from inhalation and there is concern that the carcinogenic potency of nanomaterials is higher than for the corresponding micromaterials. In this research project, long term studies with nanomaterials were used to identify relevant indicators of toxicity of nanomaterials including possible precursors of carcinogenicity. Due to the heterogeneous characteristics of the materials and the different study types, a structured and systematic data analysis was performed by means of a relational database (PaFtox). More than 100 inhalation studies and instillation studies with rodents with Carbon Black, silicon dioxide, metals or metal oxides, and carbon-nanotubes were analysed. Effects like neutrophil number, total protein and LDH content in the bronchio-alveolar lavage fluid (BALF) are frequently measured and are sensitive indicators of toxicity of all particles investigated. In addition, infiltration of inflammatory cells in the lung and increased lung weights are often observed. The LOELs of nano-objects are generally lower than the LOELs of the corresponding larger objects and they differ by several orders of magnitude between analysed substances: Silver was identified as the most toxic nanomaterial within our selection of nanomaterials. Sustained inflammation can be seen as one possible early event in the sequence of cancer development and nanomaterials can be grouped on basis of their potential to generate inflammation. A preliminary LOEL (based on inflammatory parameters) of 0.1 mg/m^3 (exposure 24 h/d, 7 d/wk) is proposed to distinguish the so called “inert” nanomaterials (e.g. Carbon Black) from nanomaterials with specific toxicity. Our data further support to have nanotubes in a separate group.

Kurzbeschreibung

Kanzerogenitätsstudien mit verschiedenen alveolengängigen Partikeln und Fasern deuten auf ein kanzerogenes Potential bei inhalativer Exposition hin und es wird befürchtet, dass dieses Potential bei Nanomaterialen höher ist als beim entsprechenden Mikromaterial. In diesem Forschungsprojekt wurden Langzeitstudien mit Nanomaterialien analysiert, um relevante Indikatoren der Toxizität einschließlich möglicher Vorstufen der Kanzerogenität von Nanomaterialien zu identifizieren. Eine strukturiertere und systematische Analyse der heterogenen Materialeigenschaften und der unterschiedlichen Studientypen wurde mit Hilfe einer relationalen Datenbank durchgeführt. Mehr als 100 Inhalations- und Instillationsstudien mit Carbon Black, Siliziumdioxid, Metallen oder Metalloxiden an Nagern wurden analysiert. Häufig werden Effekte wie Neutrophilen-Anzahl, Gesamtprotein- und LDH-Gehalt in der bronchio-alveolären Lavageflüssigkeit (BALF) gemessen, sie sind sensitive Indikatoren für die Toxizität der untersuchten Partikel. Zudem werden oft Infiltration von Entzündungszellen in der Lunge und erhöhtes Lungengewicht beobachtet. Die LOELs der Nano-Objekte sind tendenziell niedriger als die LOELs der entsprechenden größeren Objekte und sie unterscheiden sich durch mehrere Größenordnungen: In unserer Auswahl an Nanomaterialien hatte Silber das höchste toxische Potential. Chronische Entzündung kann als möglicher früher Vorläufer der Krebsentstehung betrachtet werden, und Nanomaterialien können anhand ihres Potentials Entzündungen zu verursachen gruppiert werden. Ein vorläufiger LOEL (basierend auf Entzündungsparametern) von 0.1 mg/m^3 (Exposition 24 h/d, 7 d/w) wird vorgeschlagen, um die sogenannten “inerten” Nanomaterialien (z.B. Carbon Black) von Nanomaterialien mit spezifischer Toxizität zu unterscheiden. Unsere Daten unterstreichen den Ansatz, dass Nanotubes in eine separate Gruppe gehören.

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List of Abbreviations

BALF	Bronchioalveolar lavage fluid
LDH	Lactate dehydrogenase
LOEL	Lowest observed effect level
MMAD	Mass median aerodynamic diameter
NOEL	No observed effect level
VSSA	Volume-specific surface area
PaFtox	Particle and Fibre toxicity

1 Introduction

1.1 Hazard and risk of nano-objects

The general population is exposed to nanoparticles mainly in larger cities in form of fine dust. Workers can be additionally exposed during the production and processing of nano-objects. Furthermore, the number of consumer products based on nanotechnology is raising (e.g. shoe sprays).

Nano-objects differ regarding their physico-chemical properties (e.g. size, specific surface, zeta potential) from larger particles or fibres, but cause similar effects (e.g. inflammation in the lung or other toxic effects including cancer). However, based on the same particle mass, they are more toxic, among other reasons due to their larger particle surface (Oberdörster et al., 2005). Therefore there is concern for higher carcinogenic potency of nano-objects.

The data basis concerning carcinogenicity of nano-objects is very limited with respect to inhalation studies. Also intratracheal studies as surrogate for inhalation studies, are limited. To our knowledge nanoparticles and fine particles were carcinogenic in all existing inhalation and intratracheal studies with exposure durations longer than 2 years (Roller, 2009). Most of them caused tumours at all concentrations tested. However, even at the lowest dose levels often unrealistic high dose levels were used that overwhelm the defence mechanisms in the lung (overload). Similarly, in vitro studies on genotoxicity or ROS-generation are virtually all positive, in many cases at cytotoxic concentrations (Gonzalez et al., 2008; Roller, 2011; Ziemann et al., 2011). In addition, no clear correlation of the probability of a positive in vitro test with carcinogenicity was seen (Roller, 2011).

Recent studies with (nano)materials have shown, that cell proliferation in the lung as a consequence of inflammation may be an important precursor of cancer. Histochemical analyses at Fraunhofer ITEM have shown a high correlation between tumour frequencies and cell proliferation as well as genotoxicity in lung epithelial cells (Rittinghausen et al., 2013) for carbon black, amorphous and crystalline silica. Another study showed a relationship between tumour frequencies and inflammation, fibrosis, epithelial hyperplasia and squamous metaplasia for amorphous and crystalline silica, carbon black and coal dust (Kolling et al., 2011).

These correlating parameters can be determined in studies of much shorter duration. The amount of these studies with nanomaterials is considerably higher than the amount of long term studies and some of these studies are conducted under non overload conditions with several dose levels. Therefore these studies provide the possibility of deriving NOELs and LOELs of the nano-objects and the possibility of assessing dose response and risks of nanomaterials as a basis for regulation.

In this research project repeated dose toxicity studies (including carcinogenicity studies) with different nano-objects are analysed with the purpose to identify the precursors of carcinogenicity in these studies, to compare the toxicity of different types of nano-objects and to compare the toxicity of nano-objects with objects of larger size. Based on these analyses, already existing proposals for grouping of nanomaterials are evaluated and extended.

For a better overview of the complex data studies were entered into the relational database PaFtox (Particle and Fibre toxicity database). This approach allows comparison of many parameters including statistical analyses.

1.2 Nanomaterials –Definitions

Efforts have been made to develop a consistent terminology for nanomaterials. According to ISO/TR 12802: 2010 two types of nanomaterials are distinguished: nano-objects (external nanoscale dimension) and nanostructured materials (internal nanoscale structure or surface structure). These are subdivided into further subgroups. Nano-objects have at least one dimension below 100 nm and cover nanoparticles (3D nano), nanofibres (2D nano) and nanoplates (1D nano) as well as nanocrystals (quantum dots, those exhibit size-dependent properties due to quantum confinement effects on the electronic states, see Fig 1). Nanofibres are again subdivided into nanorods (solid fibre), nanotubes (hollow fibre) and nanowires (electrically conducting or semiconducting nanofibres.). Nanotubes are currently distinguished between single-wall carbon nanotubes (SWCNT) - consisting of a single cylindrical graphene layer, double-wall nanotubes (DWCNT) - composed of two nested, concentric single-wall carbon nanotubes and multi-wall carbon nanotubes (MWCNT) - composed of nested, concentric or near-concentric graphene sheets with interlayer distances similar to those of graphite (ISO/TS 80004-3:2010).

Nanostructured materials have an internal or surface structure with a significant fraction of features, grains, voids or precipitates in the nanoscale (like nanostructured powders, nanocomposites, nanodispersions, nanoporous material). However, nanodispersions without any measureable interaction between nano-objects and medium (the medium is just background) are actually considered as cluster/accumulation of nano-objects. Articles that contain nano-objects or nanostructured materials are not necessarily nanostructured materials themselves (ISO/TS 80004-4:2011).

Most of the commercially produced nanoproducts are aggregates and / or agglomerates of nano-objects, which may be released under energy uptake (BAuA, 2007; BAuA/BfR/UBA, 2007; Creutzenberg et al., 2012). Also, under typical experimental conditions, the majority of the nano-objects in the exposure milieu are aggregated or agglomerated.

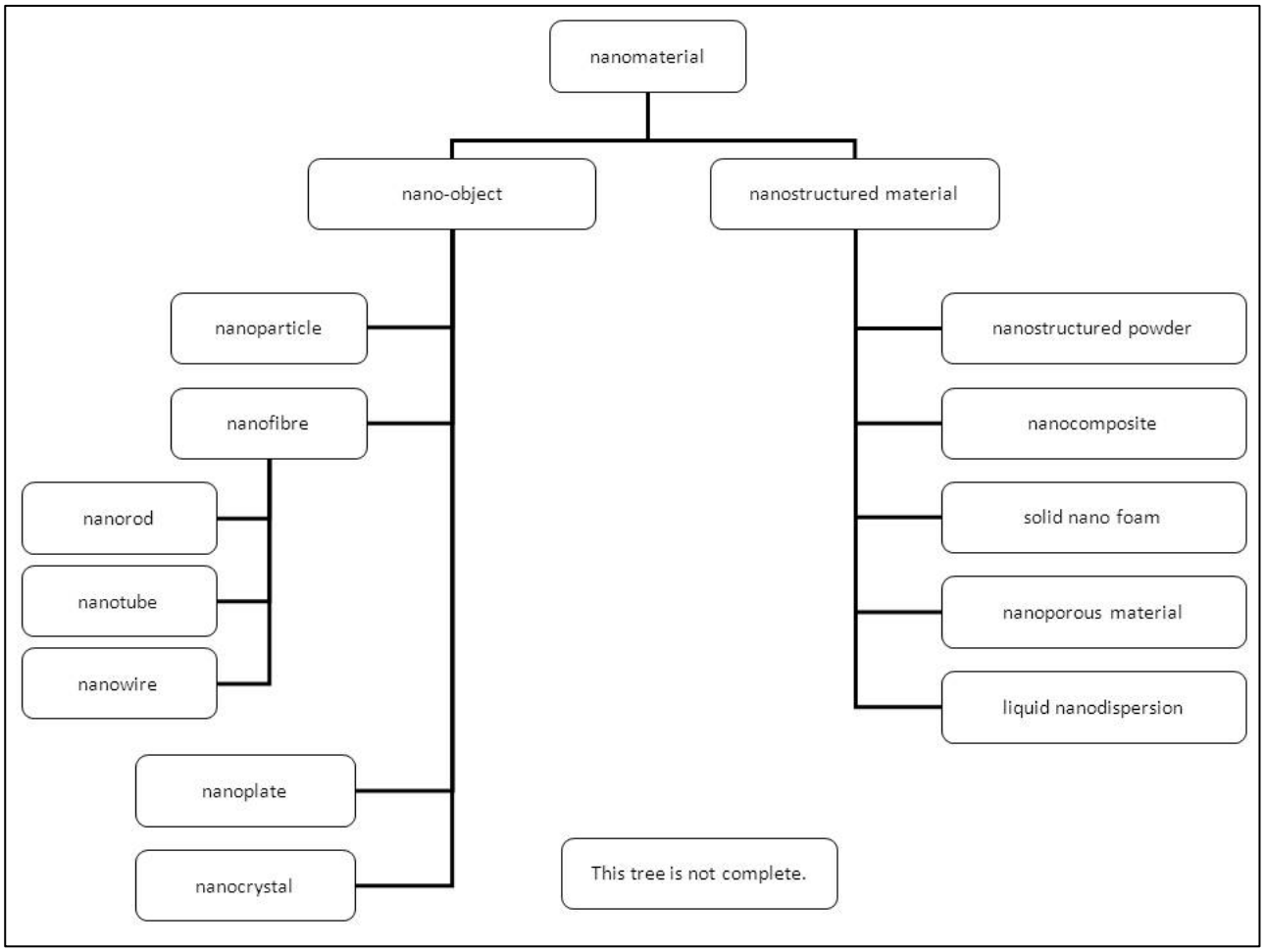

Fig 1: Taxonomy of Nanomaterial; adapted from ISO/TR 12802:2010 & ISO/TS 80004-4:2011

The European Commission (EC, 2010) proposes to define a material as a nanomaterial when it has a specific surface area by volume greater than 60 m^2/cm^3, excluding materials consisting of particles with a size lower than 1 nm. The volume-specific surface area (VSSA) of a material is generally calculated from its bulk density and its mass specific surface area. The latter is usually determined by gas absorption methodology called the BET-method (Brunauer et al., 1938) that allows surface area or porosity measurements for nanoparticles as small as 1 nm. From a 3D reconstruction of a nanomaterial, its surface area and its volume can, in principle, be estimated directly, such that its VSSA can be calculated, even on a per particle basis (Van Doren et al., 2011).

2 Methods

2.1 Literature search

2.1.1 Approach

The literature search was a tiered approach. First a most comprehensive literature search was performed. The different terms used for nano-objects, possible relevant effects and the application routes (Tab. 1) were used to perform a combined research in the databases Pubmed, Toxline and Web of Science.

Tab. 1: Concept of literature search

Search for effects			
Lung effects	Organ synonyms	Effects	
	Lung airway pulmonary respirat*	toxicity disorder disease function inflammation carcinogenic or carcinoma mesotheliom* damage	genotox* mutagen tumour fibrosis or fibrinogen cancer oxidative
Search for nano-objects			
nanoparticles	nanoparticle* / nano particle* nanotube* / nanofibre* / nanowire* nanoscale* particulates nanomaterial*		
fine particles	fine dust* fine particle*		
ultrafine particles	ultrafine dust* ultrafine particle*		
Search for application route			
(via air)	inhal* respirat* airway		
intratracheal			
intraperitoneal			

Search terms as “repeated or chronic or subchronic” were tested but led to very few hits as these words are seldom used in the title, abstract or key words. The search results were collected in EndNote. Here, medical related studies were excluded by searching for key words “drug delivery and “dry powder inhaler”. Based on the abstracts, a preliminary list of potential relevant references was established. This list was subsequently enhanced by cross references identified in the publications during the actual data entry in the database.

2.1.2 Results

The literature research led to 1343 hits in Pubmed, 1348 in Toxline and 1003 in Web of Science (January 2011). After pooling in EndNote, deleting the doubles and the medical related publications as well as screening the abstracts, about 453 publications were ordered. A lot of these publications are reviews or mini-reviews which is consistent with current great interest in the scientific and regulatory community. However, the reviews were also screened to find additional references. About one third are studies with study durations from one to seven days which were separated for potential later projects. About 200 are potentially suitable

(see criteria under point 2.2.3) and up to now, 87 publications from 41 different institutions containing 131 studies were entered into the database.

After discussion with UBA, the following material types were selected for entry into the database

“Inert” particles (granular biopersistant dusts)

- Carbon Black
- Titanium dioxide
- Aluminium oxide

Silicon dioxide

Heavy Metals (elemental or oxides)

- Silver
- Manganese
- Nickel
- Iron
- Cerium

Carbon Nanotubes

- Single wall carbon nano tubes
- Multi wall carbon nano tubes

2.2 Data analysis by means of a relational database

2.2.1 Database Structure

The structure of the database is based on the already existing database for chemicals, RepDose (www.fraunhofer-repdose.de, Bitsch et al., 2006) that has been developed at Fraunhofer ITEM. Particles and fibres are characterised by other physico-chemical properties than chemicals. Therefore, the structure was adapted and extended in this part of database (in the following **P**article **a**nd **F**ibre **tox**icity, PaFtox database). It was developed in Microsoft Access®. This software has been selected because it is commonly available and can be easily handled also by non-experts. The database consists of three parts, the nano-object characterisation, the study design and the effect related part. Generally, there are several possibilities for data entry, picklist (fixed or expandable), free text or numerical figures.

For a comprehensive analysis of particle characteristics and toxic effects the database entries have to be uniform and standardized. For this purpose glossaries were used for all fields, which can be addressed by queries for all relevant information such as particle characterisation, study design and effect data.

Descriptions of identical observations differ between studies. As a general ontology for toxicological studies is currently not available (Tcheremenskaia et al., 2012), the documentation of toxicological study data in a database requires the development of a standardised vocabulary. For this purpose in the PaFTox database observations, described by different terms were collected. If possible, one term was selected and entered into the respective glossaries. Data entry guidance assures that the right synonyms are used subsequently for data entry.

Glossaries were defined as picklists, e.g. for effects and targets. Picklists are further used to document: surface property, sample preparation, distribution, unit of exposure/dose, species, strain, sex, application route, parameter unit, score (severity of damage) and significance.

In contrast to the “defined fields” mentioned above “free text fields” are problematic, as users tend to include typos and do not use standardised text, which hampers database queries. The current PaFTox database therefore, does only include few “free text” fields. A typical example is the field “effect additional”, where the user can enter details e.g. if more details on the location of the effect are available, one gender was more sensitive than the other etc.

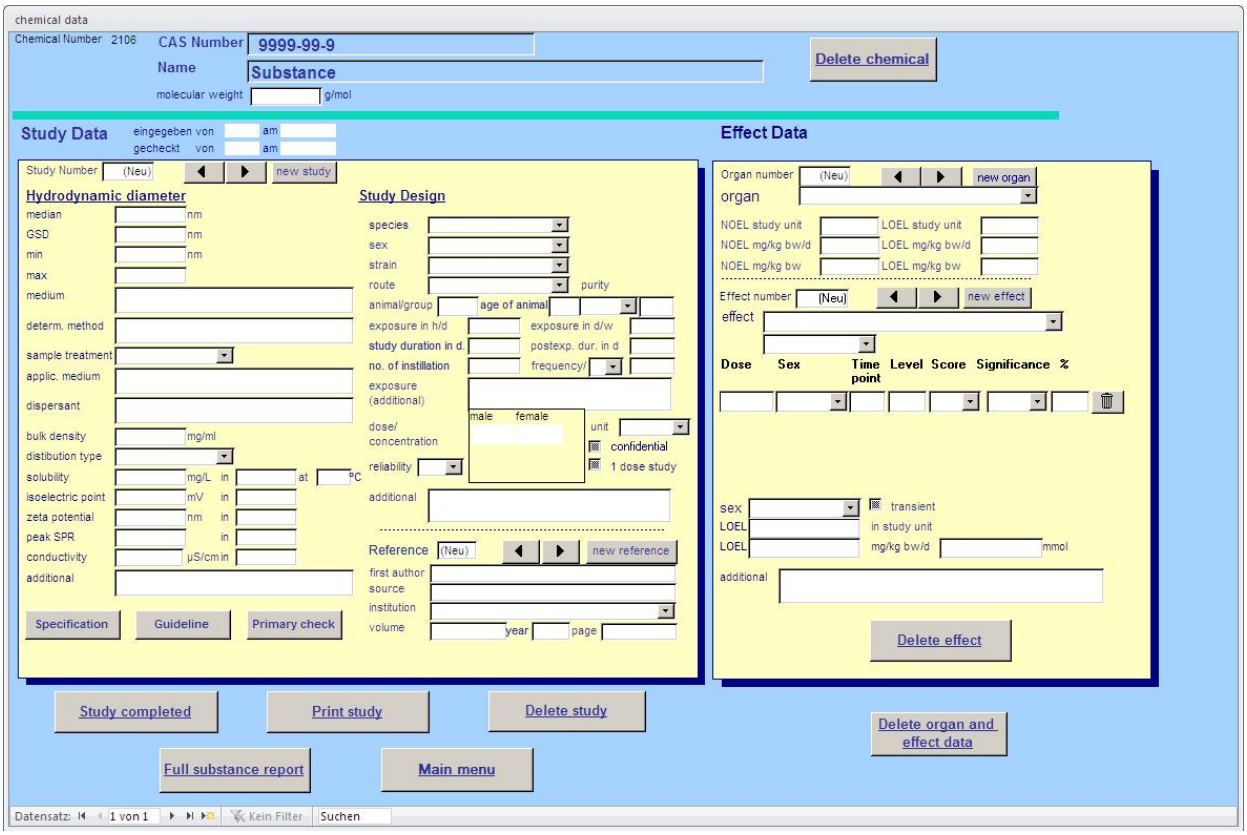

Fig 2: **Data entry mask of the PaFtox database**

2.2.2 Particle and fibre characterisation

In the guidance on physico-chemical characterisation of engineered nanoscale materials for toxicological assessment, it is stated that it is not sufficient to rely on a supplier's commercial characterisation, as that information is tailored to customer applications (ISO/TR 13014:2012). Nanodispersion (aerosol or liquid dispersion) are usually instable as nano-objects (particles and fibres) tend to aggregate or agglomerate. Therefore, it is recommended to test the material "as received" and "as administered"(ISO/TR 13014:2012).

Thus, there are three different types of data on nanomaterial characterisation, which all can be entered into the database.

- Specification of primary object by producer / supplier (as produced)
- Specification of primary object by authors (as received)
- Specification of secondary objects by authors (as administered, exposure media)

In line with ISO/TR 13014:2012, the following information can be entered into the database:

- size / distribution
- aggregation/agglomeration state in the exposure media
- shape
- specific surface area
- composition / purity
- surface chemistry
- solubility / dispersibility
- surface charge

2.2.3 Selection criteria for studies and materials

The particles and fibres which have been entered into the PaFtox database have been selected by availability of suitable and reliable data. In general, five criteria were used for the selection of studies: application route, nanoscale dimension, reliability, species and study duration.

The highest priority for entering into the database was given to inhalation studies (whole body or head/nose only). As the number of inhalation studies is limited for nano-objects also intratracheal and pharyngeal studies were included.

Generally studies with nanoscaled particles were preferred, but additionally larger particles and carbon nanotubes were included for comparison reasons.

As guideline studies are currently seldom available, this criterion couldn't be used for the selection. Non-guideline studies may address special questions. Therefore, the reliability of all publications entered in the database was assessed based on internal quality criteria (similar to RepDose, (Bitsch et al., 2006)) and resulted in reliability classes A (comprehensive study design and scope, high reliability) to C (quality not accessible, preliminary).

The majority of repeated dose toxicity studies for nano-objects were performed in rats or mice. The study selection was initially restricted to these two species, to get comparable data on target organs, mechanisms of toxicity, and LOELs for different nano-objects.

In general, studies with study durations from 28 days up to lifetime exposure were selected. Occasionally, shorter parallel studies of long-term studies (same publication) were entered into the database. For some substances several studies were included in the database. This might be useful to cover different endpoints and to analyse the influence of the study duration on several endpoints. Moreover, contradictory results can be revealed by this selection strategy.

2.2.4 Study design

In the study part, information about the study design and exposure related information can be entered.

- application type
- exposure duration (hours per day, days, days per week)
- instillation (number, frequency)
- post-exposure duration
- species/strain/sex
- number of animals
- particle and fibre characteristic as administered (secondary object)
- reference
- reliability
- scope of study

This database contains four different application types, exposure via air (whole body and nose / head only) and via instillations (intratracheal and pharyngeal). The airborne and instillation studies comprise a fundamental different exposure regime. The standard exposure protocol for inhalation studies via air is 6 hours a day 5 days a week and 28, 91 or 730 days for subacute, subchronic or cancer studies. Instillation studies are mostly performed with one instillation and the corresponding number of days for observation (post-exposure in this database).

To enable a standardised data entry in the PaFtox database, one study is defined by the application type, the exposure conditions and the species/strain. Due to this definition it occurs that one publication often contains more than one study, but it happens also that one study was distributed over several publications. Therefore, the number of publications in the database (appendix 7.1) is different from the number of studies.

Nano-objects in aerosols or in dispersions tend to aggregate or agglomerate. This tendency depends on several physico-chemical properties of the nano-object and of the exposure media. A lot of information is gathered in our report for the BAuA project F2133 (Schaudien et al., 2011). To enable retrospective conclusions, not only the metric information (e.g. MMAD) of the administered objects is a data field of this database but also the descriptive information on the exposure medium and conditions were collected.

As described under chapter 2.2.3, only data for rodents were entered into the database. Studies could have been performed with both sexes and one sex. Two free text fields allow entering more study specific details: the exposure additional and the study additional. The exposure additional describes more specific details of the exposure regimen and the field study additional is related to the study design such as a special focus, unexpected death in single dose groups, description of interim sacrifices (number of animals per time point).

The number of examinations performed in particle studies may differ considerably, because there are currently no guidelines, which define the endpoints to be investigated. The comparison of toxicological potency/effects in different studies is difficult, if it is not distinguished between absence of an effect and absence of examination. Therefore, the scope of investigation is included in the database, where every single investigation e.g. level of specific cytokine is documented.

2.2.5 Toxicological data

In any study a certain number of targets/organs is examined. In each target/organ numerous effects may be investigated using different methods. The database aims to describe all toxicological effects observed in the in vivo study. In PaFtox, effects are not related to the type of examination e.g. necropsy, histopathology or organ weight, but assigned to the target/organ where it occurred. The standardized glossary of targets/organs was adopted from the RepDose database. RepDose contains repeated dose studies of different exposure duration with chemicals for oral and inhalation exposure. However in particle and fibre studies, lung is mainly investigated but here more comprehensively as in similar chemical studies. Therefore, in addition to the already documented targets of the respiratory tract in RepDose, PaFtox includes some more specific targets, which are pleura and bronchio-alveolar lung fluid (BALF). The effect glossary was extended by new effects, which are particle specific or are coming from specific examinations, e.g. cytokine levels that are not common in repeated dose toxicity studies as they are not required in the corresponding guidelines. New entries can easily be added to the glossary. Tab. 2 shows examples for effects covered in the glossary for bronchio-alveolar lavage fluid (BALF) and lung.

It can be distinguished between general effects, which can occur in several organs, e.g. hyperplasia, collagen or burden, and organ specific effects, e.g. bronchiolo-alveolar carcinoma (lung).

For each effect the lowest observed effect level (LOEL) is documented, as well as the sex being affected. The field “effect additional” (2.2.1) further allows documenting some more details, if given in the study report. Further the grading of the effect is documented in the newly developed effect level table.

This table may contain quantitative data (measured parameter), semiquantitative data (scores - mainly histopathology) and qualitative data (yes/no). In any case, the information on dose, sex and time point is given and the parameter level for quantitative data, the score levels minimal, mild, medium, severe, very severe for semiquantitative data and ‘changed’ or ‘no change’ for qualitative data. If available, the corresponding significances are entered. Sometimes effect data were only available in figures. In these cases the values were estimated by a standardized read out system.

The detailed reports provided in the appendix 7.3 demonstrate the level of detail for each individual study.

2.2.6 Quality assurance

Every entry was double checked according to the 4 eyes principle by another scientist of the group. To improve the speed of the data entry, an import module was programmed which allows combining of different versions of the database. Further, for better overview it is possible to print a report on each study entered into the database and on each material (see appendix 7.2 for examples).

Several queries have been made to assure consistency of entries e.g.:

- Are all relevant fields filled with information? (E.g. all fields of study design)
- Do doses correspond to effect LOEL values?
- Do calculated values correspond in all fields of the database, e.g. number of decimals equal?

- Are effects documented in a comprehensive way?
- Are effects documented twice? If so, for which reasons?
- Are effects in the glossary redundant?
- Which effects are rare? Which grade of detail is needed?
- Control for synonyms.

Tab. 2: Examples for effects in BALF and lung

Target / Organ	Parameter / Effect
BALF	Total protein
	Lactate dehydrogenase (LDH)
	PMN
	Total cells
	Neutrophils
	Alveolar macrophages
	Lymphocytes
	Leukocytes
	Reactive oxygen species (RNS) ex vivo
	Alkaline phosphatase (AP)
	β-glucuronidase
	Gamma-glutamyl transferase (γGT)
	Burden
	Tumour necrosis factor protein (TNF-α)
	Reactive oxygen species (ROS) ex vivo
Lung	8-OHdG
	Adenosquamous carcinoma
	Alveolar bronchiolization
	Clearance
	Alveolar proteinosis
	Apoptose
	Benign cystic keratinizing squamous-cell tumour
	Bronchiolo-alveolar adenocarcinoma
	Bronchiolo-alveolar adenoma
	Bronchiolo-alveolar carcinoma
	Burden
	Cell depletion
	Cell division cycle mRNA (Cdc2a)
	Cell proliferation
	Changes in organ structure
	Chemokine mRNA (CCL2)

2.2.7 Database queries

NormDose: Exposure conditions in inhalation studies differ in dose level, exposure duration in hours per day and days per week. Therefore the original dose mostly provided for 6 h per day, 5 days per week in inhalation studies was normalised to 24 h/day and 7 days/week.

CumDose: If instillation studies used more than one instillation, the time point of effect determination was compared with the instillation regime and the corresponding cumulative dose for that time point was calculated.

Categories for time points: The studies differ also regarding their time regime. Thus the determination of effects occurred on a number of different days. Therefore, to simplify data analyses, the time points were categorised.

Statistical analyses were performed with STATISTICA® and Microsoft excel®.

To better illustrate differences in numbers of studies or LOELs identified, the three colour code offered in Excel was used and upper and lower limit (10th percentile, 90th percentile) were assigned to the traffic light code; green means better results or more information and red means worse results or less information.

By analysing the minima of doses where an effect was positive, LOELs can be determined. Depending on the conditions, chosen for the query, different LOELs have to be distinguished: effect-LOEL, organ-LOEL, study-LOEL, substance-LOEL and object-LOEL or categories thereof. If several studies were available with the same route and duration for a specific substance, for some analyses the lowest LOEL, i.e. the substance-LOEL was used.

3 Data analysis

3.1 Particle and fibre characterisation

Due to dimensional differences between nanoparticles and nanotubes the data about these two types of nano-objects are analysed separately. Overall relatively little information about nano-objects characteristics is provided in the toxicological studies entered in the PaFtox database. General no information is provided for solubility, neither in water nor in the application media.

3.1.1 Specification of primary particles and fibres by producer / supplier (as produced) or by authors (as received)

Overall, the amount of data related to primary characterisation is very low, see summary statistics about the primary characterisation (Tab. 3).

Tab. 3: Primary characterisation in studies with nano-objects

	Specification by producer / supplier	Specification by authors	Specification by producer/supplier or author
Diameter (mean)	93	25	114
Diameter (min)	15	3	16
Diameter (max)	17	4	19
Inner diameter *	0	0	0
Outer diameter (mean)*	4	2	5
Outer diameter (min)*	3	3	6
Outer diameter (max)*	3	3	6
Length (mean)*#	5	2	5
Length (min) *#	4	2	5
Length (max) *#	3	1	4
Medium	2	8	9
Determination method	17	33	46
Distribution type	3	11	11
Cristal structure	63	16	79
Shape	15	16	29
Solubility	0	0	0
Specific surface	55	59	114
Surface property	27	23	44
Particle density	27	22	45

Overall number of studies: 131, *for tubes; # for tubes and rods

Tab. 4 demonstrates that even if information is available, there are differences for the frequently used reference materials. Different batches or producing sites could cause these differences.

Tab. 4: Available information for reference material "Min-U-Sil 5"

		Specification by supplier			Specification by authors		
		Diameter [nm]			Diameter [nm]		
		Median	Minimum	Maximum	Median	Minimum	Maximum
Kobayashi, et al. 2009	Toxicology				Cites Warheit et al., 2007a and Kajiwara et al., 2007		
Kobayashi, et al. 2010	Toxicology				Cites Warheit et al., 2006, 2007a,b; Kobayashi et al., 2009		
Shvedova , et al. 2005	Am J Physiol Lung Cell Mol Physiol				2140		
Ogami, et al. 2009	Inhalation Toxicology		1600	10000			
Warheit, et al. 2004	Tox Sciences		1000	3000			
Warheit, et al. 2007a	Tox Sciences		300	2000	534	300	700
Warheit, et al. 2007b	Toxicology		200	2000	480		

3.1.2 Specification of secondary particles and fibres by authors (as administered)

Tab. 5 shows the number of studies where information on secondary characterization (as administered) was available. No information was provided in the publications about solubility in the used medium, the isoelectric point, the conductivity and almost nothing about zeta potential or spectra data which allow some inferences to the dimension and appearance of the nano-objects in the media.

Tab. 5: Secondary characterisation in studies with nano-objects

Number of studies	Inhalation studies: Number of available data	Percentage of Inhalation studies (n=38)	Instillation studies: Number of available data	Percentage of Instillation studies (n=93)
Diameter (median)	26	68%	24	26%
Diameter (GSD)	23	61%	1	1%
Diameter (min)	3	8%	9	10%
Diameter (max)	3	8%	10	11%
Medium	4	11%	31	33%
Determination method	26	68%	31	33%
Sample treatment	2	5%	66	71%
Application medium	2	5%	90	97%
Dispersant	0	0%	26	28%
Bulk density	4	11%	0	0%
Distribution type	38	100%	92	99%
Zetapotential	2	5%	1	1%
Solubility in medium	0	0%	0	0%
Isoelectric point	0	0%	0	0%
Conductivity	0	0%	0	0%

Diameter means mass median aerodynamic diameter and hydrodynamic diameter for inhalation and instillation studies, respectively.

A comparative analysis between MMAD or hydrodynamic diameter and primary diameter was performed (Fig 3). A dependency could not be identified. However, MMADs cover several orders of magnitudes. MMAD is a double critical parameter, first its size determines the deposition behaviour and fate (Oberdörster et al., 2005) and second it's difficult to standardize these size measurements, currently three different techniques lead to three different results (Pauluhn, 2010). This issue of primary nano diameter versus micro MMAD or hydrodynamic diameter contains the problem that in many studies the nano-objects are applied as larger aggregates, only slightly different from original larger particles. As disaggregation seldom occurs, only minor differences in effects could be expected between nano and larger particles.

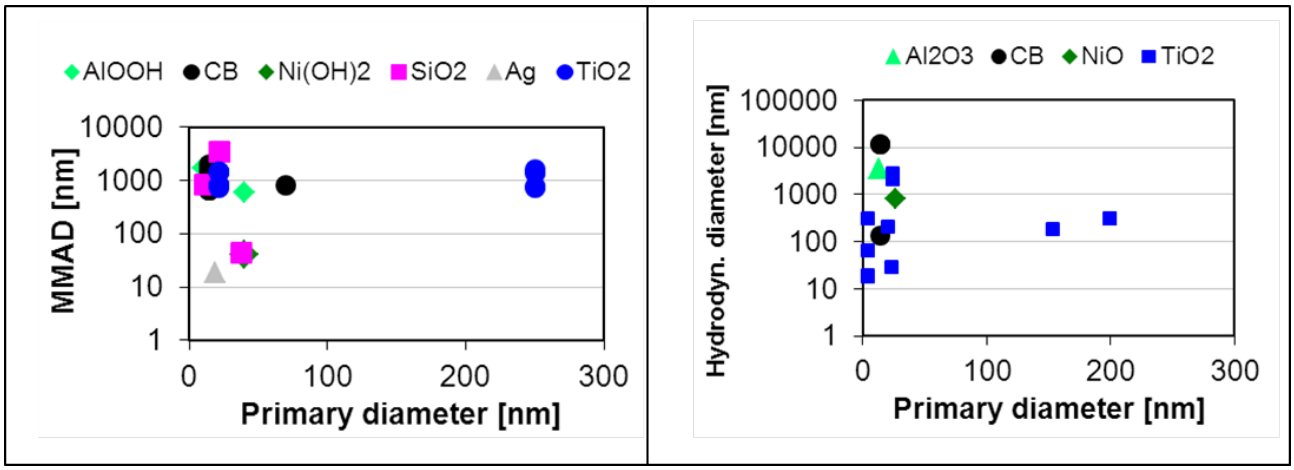

Fig 3: MMAD in inhalation studies (left) and hydrodynamic diameter in instillation studies (right)

3.2 Studies included

3.2.1 Routes and study durations

In the public literature a huge variety of study designs are published. Tab. 6 and Tab. 7 give an overview of the design of inhalation and instillation studies. The inhalation studies differ with respect to the type of inhalation (nose / head only), study duration, duration of postexposure period and duration of exposure per day and per week. Similarly instillation studies differ with respect to frequency of instillations and time after instillation. The study designs encountered most frequently are highlighted in bold. Within the different studies, investigations have been performed at interim time points, especially in instillation studies.

Tab. 6: Study design of inhalation studies

route	Category of study duration	Exposure duration in days	Post-exposure in days	Exposure time in h/d	Exposure duration in d/wk	Number of studies
Nose / head only	Subacute	23	90	6	5	2
		28	91	6	5	1
		28	546	0.66	7	1
	Subchronic	90	1	6	5	1
		91	180	6	5	1
Whole body	Subacute	10	0	6	7	2
		24	0	6	5	1
		28	0	2	3	2
		28	0	6	5	1
		28	2	6	5	1
	Subchronic	84	364	6	5	2
		91	0	6	5	1
		91	224	6	5	1
		91	240	6	5	1
		91	335	6	5	3
		91	364	6	5	8
	Chronic	152	1	5	5	1
		411	288	18	5	2
		547	180	18	5	1
		547	183	18	5	1
	Cancer	730	0	6	5	1
		730	50	16	5	1
		730	180	18	5	1
		730	182	18	5	1

Tab. 7: Study design of instillation studies

Application route	Category of study duration	Exposure duration in days	Post-exposure in days	Number of instillation	frequency	per	Number of studies
Intratracheal	Cancer	1	874	1			1
		28	872	5	1	wk	5
		63	812	10	1	wk	1
		63	827	10	1	wk	1
		63	837	10	1	wk	4
		64	826	10	1	wk	1
		98	802	15	1	wk	1
		105	695	16	1	wk	2
		133	767	20	1	wk	2
		203	697	30	1	wk	1
		406	469	30	2	wk	1
	Chronic	1	98	1			2
		1	181	1			**6**
		1	274	1			1
		2	456	2	1	d	3
		63	393	10	1	wk	1
		84	77	4	0.5	d	1
		266	14	20	2	wk	1
	Subacute	1	3	1			1
		1	7	1			1
		1	13	1			1
		7	21	2	1	wk	1
		8	2	2	1	wk	1
		21	0	15	5	wk	1
		21	7	4	1	wk	1
		1	28	1			**8**
	Subchronic	1	30	1			2
		1	42	1			4
		1	60	1			**6**
		1	90	1			**17**
		35	0	6	1	wk	2
		35	1	6	1	wk	2
		42	0	30	5	wk	1
		42	1	7	1	wk	1

Application route	Category of study duration	Exposure duration in days	Post-exposure in days	Number of instillation	frequency	per	Number of studies
		61	30	3	1	mo	3
		63	0	45	5	wk	1
Pharyngeal	Subchronic	1	28	1			1
		1	60	1			3

3.2.2 Species

Usually studies were performed with rats or mice (Tab. 8). Due to the high spontaneous lung tumour rate in mice, the rat is the preferred species especially for long term studies with particles. This is also reflected by the species distribution in the database where 80% are rat studies. However, especially for the shorter study duration, where inflammation endpoints are mainly investigated, mice are also used (see Tab. 8).

Tab. 8: Types of studies in rats and mice

Category of study duration	Application route	Number of mouse studies	Number of rat studies
Subacute	Nose /head only		4
	Whole body	3	4
	Intratracheal	**7**	12
	Pharyngeal	1	
Subchronic	Nose /head only		2
	Whole body	3	13
	Intratracheal	5	**32**
	Pharyngeal	3	
Chronic	Whole body	3	6
	Intratracheal	1	**32**
Total number species	All routes	26	105

Bold: Highest number of studies

3.2.3 Dose levels, NOELs and LOELs

According to the respective OECD guidelines repeated dose toxicity studies are performed with 3 dose levels and a control group. The lowest dose group is intended to show no effects (NOEL), the medium dose group should reveal slight toxicity (LOEL) and the highest dose group should have clear toxic effects, but no increased mortality. For toxicological evaluations effects at the LOEL are most important, as these reflect effects at low dose levels. As demonstrated in Tab. 9, 74 studies investigated only one dose level, 22 studies two dose levels and 35 studies used three or more dose levels.

Tab. 9: Number of different dose levels

		Number of studies		
Route	**Category time point**	**1 Dose Level**	**2 Dose Levels**	**3 and more dose levels**
Nose /head only	37-99			1
Nose /head only	100-189			3
Nose /head only	190-365			1
Nose /head only	366-912	1		
Whole body	1-10	2		
Whole body	11-36	4		1
Whole body	37-99			1
Whole body	100-189	1		
Whole body	190-365	1		1
Whole body	366-912	12	1	8
Intratracheal	1-10	1		2
Intratracheal	11-36	10	4	4
Intratracheal	37-99	14	12	9
Intratracheal	100-189	5	1	1
Intratracheal	190-365	2		
Intratracheal	366-912	19	4	1
Pharyngeal	11-36			1
Pharyngeal	37-99	2		1

3.3 Particles included

Currently, the PaFtox database contains 17 different materials. For titan dioxide and Carbon Black numerous studies with different particle sizes were available. An overview is provided in Tab. 10.

Tab. 10: Particles included

Substance	**Titan dioxide**							**Carbon Black**							
Diameter (mean) in nm	4.9	20-25	154	180	200	250	1000	14	15	37	56	70	95	120	260
Specific Surface in m2/g	316	32-66	10	9.9	8.8	6.5	2.34	271-337	230	43	45	37	22	n.g.	n.g.

Not all particles are available for all application routes

The distribution of particles over the different application routes is illustrated in Tab. 11.

Tab. 11: Number of studies for different substances and particle diameter categories and application routes

Substance	Category of diameter (mean)in nm	Number of studies			
		Intra-tracheal	Nose/ head only	Pharyngeal	Whole body
Aluminium oxide C	50	2			
Aluminium trioxide	50	1			
Aluminium trioxide	500	1			
Aluminium oxyhydroxide	50		2		
Carbon Black	50	13		1	7
Carbon Black	100	1			1
Carbon Black	500	1			1
Cerium(IV) oxide	50	1			
Iron(II,III) oxide	10	1			
lamp black 101+diesel soot	100	3			
Manganese(IV) oxide	50	3			
Nickel	50	1			
Nickel hydroxide	50				1
Nickel oxide	50	2			
Nickel oxide	3000	1			
Silicon dioxide	50	16	2		4
Silicon dioxide	500	6			
Silicon dioxide	1500	8			
Silicon dioxide	3000	1		1	
Silicon dioxide	5000	1			
Silicon dioxide	8000				1
Silver	50				2
Titanium dioxide	10	4			
Titanium dioxide	50	11			7
Titanium dioxide	500	6			5
Titanium dioxide	1500	1			
Titanium dioxide	3000				1
Toner	500	1			

Total number of studies: 122

3.4 Parameter / Effect analysis

3.4.1 Target / organs and effects

Fig 4 presents an overview of affected targets / organs, distinguished between the different application routes. Generally, lung and BALF are the main targets, followed by lymph nodes.

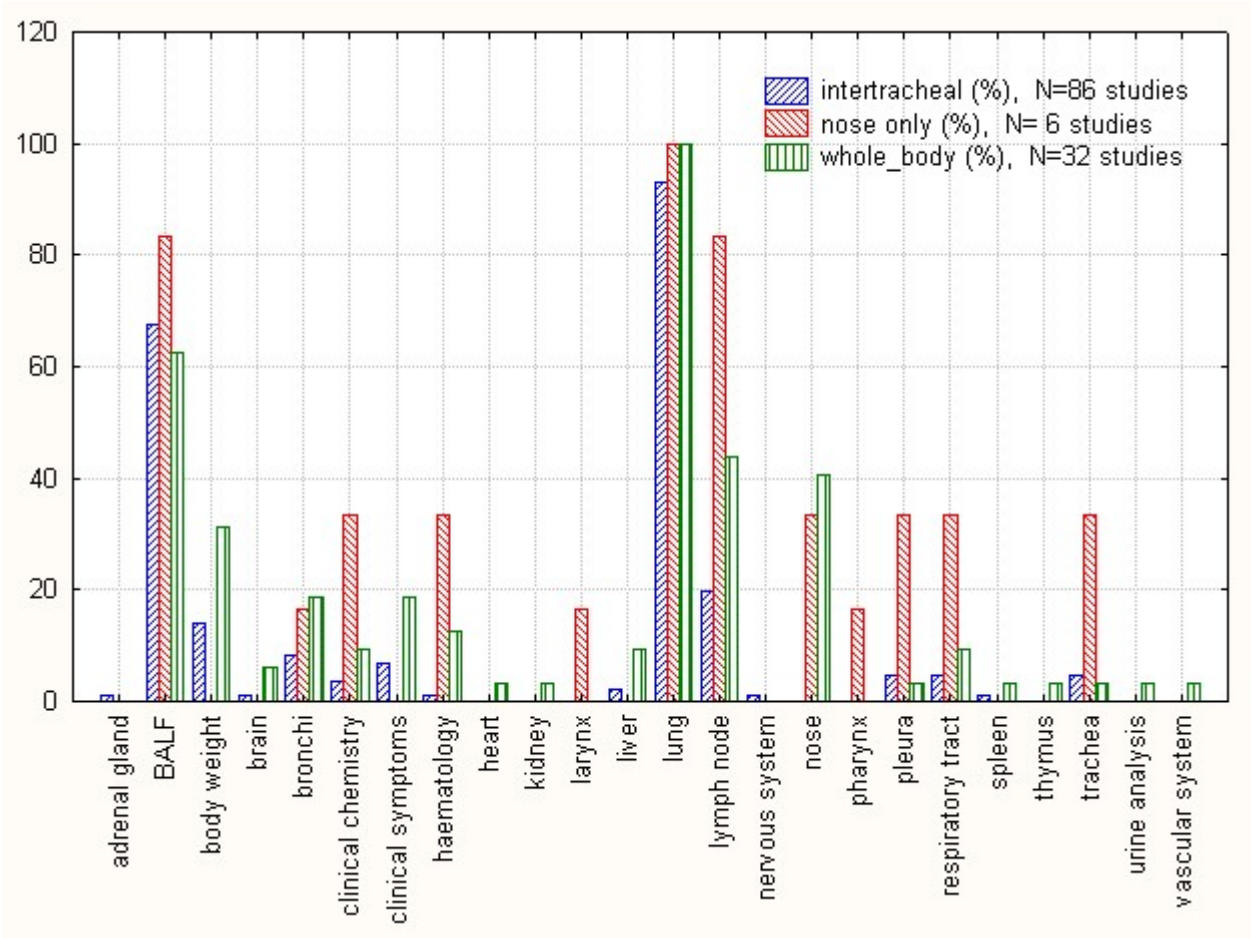

Fig 4: Overview effected targets / organs

Tab. 12 provides an overview of the effects found in all studies, irrespective of the route of application, dose and time point. It shows that some effects appear very frequently, such as lactate dehydrogenase (LDH), total protein, PMN in BALF or macrophage infiltration, fibrosis and inflammation in the lung.

Tab. 12: Observed effects

Target / Organ	Parameter / Effect	Parameter / Effect observed (no of studies)
BALF	Lactate dehydrogenase (LDH)	50
BALF	Total protein	50
BALF	PMN	50
Lung	Macrophage infiltration	48
Lung	Weight	46
BALF	Total cells	41
BALF	Neutrophils	40
BALF	Alveolar macrophages	36
Lung	Fibrosis	34
Lung	Inflammation	28
Lymph node	Burden	28
BALF	Lymphocytes	24
Lung	Hyperplasia	22
Body weight	Weight decreased	21
Lung	Collagen	20
Lung	Bronchiolo-alveolar adenoma	18
Lung	Tumour (other)	17
Lung	Squamous cell carcinoma	16
Lung	Alveolar type II cells	16
Lung	Granuloma	16
Lung	Alveolar proteinosis	15
Lung	Cell proliferation	15
BALF	Leukocytes	13
Lung	Macrophages foamy	13
Lung	Bronchiolo-alveolar carcinoma	13
Lung	Cystic keratinizing epithelioma	13

Total number of studies: 131

As indicated in Fig 4 most effects are local effects in the respiratory tract. Tab. 13 shows that effects in the lymph nodes, spleen, blood and pleura have been found especially in inhalation studies and indicate migration of the nano-objects.

Tab. 13: Parameter / Effects beyond the respiratory tract

Target / Organ	Effect	Nose / head only	Whole body	Intratracheal
Adrenal gland	Weight			1
Brain	Burden		2	1
Brain	Functional disorders			1
Clinical symptoms	Behaviour abnormal			1
Blood	B cells			3
Blood	Erythrocytes		8	
Blood	Granulocytes	1		
Blood	Haematocrit		2	
Blood	Haemoglobin		2	
Blood	Leukocytes total	1		
Blood	Lymphocytes total	3		
Blood	Natural killer cells (NK)			3
Blood	Natural killer T cells (NKT)			3
Blood	Neutrophils total	2	6	
Blood	T cells			3
Blood	T cells CD4+/CD8+			3
Heart	Blood pressure		2	
Heart	Heart rate		1	
Kidney	Burden		1	
Liver	Burden		2	
Liver	Hyperplasia		1	
Liver	Necrosis		2	
Liver	Vacuolization		1	
Liver	Weight			2
Lymph node	Burden	25	40	23
Lymph node	Cell proliferation			1
Lymph node	Changes in organ structure	4		
Lymph node	Discoloration	2		
Lymph node	Fibrosis		1	4
Lymph node	Granuloma	1		5
Lymph node	Histiocytosis	3		1
Lymph node	Hypercellularity	1		
Lymph node	Hyperplasia	2	8	6
Lymph node	Hypertrophy			3
Lymph node	Infiltration			8

Target / Organ	Effect	Nose / head only	Whole body	Intratracheal
Lymph node	Inflammation			4
Lymph node	Macrophage accumulation	9	3	7
Lymph node	Macrophage damage		3	
Lymph node	Macrophage infiltration			4
Lymph node	Weight	13	1	
Nervous system	Functional disorders			1
Pleura	Changes in organ structure	1		
Pleura	Collagen			6
Pleura	Fibrosis			1
Pleura	Inflammation		1	1
Pleura	Thickening	4		
Spleen	Burden		1	
Spleen	Weight			1
Thymus	Weight		1	
Urine	Protein		1	
Vascular system	Burden		1	

3.4.2 Reversibility of effects

Tab. 14 documents the effects per target organ for studies with whole body exposure which include in addition to the investigations at the end of the exposure period also investigations after a postexposure period. The postexposure durations ranged from 2 to 364 days.

In total 755 effects were observed, 407 effects within the treatment period of the studies and 348 effects in addition in the post exposure period. The number of effects (at lowest observed effect level) were counted per time point and are subdivided into two groups: effect observed within exposure duration (termed (-) postexposure) or the effect was observed in the postexposure time (termed (+) postexposure). In most studies interim sacrifices were performed, so that effects for several time points can be distinguished. Time points were grouped into six time categories, evaluations <= 10 days (termed 10 days), <=36 days (termed 28 days), <100 (termed 90) <= 189 days (termed 182 days), <= 365 days (termed 365 days) and > 365 days (termed 700 days). All effects of the treatment period were also observed in the postexposure period. To obtain comparable results for the different groups, the number of positive effects was normalized to the number of studies of the corresponding subgroup.

Tab. 14: Parameter / Effect of study evaluation (+) or (-) postexposure in whole body inhalation studies

		Percentage of positive effects per total study number (n = 22)								Ratio (+)postexposure / (-)postexposure			
		(-) postexposure				(+) postexposure							
Target / Organ	**Parameter / Effect**	**90**	**182**	**365**	**700**	**90**	**182**	**365**	**700**	**90**	**182**	**365**	**700**
BALF	AM relative	23				23	32	9	23	1			
BALF	AM total	9		5	23	9	9	9	45	1		2	2
BALF	Burden	9				9		5	5	1			
BALF	LDH	23		5	18	23	14	9	32	1		2	1.75
BALF	Neutrophils relative	14				14	18	9	14	1			
BALF	Neutrophils total	9		5		9	18	9	5	1		2	
BALF	PMN relative	27				27	14	5	14	1			
BALF	PMN total	9			9	9		5	18	1			2
BALF	β-glucuronidase	23		5	18	23	9	9	32	1		2	1.75
BALF	Total cells	41				41	9	5	14	1			
BALF	Total protein	23			9	23	9	5	23	1			2.5
Body weight	Weight	5		9	23	5	9	18	45	1		2	2
Clinical symptoms	Mortality				27				41				1.5
Lung	Alveolar proteinosis	14		5	9	14	9	5	27	1		1	3
Lung	mRNA (CCL2)	9				9	5	5		1			
Lung	Deposits			5	9		5	5	14			1	1.5
Lung	Fibrosis	5	14	18	27	5	32	32	68	1	2.33	1.75	2.5
Lung	Hyperplasia alveolar type II cells	23	5	5	14	23	14	5	32	1	3	1	2.3
Lung	Infiltration	23		5	5	23	14	9	18	1		2	4
Lung	Inflammation	9	5	5	14	9	5	5	18	1	1	1	1.3
Lung	Macrophage damage	14			9	14	9		23	1			2.5
Lung	Macrophage infiltration	32	9	9	23	32	50	27	50	1	5.5	3	2.2
Lung	Macrophages interstitial	9	5	9	9	9	27	23	32	1	6	2.5	3.5
Lung	Metaplasia			5	9			5	14			1	1.5
Lung	Weight	27	14	23	32	27	23	23	45	1	1.67	1	1.4
Lymph node	Burden	27	5	5	27	27	18	23	45	1	4	5	1. 7
Nose	Degeneration	5	18	18	18	5	18	18	45	1	1	1	2.5
Nose	Hyperplasia	5	9	9		5	14	9	18	1	1.5	1	
Nose	Inflammation			9	18			9	27			1	1.5
Nose	Squamous cell metaplasia			9	18			9	27			1	1.5

Traffic light code used for illustration lower limit: 10 percentile set to green and upper limit: 90 percentile set to red;
AM - alveolar macrophages

Tab. 14 also depicts in the last column the ratio between the number of effects during the treatment and postexposure period of studies. If this value is higher than 100% it means, that effects are occurring more frequently at the LOEL of the study in the subgroup with postexposure.

No effect disappeared, indicating that no effect was fully reversible. This analysis does however, not specify differences in grade and severity of effects and thus their in- or decrease during the postexposure period. In addition one effect in BALF and eight effects in lung are only seen in the postexposure period (Tab. 15).

Tab. 15: Parameter / Effects (number) appearing only in the postexposure period at LOEL per time point in studies with whole body exposure.

		Category time point	
Target / Organ	**Parameter / Effect**	**100-189**	**366-730**
BALF	Glutathione peroxidase	1	2
Lung	Adenosquamous carcinoma		1
Lung	Benign cystic keratinizing squamous-cell tumour		2
Lung	Bronchiolo-alveolar adenocarcinoma		3
Lung	Gamma-glutamylcysteine synthetase (γ-glutamGCL)		2
Lung	Manganese superoxide dismutase (Mn SOD)		2
Lung	Squamous metaplasia		1
Lung	Thickening		1
Lung	Tumour supressor protein p53		1

3.4.3 Parameters affecting toxicity of particles and fibres

In a preliminary analysis the influence of different parameters discussed to influence the toxicity of nano-objects was investigated. The following parameters were analysed:

- the composition of the (nano) particle
- the mean diameter
- the specific surface

Studies with whole body exposure and intratracheal studies were analysed, because most studies were available for these application types. In most studies interim sacrifices were performed, so that effects for several time points can be distinguished. Time points were again grouped into six time categories, evaluations <= 10 days (termed 10 days), <=36 days (termed 28 days), <100 (termed 90) <= 189 days (termed 182 days), <= 365 days (termed 365 days) and > 365 days (termed 700 days).

While in intratracheal studies investigations are already performed at early time points, the study design of inhalation studies focus on analyses at later time points. However in both routes, not every time point is covered.

3.4.3.1 Composition

Tab. 16 shows LOELs derived from different studies for different substances and for the different application routes. There are considerable differences in toxicity between substances, while the exposure duration has a minor influence on the toxicity.

Tab. 16: LOELs per route, substance and time point

Application route	Name	Min LOEL per time point					
		10	28	90	182	365	700
Nose / head only	Aluminum oxyhydroxide	5E+00	4E+00	5E+00	6E-01		
	MWCNT			2E-02	2E-02	8E-02	
	Silicon dioxide		7E-01	9E+00	9E+00	3E+01	
Whole body	Carbon Black		3E+00	2E-01	2E-01	1E+00	2E-01
	MWCNT		6E+00				
	Nickel hydroxide			2E-02	2E-02		
	Silicon dioxide			2E-01	2E-01	2E-01	2E-01
	Silver		9E-05	9E-03			
	Titanium dioxide	6E+00	4E-01	9E-02	9E-02	9E-02	9E-02
Intratracheal	Aluminium oxide C				1E+02		1E+02
	Aluminium trioxide	2E+00	2E+00	2E+00			
	Carbon Black	2E-01	1E+01	4E-01	7E+01	2E+02	1E+01
	Cerium(IV) oxide	2E-01	5E-01				
	Iron(II,III) oxide	3E-01	1E+00				
	lamp black 101+diesel soot						1E+02
	Manganese(IV) oxide		6E+01	1E+02			
	MWCNT	4E-02	4E-02	4E-02	2E-01		
	Nickel	5E-01	5E+00				
	Nickel oxide	3E-01	3E-01	3E-01	3E-01		
	Silicon dioxide	8E-03	8E-01	8E-01	5E+00	1E+01	1E+01
	SWCNT	1E+00	5E+00	1E+00			
	Titanium dioxide	8E-01	8E-01	8E-01			1E+01
	Toner	2E+02	2E+02	2E+02			
Pharyngeal	SWCNT	3E-01	5E-01	5E-01			

LOELs normalised doses for inhalation studies in mg/m³ and cumulative doses in mg/kg bw for instillation studies; Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

To further investigate the influence of particle composition; effects were identified that were either caused by reference particles (titanium oxide, silicon oxide, aluminium oxides and Carbon Black) or by heavy metals and oxides (silver, nickel and oxide, iron oxide, cerium oxide and manganese oxide). Reference particles cause overall 178 different effects (data not shown). The high number of effects is probably due to the fact

that especially Carbon Black and titanium dioxide are well investigated with numerous endpoints. A lower number of unique effects have been observed for the heavy metal based particles (Tab. 17). Interestingly, only one effect is caused by two different substances, but both are Nickel compounds. All other effects are unique per substance.

Tab. 17: Parameter / Effects occurring only with heavy metals and oxides

Target / Organ	Parameter / Effect	Substance
Adrenal gland	Weight	Manganese(IV) oxide
BALF	Apoptosis	Cerium(IV) oxide
BALF	Arginase-1 mRNA ex vivo	Cerium(IV) oxide
BALF	Caspase protein (CASP3) ex vivo	Cerium(IV) oxide
BALF	Caspase protein (CASP9) ex vivo	Cerium(IV) oxide
BALF	Cell cycle: G1 phase	Iron(II,III) oxide
BALF	Cell cycle: S phase	Iron(II,III) oxide
BALF	Chemokine mRNA (CCL3)	Nickel oxide
BALF	**Chemokine protein (CCL2)**	**Nickel hydroxide**
BALF	**Chemokine protein (CCL2)**	**Nickel oxide**
BALF	Cytokine-induced neutrophil chemoattractant protein (CINC-1)	Nickel oxide
BALF	Cytokine-induced neutrophil chemoattractant protein (CINC-2 αβ)	Nickel oxide
BALF	Interleukin protein (IL-1)	Iron(II,III) oxide
BALF	Interleukin protein (IL-12)	Iron(II,III) oxide
BALF	Interleukin protein (IL-12) ex vivo	Cerium(IV) oxide
BALF	Interleukin protein (IL-4)	Iron(II,III) oxide
BALF	Interleukin protein (IL-5)	Iron(II,III) oxide
BALF	Lipid peroxides (LPO)	Nickel
BALF	Nuclear factor mRNA (NF-κB) ex vivo	Cerium(IV) oxide
BALF	Phospholipids	Cerium(IV) oxide
BALF	Suppressor of chemokine signalling mRNA (SOCS-1) ex vivo	Cerium(IV) oxide
Brain	Functional disorders	Manganese(IV) oxide
Clinical chemistry	Calcium	Silver
Clinical chemistry	IgE	Iron(II,III) oxide
Clinical chemistry	Interleukin protein (IL-1)	Iron(II,III) oxide
Clinical chemistry	Interleukin protein (IL-12)	Iron(II,III) oxide
Clinical chemistry	Interleukin protein (IL-4)	Iron(II,III) oxide
Clinical chemistry	Interleukin protein (IL-5)	Iron(II,III) oxide
Clinical chemistry	Total protein	Silver
Clinical chemistry	Tumour necrosis factor protein (TNF-α)	Iron(II,III) oxide
Clinical symptoms	Behaviour abnormal	Manganese(IV) oxide

Target / Organ	Parameter / Effect	Substance
Blood	B cells	Iron(II,III) oxide
Blood	Natural killer cells (NK)	Iron(II,III) oxide
Blood	Natural killer T cells (NKT)	Iron(II,III) oxide
Blood	T cells	Iron(II,III) oxide
Blood	T cells CD4+/CD8+	Iron(II,III) oxide
Liver	Hyperplasia	Silver
Liver	Necrosis	Silver
Liver	Vacuolization	Silver
Liver	Weight	Manganese(IV) oxide
Lung	Cytokine-induced neutrophil chemoattractant protein (CINC-1)	Nickel oxide
Lung	Cytokine-induced neutrophil chemoattractant protein (CINC-2 αβ)	Nickel oxide
Lung	Glycoprotein mRNA (OPN)	Cerium(IV) oxide
Lung	Heat shock protein mRNA (HSP1a)	Iron(II,III) oxide
Lung	Heat shock protein mRNA (HSP8)	Iron(II,III) oxide
Lung	Interleukin mRNA (IL-1 α)	Nickel hydroxide
Lung	Interleukin protein (IL-1 α)	Nickel oxide
Lung	Interleukin protein (IL-2)	Nickel oxide
Lung	Lipidosis	Cerium(IV) oxide
Lung	Matrix metalloproteinase mRNA (MMP-12)	Iron(II,III) oxide
Lung	Matrix metalloproteinase mRNA (MMP-19)	Iron(II,III) oxide
Lung	Matrix metalloproteinase mRNA (MMP-23)	Iron(II,III) oxide
Lung	MHC II mRNA (H2-Eb1)	Iron(II,III) oxide
Lung	MHC II mRNA (H2-T17)	Iron(II,III) oxide
Lung	MHC II mRNA (H2-T23)	Iron(II,III) oxide
Lung	Minute volume	Silver
Lung	Peak inspiration flow	Silver
Lung	PMN total	Cerium(IV) oxide
Lung	Proteinase inhibitor mRNA (SLPI)	Iron(II,III) oxide
Lung	Serum amyloid mRNA (SAA3)	Iron(II,III) oxide
Lung	Tidal volume	Silver
Nervous system	Functional disorders	Manganese(IV) oxide
Urine analysis	Protein	Silver

Silicon dioxide is frequently used as reference material in toxicity studies of particles. Therefore we analysed if there are silicon dioxide specific effects. Tab. 18 shows silicon dioxide specific effects and the size and

crystal structure responsible for the effect. Both types can cause significant effects in the lung, pleura and lymph nodes of different severity. The weight gain of thymus found for crystalline silica was transient.

Tab. 18: Parameter / Effects occurring only with silicon dioxides

Target / Organ	Parameter / Effect	Size & Crystal structure
Haematology	Neutrophils total	Nano amorphous or micro crystalline
Lung	Spongiosis	Nano amorphous or micro crystalline
Lung	Swelling	Nano amorphous or micro crystalline
Lymph node	Fibrosis	Nano amorphous or micro crystalline
Lymph node	Infiltration	Nano amorphous or micro crystalline
Lymph node	Inflammation	Nano amorphous or micro crystalline
Nose	Necrosis	Nano amorphous or micro crystalline
BALF	Chemotaxis	Micro crystalline
BALF	Phosphatidylglycerol/phosphatidylinositol (PG/PI)	Micro crystalline
Bronchi	Hyperplasia mucus cell	Micro crystalline
Clinical chemistry	Alanine aminotransferase (ALAT)	Micro crystalline
Clinical chemistry	Alkaline phosphatase	Micro crystalline
Lung	8-oxoGua	Micro crystalline
Lung	Hyperplasia alveolar type II cells	Micro crystalline
Lung	p53 mutations	Micro crystalline
Lymph node	Hypertrophy	Micro crystalline
Pleura	Fibrosis	Micro crystalline
Thymus	Weight	Micro crystalline
Bronchi	Apoptosis	Nano amorphous
Bronchi	Necrosis	Nano amorphous
Clinical chemistry	Globulin	Nano amorphous
Clinical chemistry	Histamine	Nano amorphous
Clinical symptoms	Respiratory distress	Nano amorphous
Haematology	Haematocrit	Nano amorphous
Haematology	Haemoglobin	Nano amorphous
Lung	Chemokine protein (CXCL2)	Nano amorphous
Lung	Interferon gamma mRNA (IFN-γ)	Nano amorphous
Lung	Interleukin mRNA (IL-4, IL-8, IL-10, IL-13)	Nano amorphous
Lung	Interleukin protein (IL-1β, IL-4, IL-6, IL-8, IL-	Nano amorphous

Target / Organ	Parameter / Effect	Size & Crystal structure
	10, IL-13)	
Lung	Laminin	Nano amorphous
Lung	Matrix metalloproteinase mRNA (MMP-9, MMP-10)	Nano amorphous
Lung	Matrix metalloproteinase protein (MMP-9)	Nano amorphous
Lung	Metallopeptidase inhibitor protein (TIMP-1)	Nano amorphous
Lung	Necrosis	Nano amorphous
Lymph node	Cell proliferation	Nano amorphous
Lymph node	Histiocytosis	Nano amorphous
Lymph node	Macrophage damage	Nano amorphous
Lymph node	Macrophage infiltration	Nano amorphous
Pleura	Changes in organ structure	Nano amorphous

Crystalline covers also mainly crystalline.

3.4.3.2 Tubes versus particles

To investigate also the influence of the shape on toxicity an analysis was performed if carbon nano tubes (CNTs) cause other effects than titanium oxide, silicon oxide, aluminium oxides, Carbon Black or heavy metal compounds. Tab. 19 shows effects identified only for tubes but not with „inert" particles, silicon dioxide or heavy metal based particles. Interestingly, all tube specific effects are unique per CNT type. Besides changes in cytokine levels other parts of the respiratory tract such as nose, pharynx and trachea are affected. Further thickening of the pleura was found, an effect that is consistent with the hypothesis that carbon nanotubes migrate to the pleura like asbestos fibres.

Tab. 19: Parameter / Effects occurring only with carbon nanotubes

Target / Organ	Parameter / Effect	Substance
BALF	Collagen	MWCNT
BALF	Growth factor protein (TGF-β1)	SWCNT
BALF	Interleukin protein (IL-23)	SWCNT
BALF	Myeloperoxidase (MPO)	SWCNT
BALF	Reactive oxygen species (ROS)	SWCNT
Bronchi	Hypertrophy	MWCNT
Bronchi	Weight	SWCNT
Blood	Granulocytes	MWCNT
Blood	Leukocytes total	MWCNT
Larynx	Changes in organ structure	MWCNT
Lung	Chemokine protein (CCL11)	SWCNT

Target / Organ	Parameter / Effect	Substance
Lung	Chemokine protein (CCL17)	SWCNT
Lung	Chemokine protein (CCL22)	SWCNT
Lung	Cytokeratin	SWCNT
Lung	Expiratory time	SWCNT
Lung	Glutathione (GSH)	SWCNT
Lung	Histiocytosis	MWCNT
Lung	Interferon gamma protein (IFN-γ)	SWCNT
Lung	Interleukin protein (IL-17A)	SWCNT
Lung	Interleukin protein (IL-23)	SWCNT
Lung	Interleukin protein (IL-33)	SWCNT
Lung	Pentosidine	SWCNT
Lymph node	Hypercellularity	MWCNT
Nose	Changes in organ structure	MWCNT
Nose	Eosinophilic structures	MWCNT
Nose	Infiltration	MWCNT
Pharynx	Hypercellularity	MWCNT
Pharynx	Mucus	MWCNT
Pleura	Thickening	MWCNT
Trachea	Hypercellularity	MWCNT
Trachea	Mucus	MWCNT

3.4.3.3 Diameter and specific Surface

There is concern, that particles with nanoscale diameter have a higher toxicity than particles of larger scale, due to their higher surface, inter alia. Therefore, we investigated, whether we can reproduce these results with queries in the PaFtox database.

The LOELs were analysed for up to four different subgroups regarding their diameter and for two different subgroups regarding their specific surface for six categories of study durations from 10 to 700 days in whole body and intratracheal studies. The results are presented in Tab. 20 for the different diameters and in Tab. 21 for different specific surfaces. Generally the LOELs are lower for the particles with the smaller diameter and the higher specific surface. The difference in LOEL for the study with the lowest LOEL for the respective substances and time point is up to two orders of magnitude. The LOELs differ for particles within the same diameter or surface category, confirming substance specific toxicity. Silver nanoparticles are by far the most toxic nanoparticles.

Tab. 20: Influence of particle diameter on LOELs

Application route	Substance	Diameter	Min LOEL per time point					
			10	28	90	182	365	700
Nose / head only	Aluminium oxyhydroxide	< 56 nm	5E+00	4E+00	5E+00	6E-01		
	MWCNT	< 56 nm			2E-02	2E-02	8E-02	
	Silicon dioxide	< 56 nm		7E-01	9E+00	9E+00	3E+01	
Whole body	Carbon Black	< 56 nm		9E+00	2E-01	2E-01	1E+00	2E-01
		> 56 nm		3E+00	9E+00	9E+00		9E+00
	MWCNT	< 56 nm		6E+00				
	Nickel hydroxide	< 56 nm			2E-02	2E-02		
	Silicon dioxide	< 56 nm			2E-01	2E-01	2E-01	2E-01
		> 56 nm			1E+01	1E+01	1E+01	1E+01
	Silver	< 56 nm		9E-05	9E-03			
	Titanium dioxide	< 56 nm	6E+00		9E-02	9E-02	9E-02	9E-02
		> 56 nm		4E-01	2E+00	2E+00	2E+00	2E+00
Intratracheal	Aluminium oxide C	< 56 nm				1E+02		1E+02
	Aluminium trioxide	< 56 nm	2E+00	2E+00	2E+00			
		> 56 nm	2E+00	2E+00	2E+00			
	Carbon Black	< 56 nm	2E-01	1E+01	4E-01	7E+01	2E+02	1E+01
		> 56 nm	6E+00		2E+01	7E+01		
	Cerium(IV) oxide	< 56 nm	2E-01	5E-01				
	Iron(II,III) oxide	< 56 nm	3E-01	1E+00				
	Lamp black 101+diesel soot	> 56 nm						1E+02
	Manganese(IV) oxide	< 56 nm		6E+01	1E+02			
	MWCNT	< 56 nm	4E-02	4E-02	4E-02	2E-01		
	Nickel	< 56 nm	5E-01	5E+00				
	Nickel oxide	< 56 nm	3E-01	3E-01	3E-01	3E-01		
		> 56 nm		7E+00	7E+00	7E+00		
	Silicon dioxide	< 56 nm	8E-03	8E-01	1E+00		5E+01	7E+01
		> 56 nm	1E+00	8E-01	8E-01	5E+00	1E+01	1E+01
	SWCNT	< 56 nm	1E+00	5E+00	1E+00			
	Titanium dioxide	< 56 nm	8E-01	8E-01	8E-01			4E+01
		> 56 nm	5E+00	5E+00	2E+01			1E+01
	Toner	> 56 nm	2E+02	2E+02	2E+02			
Pharyngeal	SWCNT	< 56 nm	3E-01	5E-01	5E-01			

LOELs normalised doses for inhalation studies in mg/m^3 and cumulative doses in mg/kg bw for instillation studies; Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

Tab. 21: Influence of specific surface on LOELs

Application route	Name	Category specific surface	Min LOEL per time point					
			10	28	90	182	365	700
Nose / head only	Aluminum oxyhydroxide	400	5E+00	4E+00	5E+00	6E-01		
	MWCNT	400			2E-02	2E-02	8E-02	
	Silicon dioxide	70		7E-01				
Whole body	Carbon Black	70			1E+00	1E+00	1E+00	1E+00
		400		9E+00	2E-01	2E-01	1E+00	2E-01
	MWCNT	400		6E+00				
	Nickel hydroxide	70			2E-02	2E-02		
	Silicon dioxide	70			1E+01	1E+01	1E+01	1E+01
		400			2E-01	2E-01	2E-01	2E-01
	Titanium dioxide	70	6E+00	4E-01	9E-02	9E-02	9E-02	9E-02
Intratracheal	Aluminium oxide C	400				1E+02		1E+02
	Aluminium trioxide	400	2E+00	2E+00	2E+00			
	Carbon Black	70		1E+01		7E+01		
		400	2E-01	1E+01	4E-01	7E+01	2E+02	1E+01
	Cerium(IV) oxide	70	2E-01	5E-01				
	lamp black 101+diesel soot	70						1E+02
	Manganese(IV) oxide	70						
	MWCNT	70	4E-02	4E-02	4E-02	2E-01		
	Nickel	70	5E-01	5E+00				
	Nickel oxide	70	7E+00	7E+00	7E+00	7E+00		
		400	3E-01	3E-01	3E-01	3E-01		
	Silicon dioxide	70	1E+00	8E-01	8E-01	5E+00	1E+01	1E+01
		400	8E-03	8E-01	1E+00		5E+01	7E+01
	Titanium dioxide	70	8E-01	8E-01	8E-01			1E+01
		400	5E+00	5E+00	5E+00			
Pharyngeal	SWCNT	400	3E-01	5E-01	5E-01			

LOELs normalised doses for inhalation studies in mg/m³ and cumulative doses in mg/kg bw for instillation studies; Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

3.4.4 Genotoxicity of nano-objects in vivo

An important question is, whether carcinogenicity of nano-objects can be predicted by studies on genotoxicity in vitro. In vitro studies are not subject of the PaFtox database. However, in some in vivo studies endpoints indicating genotoxicity have been investigated, such as 8-OHdG or HPRT mutations (Tab. 22). As Tab. 22 shows, only few studies are available. Parameter / Effects were found in 33 to 100 % of the studies for silicon dioxide, Carbon Black, titanium dioxide and SWCNT indicating the potential for

genotoxicity also for „inert“ particles. Genotoxicity has been found also in other recent in vivo studies (Rittinghausen et al. 2012) with a high correlation with cell proliferation. Thus genotoxicity may arise as primary effect but more probably as secondary effect following cell proliferation.

Tab. 22: Genotoxic effects (measured and percentage positive effects)

		Number of studies effect measured						Percentage positive effects (positive/measured)					
	Category time point	10	36	99	189	365	730	10	36	99	189	365	730
Whole body inhalation - Lung													
8-OHdG	Carbon Black			3			3			67			67
HPRT mutations	Carbon Black			3	3	3				67	33	33	
	Silicon dioxide			1									
PAH-derived DNA adducts	Carbon Black			3						33			
Whole body inhalation - BALF													
HPRT mutations	Carbon Black			6	5		5			67	60		60
Intratracheal instillation - Lung													
8-OHdG	Carbon Black		2						100				
	Silicon dioxide	3	2					100	100				
	SWCNT			1						100			
8-oxoGua	Silicon dioxide		5	6					100	50			
HPRT mutations	Carbon Black						2						50
	Silicon dioxide						2						100
	Titanium dioxide						2						50
p53 mutations	Silicon dioxide		5	5					40	40			
Intratracheal instillation - BALF													
HPRT mutations	Carbon Black						2						50
	Silicon dioxide						2						100
	Titanium dioxide						2						0

Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

Tab. 23: "LOELS" for positive genotoxic effects

		Category time point	10	36	99	189	365	730
Target / Organ	**Parameter / Effect**	**Substance**						
Whole body inhalation								
Lung	8-OHdG	Carbon Black			1.4			1.4
	HPRT mutations	Carbon Black			1.3	9.4	9.4	
		Silicon dioxide						
	PAH-derived DNA adducts	Carbon Black			3.1			
BALF	HPRT mutations	Carbon Black			1.4	1.4		1.4
Intratracheal instillation								
Lung	8-OHdG	Carbon Black		9.7				
		Silicon dioxide	0.8	0.8				
		SWCNT			11.3			
	8-oxoGua	Silicon dioxide		0.75	3			
	HPRT mutations	Carbon Black						100
		Silicon dioxide						10
		Titanium dioxide						100
	p53 mutations	Silicon dioxide		0.75	6			
BALF	HPRT mutations	Carbon Black						100
		Silicon dioxide						10
		Titanium dioxide						-

LOELs normalised doses for inhalation studies in mg/m^3 and cumulative doses in mg/kg bw for instillation studies; Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

3.4.5 Carcinogenicity

Fig 5 shows an analysis for the chain of effects: inflammation, hyperplasia, metaplasia and tumour. The effects were counted for the lowest dose and the earliest time point the effect was observed for all studies. This graphic gives an indication what effect can be detected at which time point. While inflammation and hyperplasia were observed within one or three months, metaplasia, cysts, adenoma, carcinoma were determined at later stages, after one or two years. Similar observations were seen for intratracheal studies (data not shown).

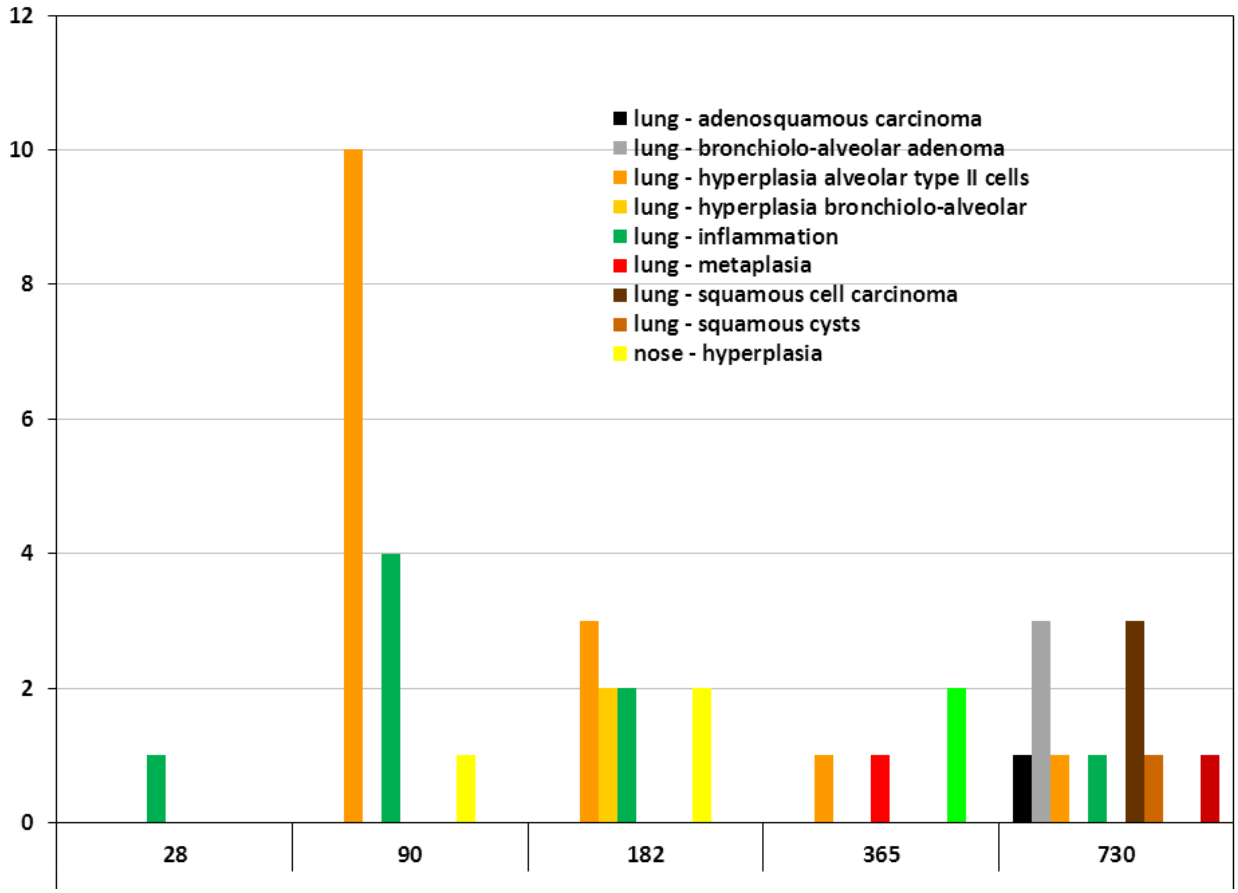

Fig 5: Number of effects per category time point in whole body inhalation studies on carcinogenicity (query counted effects at minimum dose and earliest time point)

Fig 6 shows the LOELs for the same effects (inflammation, hyperplasia, metaplasia and tumour) for two or three different particles sizes of Carbon Black, silicon dioxide and titan dioxide. According to this preliminary analysis, considering the relevance of the effects (inverse the chain of effects) and the corresponding LOELs, the toxicity could be ranked as follows CB 50 ~ TiO2 500 > SiO2 50 > TiO2 50 > CB 100 > CB 500 > SiO2 8000. However, the data are limited to only few data points and do not allow any conclusion regarding the carcinogenicity of nano-objects.

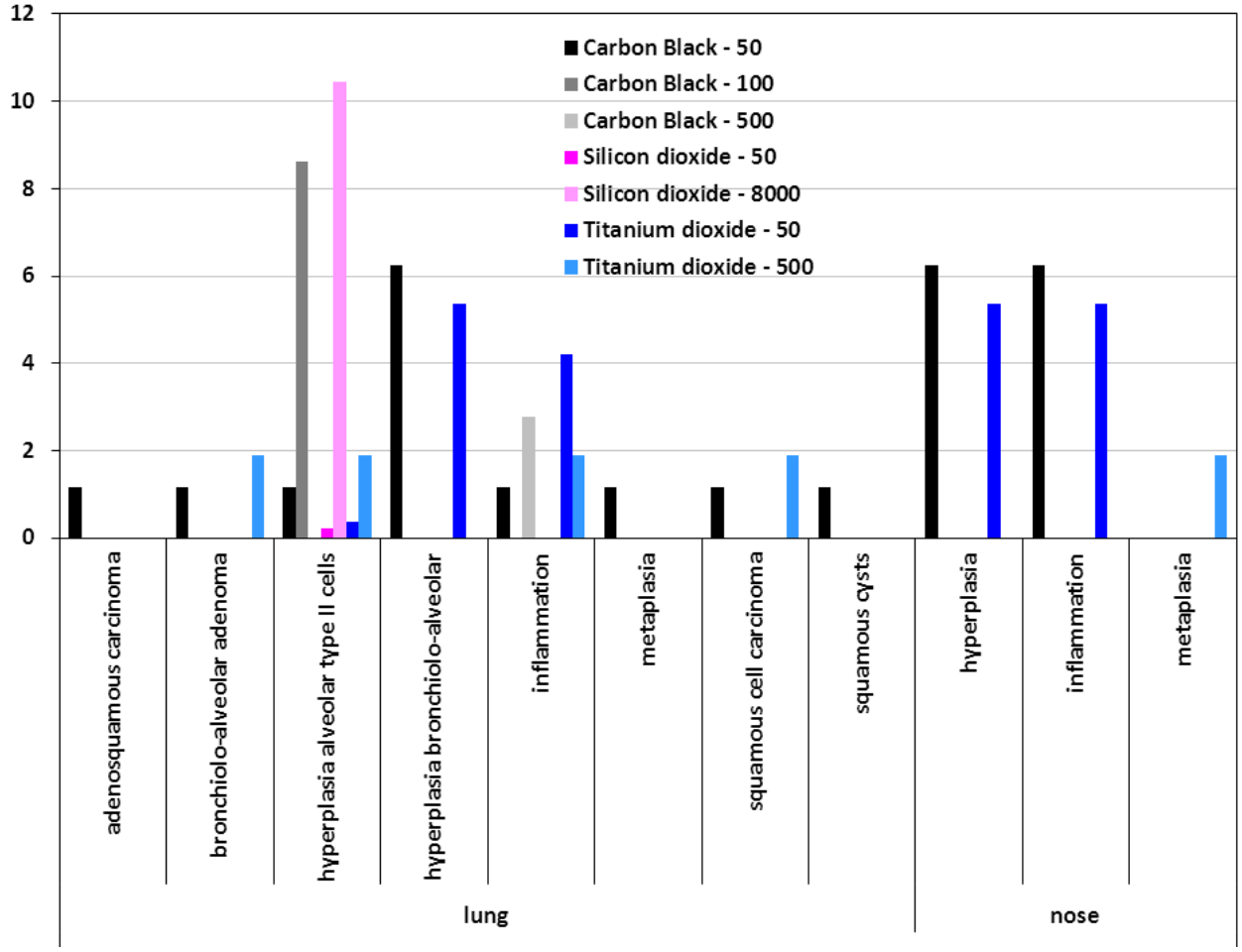

Fig 6: Effect – "LOELs" per particle in whole body inhalation studies on carcinogenicity (query counted effects at min dose and first time point)

3.5 Correlations

3.5.1 Cell counts

Changed counts of different types of lymphocytes are often observed in BALF (Tab. 12). They are related to (acute) inflammation in the respiratory tract. In the PaFtox database they account for about 15% of all effects and are mainly determined in BALF (Tab. 24). Frequently affected cell types are alveolar macrophages and neutrophils. Neutrophils are also called polymorphonuclear neutrophils (PMN). And sometimes the term PMN is synonymously used to polymorphonuclear leukocytes (PML), which covers in addition to neutrophils, also eosinophil and basophil granulocytes. In the PaFtox database, both terms are separately recorded to keep the possibility to identify possible differences (Tab. 24). In the statistical analyses (Tab. 24

and Tab. 25), they are separately counted but have been combined at a later stage for effect level/power analyses.

In Tab. 25 it was investigated, if the effects in BALF occur early or late during treatment. No consistent time pattern emerges for either of the parameters investigated. No consistent pattern was found in sensitivity concerning absolute or relative cell counts. Sometimes relative counts were positive more frequently, sometimes absolute counts.

Tab. 24: Investigation of cells in different targets (statistically significant increased/measured or investigated)

Parameter / Effect	BALF	Blood	Lung	Nose
Alveolar macrophages relative	**58/73**			
Alveolar macrophages total	**75/127**			
Eosinophils total	3/8		0/2	
Erythrocytes		1/10		
Granulocytes	0/4	1/1		
Leukocytes total	23/26	1/1		
Lymphocytes relative	28/48			
Lymphocytes total	32/60	1/3		
Neutrophils relative	**87/119**			
Neutrophils total	**72/86**	**12/19**	**7/23**	**1/3**
PMN relative	**87/123**			
PMN total	**84/130**		**1/1**	

Bold marked effects were analysed in more detail.

Tab. 25: Frequency of increased / measured cell counts in BALF per time point category

	Positive / Measured								
Category time point	**1**	**3**	**10**	**21**	**36**	**99**	**189**	**365**	**730**
Nose / head only									
Alveolar macrophages total						4 / 4	4 / 4	2 / 2	
Lymphocytes relative					1 / 1				
Lymphocytes total						1 / 4	2 / 4	1 / 2	
Neutrophils total				2 / 2	2 / 2		1 / 2		
PMN relative					1 / 1	6 / 6	6 / 6	2 / 3	
PMN total			2 / 5		4 / 10	8 / 12	8 / 12	2 / 3	
Whole body									
Alveolar macrophages relative			/ 1			12 / 16	12 / 14	4 / 6	7 / 7
Alveolar macrophages total						4 / 8	8 / 16	5 / 9	13 / 19
Granulocytes									4 / 4
Leukocytes total								2 / 2	3 / 4
Lymphocytes relative			/ 1			4 / 6	7 / 9	2 / 2	7 / 8
Lymphocytes total						2 / 2	3 / 4	/ 2	1 / 1
Neutrophils relative						10 / 14	14 / 19	8 / 11	7 / 8
Neutrophils total					1 / 1	2 / 2	4 / 4	3 / 4	3 / 5
PMN relative			0 / 1		0 / 2	11 / 12	7 / 7	1 / 3	5 / 7
PMN total	1 / 1		1 / 1		0 / 1	2 / 2		1 / 1	4 / 5
Intratracheal									
Alveolar macrophages relative	6 / 7	4 / 4	4 / 4	1 / 1	2 / 4	1 / 3			5 / 6
Alveolar macrophages total	2 / 4	7 / 10	7 / 8	3 / 6	7 / 11	4 / 5	0 / 2		
Eosinophils total		1 / 2	1 / 1		0 / 3	1 / 1	0 / 1		
Leukocytes total			4 / 4		8 / 8	5 / 5		3 / 3	
Lymphocytes relative	1 / 1	4 / 4	4 / 4	1 / 1	3 / 4				6 / 6
Lymphocytes total	1 / 1	3 / 6	4 / 5	1 / 1	2 / 9	2 / 2	1 / 1		
Neutrophils relative	12 / 14	16 / 17	6 / 14	3 / 3	3 / 6	4 / 6	1 / 1		5 / 6
Neutrophils total	3 / 3	15 / 18	5 / 6	3 / 3	6 / 7	8 / 9	1 / 2		
PMN relative	13 / 15	2 / 2	12 / 17		17 / 22	13 / 16	2 / 2	1 / 1	
PMN total	16 / 18		15 / 19		11 / 13	9 / 15	1 / 1	3 / 3	
Pharyngeal									
Alveolar macrophages total	0 / 3	3 / 3	4 / 4		3 / 3	2 / 3			
Lymphocytes total	2 / 3	3 / 3	3 / 3		3 / 3	1 / 3			
Neutrophils total	3 / 3	3 / 3	3 / 3		1 / 1	2 / 3			
PMN total	3 / 5		1 / 1						

Based on this overview, the effect levels of relative PMNs and relative neutrophils combined were further investigated in dependency of different measures of dose. As was shown by Oberdörster at al. 2005 for TiO2, surface area may be a suitable dose measure for comparing effects of nanoparticles with particles of larger size. Therefore we compared both: dose as mass unit in mg/kg bw for intratracheal studies or mg/m^3 for inhalation studies and surface area (dose as mass multiplied by the specific surface if provided for the respective particles in the studies) for the time point day 1. Fig 7 shows selected trend analyses performed for all corresponding particles available for the particular application route and time point category. Further differences in substance, size and crystal structure are marked by different symbols. Fig 7 A shows relative neutrophils (incl. PMNs) for intratracheal instillations 1 day after instillation in dependency of applied dose in mg/kg bw. The Plot Fig 7 B shows the same values in dependency of particle surface. Fig 7 C and Fig 7 D show the corresponding values for the time point categories 5-10 and 11-36 days after instillations, respectively. Finally, Fig 7 E and Fig 7 F show the results for whole body exposure studies for the time point categories 37-99 and 100-189 days, respectively. Due to the larger spread in the figure, particle surface as dose measure seems to better illustrate the dose-response for different particle sizes and compositions. Therefore the following analyses have been performed solely based on surface. Additionally, a regression line has been introduced into the figures as visual aid for distinguishing effect levels for different particles.

Overall, a rough dose response relationship can be found for the different particles depending on particle surface as dose measure.

Some particles (aluminium trioxide or titanium dioxide) lie below the regression line indicating a lower toxicity than average, while silicon dioxide lies above. Interestingly, this includes also amorphous silicon dioxide particles which are generally considered to have a lower toxicity than crystalline silica.

Generally the pattern is similar with all durations and also in intratracheal studies compared to inhalation studies. The steepness of the dose-response curve is also similar. In contrast to studies with intratracheal instillation few inhalation studies are available for silicon dioxide. Rutile, the crystalline form of titanium dioxide with higher toxicity lies above the regression line.

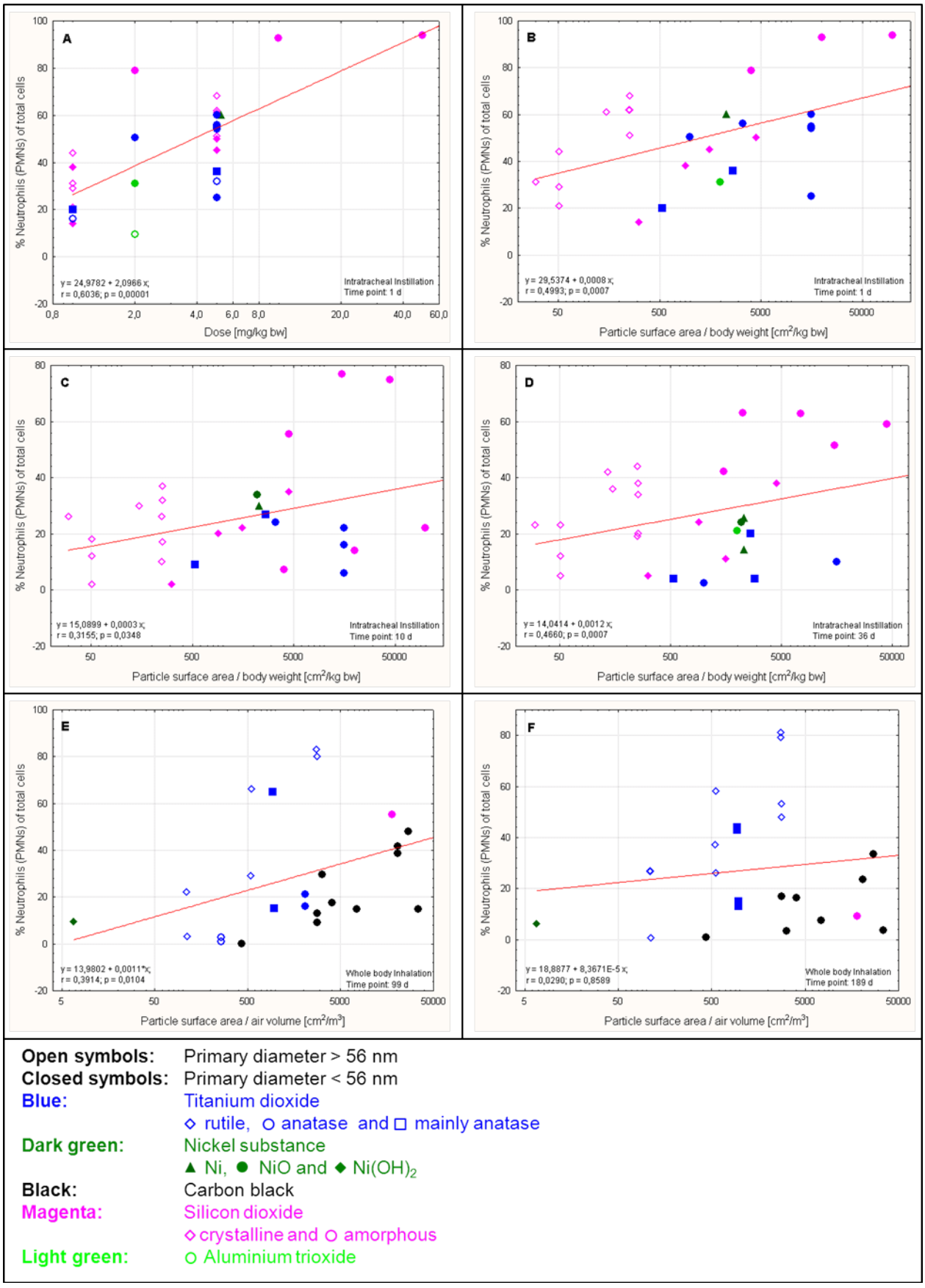

Fig 7: Correlation of relative number of neutrophils with particle mass or particle surface area

3.5.2 Alveolar macrophages

Similar analyses were performed for the relative alveolar macrophage counts in whole body studies and total alveolar macrophage counts for intratracheal studies (data not shown). A general negative trend was found, but some particles showed a positive trend: cerium oxide (4 points) and nickel oxide (2 points) in intratracheal studies at time category 11-36 days after instillation (not at time point 3 days) and titanium oxide (2 points) in whole body studies at time category 37-99 and 100-189 days (negative in intratracheal studies).

3.5.3 Total Protein and lactate dehydrogenase

As earlier mentioned (Tab. 12), the effects total protein and lactate dehydrogenase (LDH) in BALF are more frequently measured than other effects. Therefore, a detailed analysis was performed also for these endpoints. Tab. 26 shows the corresponding time related distribution of the number of measurements for both effects.

Tab. 26: Time related distribution of measurements of total protein and lactate dehydrogenase

Category time point	1	3	10	36	99	189	365	730
Whole body inhalation								
Total protein			1	1	34	29	13	23
Lactate dehydrogenase (LDH)				1	33	31	15	27
Intratracheal instillation								
Total protein	32	23	21	27	28	2	6	
Lactate dehydrogenase (LDH)	50	11	45	26	37	2	6	

Based on this overview (Tab. 26), the percentage changes of total protein measured in BALF were further investigated in dependency of particle surface area as dose measures for several time point categories: For intratracheal instillation the time point categories 1, 11-36 and 37-99 days, and for whole body exposure inhalation studies the time points 37-99 and 100-189 days (Fig 7).

Fig 8 shows selected trend analyses for percentage change of total protein in dependency of particle surface area performed for all corresponding particles available for the particular application route and time point category. Further differences in substance, size and crystal structure are marked by different symbols. Fig 8A shows the results for intratracheal instillations 1 day after instillation. The Plots Fig 8 B, Fig 8 C and Fig 8 D show the corresponding values for the time point categories 3 days, 11-36 and 37-99 days after instillation, respectively. Finally, Fig 8 E and Fig 8 F show the results for whole body exposure studies for the time point categories 37-99 and 100-189 days, respectively.

Overall, a slight dose response relationship can be found for the different particles with particle surface area as dose measure.

Unfortunately, the data amount does not allow a more detailed analysis regarding differences between different particle substances or other properties. However, two results are considered as very interesting: First, nickel led to very high protein contents in BALF at the three time point categories (1 day, 3 and 11-36 days for i.tr. studies), and second nano quartz seems to be less toxic than amorphous nano silicon dioxide. Aluminium oxide is again less toxic.

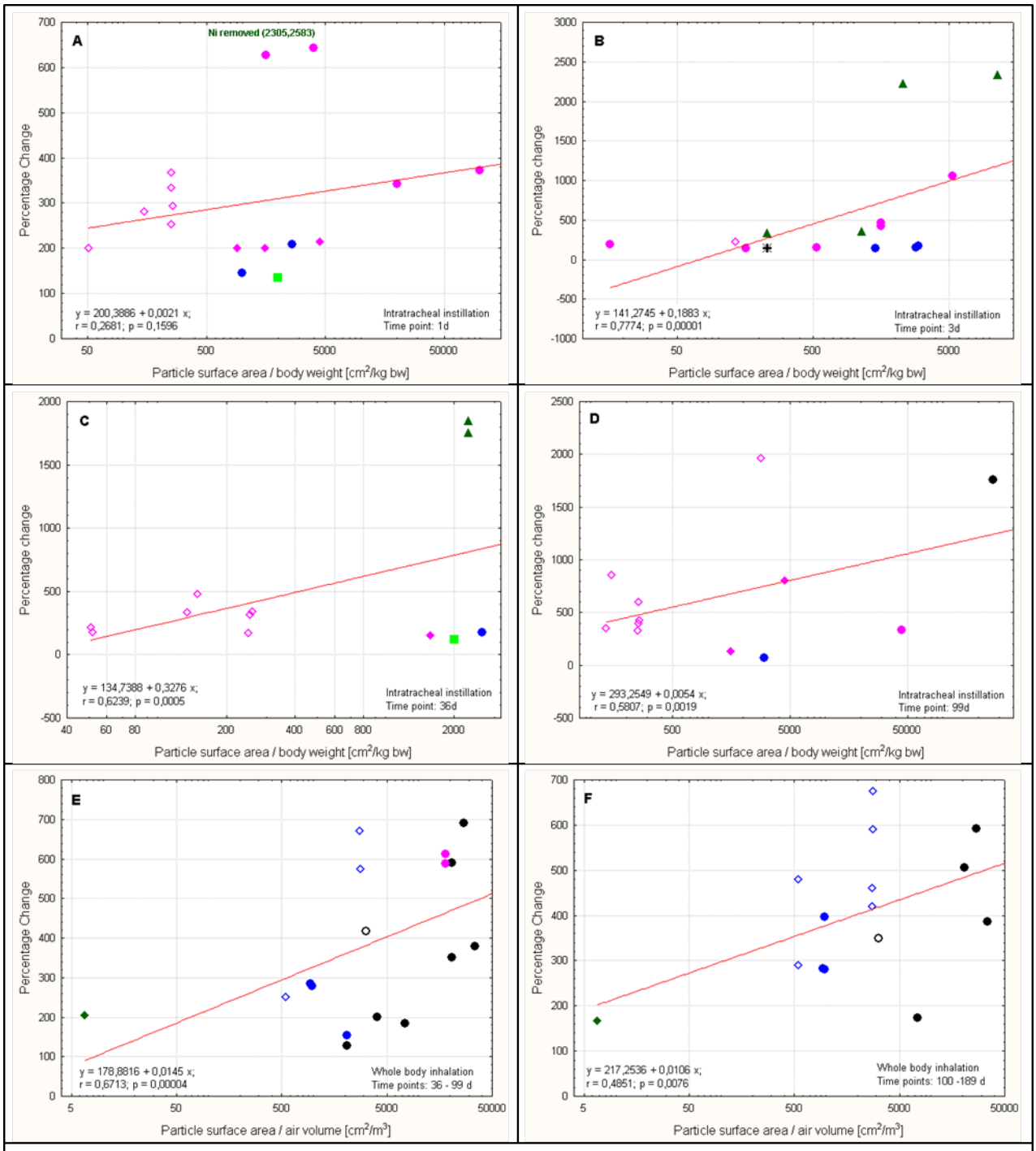

Fig 8: Correlation of total protein with particle surface area

Time or application route related differences were not identified. The trend and the effect level (with few exceptions) are similar with all durations and also in intratracheal studies compared to inhalation studies.

Based on the overview presented above (Tab. 26), the percentage changes of LDH measured in BALF were also further investigated in dependency of particle surface area as dose measures for several time point categories: For intratracheal instillation the time point categories 1, 11-36 and 37-99 days, and for whole body exposure inhalation studies the time points 37-99 and 100-189 days (Fig 9).

Fig 9 shows selected trend analyses for percentage change of LDH in dependency of particle surface area performed for all corresponding particles available for the particular application route and time point category. Further differences in substance, size and crystal structure are marked by different symbols. Fig 9 A shows the results for intratracheal instillations 1 day after instillation. The Fig 9 B and Fig 9 C show the corresponding values for the time point categories 5-10 days and 37-99 days after instillation, respectively. And the plots Fig 9 C, Fig 9 E and Fig 9 F show the results for whole body exposure studies for the time point categories 37-99, 100-189 and 366-730 days, respectively.

The results differ for each panel.

A slight dose response relationship can be found for the different particles in intratracheal studies at time point categories 1 day or 5-10 days after instillation and even more powerful correlations can be found for some substances (cerium oxide, titanium oxide (nano and fine) and Carbon Black) one day after instillation. The data at time point category 37-99 does not allow analogue conclusions. The analyses for whole body exposure studies suffer from the limited number of data. The corresponding analyses cover mainly titanium dioxide and Carbon Black and show only weak correlations (Fig 9 D to F).

Surprisingly, titanium dioxide led to significant higher effect level than cerium oxide one day after intratracheal instillation (Fig 9 A) and at time point category 5-10 days similar levels were achieved.

However, the slight trend is similar at all time point categories and also in intratracheal studies compared to inhalation studies.

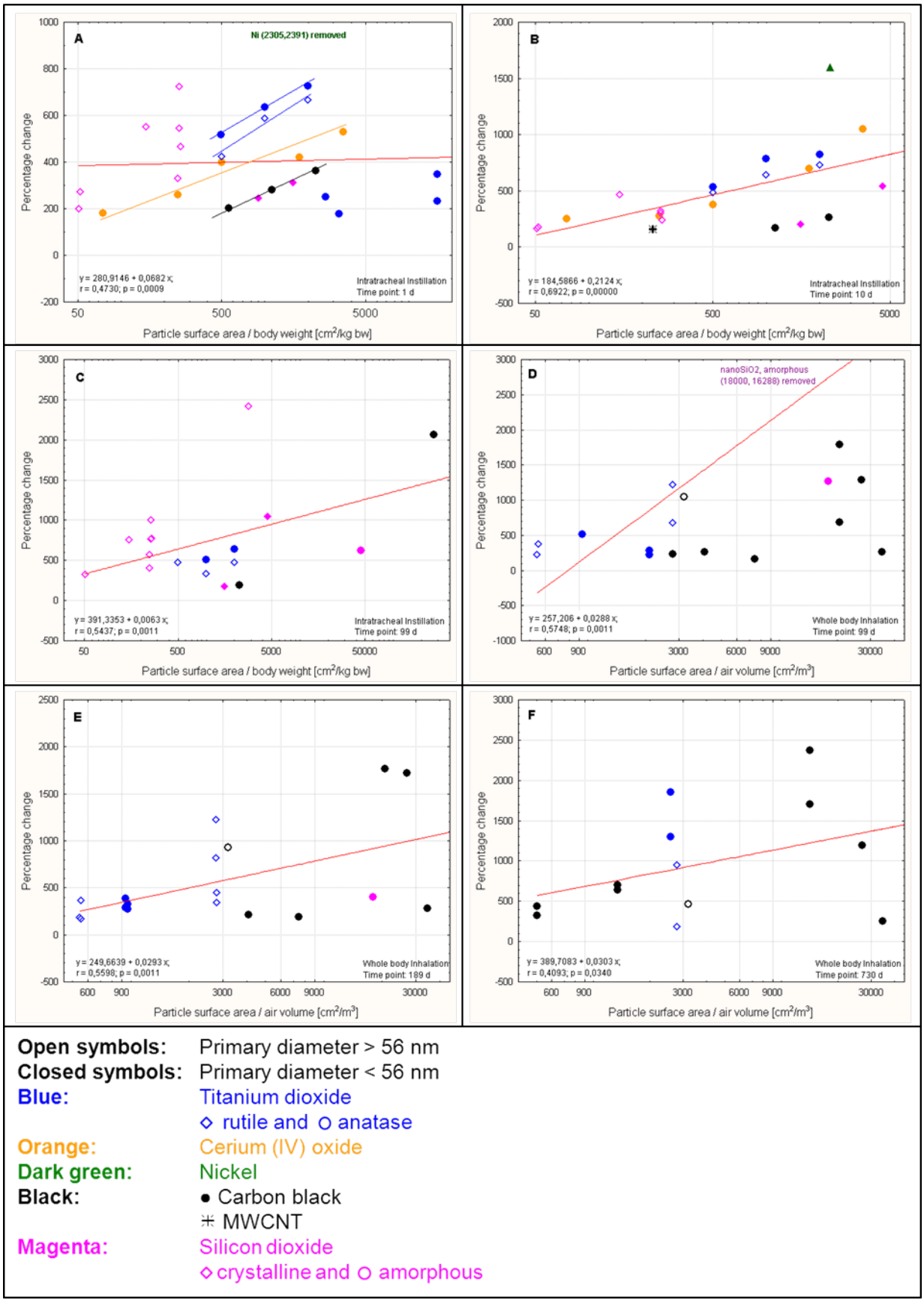

Fig 9: Correlation of LDH with particle surface area

3.5.4 Infiltration

Infiltration of inflammatory cells is the first response of the immune system. The precise description of this effect differs between the several publications. Currently two types of infiltrations are distinguished in the database. Terms like hypercellularity, infiltration of (inflammatory) cells, neutrophils, mononuclear cells (mainly lymphocytes, monocytes and plasma cells) and infiltration of polymorphonuclear leukocytes are summarised under the effect "infiltration". Beside this common term, the term "macrophage infiltration" is separately recorded.

Tab. 27: Number of studies positive infiltrations

Parameter / Effect	Category time point	1	3	10	36	99	189	365	730
Nose/head only inhalation									
Infiltration	Aluminum oxyhydroxide				2		2		
	MWCNT					1	1	1	
	Silicon dioxide				2				
Macrophage infiltration	Aluminum oxyhydroxide				2		2		
	MWCNT					1			
Whole body inhalation									
Infiltration	Carbon Black					2	2	1	4
	Nickel hydroxide						1		
	Silicon dioxide					5	4	6	1
	Silver					1			
	Titanium dioxide					3	2	1	1
Macrophage infiltration	Carbon Black					3	4	1	6
	Silicon dioxide					2	1	1	
Intratracheal instillation									
Infiltration	Carbon Black				1	1			1
	Iron(II,III) oxide	1		1	2				
	MWCNT		1	1	1				
	Nickel	1	1	1	1				
	Nickel oxide		1	1	1	2	1		
	Silicon dioxide	2	3	3	10	9	1		2
	SWCNT					1			
	Titanium dioxide	5	3	1					
Macrophage infiltration	Cerium(IV) oxide				1				
	Silicon dioxide	1	1	1	1	1			
	Titanium dioxide	5	6	5	3	1			

Traffic light code used for illustration lower limit: 1 set to red and ideal minimal upper limit: 20 set to green

Tab. 28: "LOELs" for infiltration and macrophage infiltration

Parameter / Effect	Category time point	1	3	10	36	99	189	365	730
Nose/head only inhalation									
Infiltration	Aluminum oxyhydroxide				5.1E+00		5.1E+00		
	MWCNT					8.0E-02	1.1E+00	2.9E-01	
	Silicon dioxide				6.6E-01				
Macrophage infiltration	Aluminum oxyhydroxide				5.1E+00		5.1E+00		
	MWCNT					1.8E-02			
Whole body inhalation									
Infiltration	Carbon Black					1.3E+00	2.5E+00	1.2E+00	1.2E+00
	Nickel hydroxide						1.9E-02		
	Silicon dioxide					2.3E-01	2.3E-01	2.3E-01	1.0E+01
	Silver					8.6E-03			
	Titanium dioxide					4.0E+00	4.3E+01	4.3E+01	4.3E+01
Macrophage infiltration	Carbon Black					1.2E+00	1.2E+00	1.2E+00	1.2E+00
	Silicon dioxide					9.0E+00	9.0E+00	9.0E+00	
Intratracheal instillation									
Infiltration	Carbon Black				3.0E+01	9.0E+01			2.5E+01
	Iron(II,III) oxide	1.0E+00		1.0E+00	1.0E+00				
	MWCNT		4.0E-02	4.0E-02	4.0E-02				
	Nickel	5.3E+00	5.3E+00	5.3E+00	5.3E+00				
	Nickel oxide		3.3E-01	3.3E-01	3.3E-01	3.3E-01	3.3E-01		
	Silicon dioxide	8.2E-01	8.2E-03	8.2E-01	8.2E-01	1.0E+00	5.0E+00		1.5E+01
	SWCNT					1.1E+01			
	Titanium dioxide	5.0E+00	5.0E+00	5.0E+00					
Macrophage infiltration	Cerium(IV) oxide				3.5E+00				
	Silicon dioxide	5.0E+00	5.0E+00	5.0E+00	5.0E+00	5.0E+00			
	Titanium dioxide	5.0E+00	5.0E+00	3.3E+00	5.0E+00	5.0E+00			

LOELs normalised doses for inhalation studies in mg/m³ and cumulative doses in mg/kg bw for instillation studies. Traffic light code used for illustration lower limit: 10 percentile set to red and upper limit: 90 percentile set to green

3.5.5 Lung weight

Based on our experience with RepDose, we know that lung weight is a sensible term for lung damages. In contrast to chemicals it might be possible that the lung weight gain resulted from lung burden by the nano-objects. Therefore, the percentage of burden of the lung weight was determined (see Tab. 29). In all studies lung burden contributed to a small degree to the lung weight increase, the maximum was 11 % in a

carcinogenicity study with titanium dioxide. Therefore, the lung weight gain compared to control is caused by other factors, e.g. influx of inflammatory cells, or fibrosis.

Tab. 29: Minimum and Maximum percentages of burden of the lung weight

Category time point	1-10		11-36		37-99		100-189		218-365		366-730	
	min	max	min	max	min	max	min	max	min	max	min	max
Nose/head only inhalation												
Aluminum oxyhydroxide			0.08	5.96	0.10	5.57	0.003	5.03				
MWCNT					0.03	0.08	0.005	0.08	0.02	0.05		
Silicon dioxide			0.17	0.37								
Whole body inhalation												
Carbon Black			0.15	0.21	0.14	0.61	0.25	0.82	0.29	0.74	0.18	1.02
Silicon dioxide					1E-04	3E-03	0	5E-04	4E-04	1E-03	4E-04	7E-04
Titanium dioxide					0.27	6.46	0.78	6.40	0.58	6.35	0.65	10.88
Intratracheal instillation												
Carbon Black							0.56	0.56	0.36	0.36		
MWCNT	0.11	0.19										
Silicon dioxide									0.01	0.01		

Green: 10 percentile; red: 90 percentile

Tab. 30: Minimum and maximum of percentage change of lung weight

Category time point	1-10		11-36		37-99		100-189		218-365		366-730	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Nose/head only inhalation												
Aluminum oxyhydroxide			115	117	106	106	108	115				
MWCNT					121	176	113	169	127	161		
Silicon dioxide			114	150								
Whole body inhalation												
Carbon Black			131	133	129	192	114	235	137	500	194	517
Silicon dioxide					120	230	110	405	100	430	355	420
Titanium dioxide					147	151	120	239	135	450	132	427
Intratracheal instillation												
Carbon Black							149	149	123	123		
MWCNT	122	122										
Silicon dioxide									165	165		

Traffic light code used for illustration lower limit: 10 percentile set to green and upper limit: 90 percentile set to red

Tab. 30 shows that for all particles and MWCNT lung weight is increased and the increase can be detected already at early time points, indicating that lung weight is a sensitive parameter. Lung weight was increased in 47 studies. The lung weight LOEL was at study LOEL in 85 % of these studies.

Tab. 31: "LOELs" for lung weight

Category time point	1-10		11-36		37-99		100-189		218-365		366-730	
	Norm Dose	Orig Dose	Norm Dose	Orig Dose	Norm Dose	Orig Dose	Norm Dose	Orig Dose	Norm Dose	Orig Dose	Norm Dose	Orig Dose
Nose/head only inhalation												
Aluminum oxyhydroxide			5	28	5	29	1	3				
MWCNT					0.3	1.6	0.1	0.5	0.3	2		
Silicon dioxide			9	51								
Whole body inhalation												
Carbon Black			9	50	2	7	1	2	1	2	1	2
Silicon dioxide					6	31	6	31	6	35	10	59
Titanium dioxide					5	10	5	10	5	10	5	10
Intratracheal instillation												
Carbon Black							75	1	250	5		
MWCNT	1	1										
Silicon dioxide									50	0.5		

LOELs normalised doses for inhalation studies in mg/m³ and cumulative doses in mg/kg bw for instillation studies; * Original dose in mg; Traffic light code used for illustration lower limit: 10 percentile set to green and upper limit: 90 percentile set to red.

4 Discussion

4.1 Results

4.1.1 Carcinogenicity

The original focus of this project was to derive structure activity relationships for nano-objects with respect to carcinogenicity. However, as discussed with UBA, this requires a huge data set including numerous carcinogenicity studies, which are currently not available. Carcinogenicity studies are, however, limited to 4 in inhalation studies (Titanium dioxide – 21 & 250 nm and Carbon Black – 14 & 37 nm) and 20 in intratracheal studies (Aluminium oxide C - 13 nm, Carbon Black - 14 & 95 nm, Silicon dioxide - 14 & 1100 nm, Titanium dioxide - 21, 25 & 200 nm. As Tab. 29 reveals for Titanium dioxide, the tumours were induced under severe overload conditions (lung weight contains 11% burden). Therefore, it is questionable, whether they are suitable for risk assessment at all. In addition, there is no negative study available that would allow identifying relevant precursors for the development of cancer. Thus, the statement that the potential carcinogenic risk of nanomaterials can currently be assessed only on a case-by-case basis (Becker et al., 2011) is still true.

4.1.2 Availability of studies for different particles and fibres

The nano-objects to be analysed were selected together with the sponsor and included Carbon Black and related compounds, titanium dioxide, aluminium oxide, silicon dioxide, heavy metals and their oxides (silver, nickel, manganese, iron, and cerium), single and multiwall carbon nano tubes. With respect to numbers of studies in the database, there is a strong imbalance for these nano-objects: numerous studies are available for titanium dioxide, Carbon Black and silicon dioxide for different particle sizes, application

routes and study durations, while for other particles especially the heavy metals only few studies are available.

According to our literature search more than 100 additional studies are available including several other compounds or modifications thereof that could be entered into the database.

4.1.3 Particle and fibre characterisation

Besides the chemical composition, the special properties (high specific surface area or specific volume) of the nano-objects are considered a crucial/elementary cause for hazard differences compared to larger particles. Therefore the particle characterisation is as important as the determination of the chemical composition. However particle characterisation itself in the nanoscale dimension is a relatively new area of research and technical development. Difficulties arise as the values from different measurement techniques are difficult or hardly to compare. The importance is recognized by international standardisation organisation which implemented technical committees (TC) to derive standard protocols for characterisation of nanomaterials (nano-objects and nanostructured materials) as well as the surface chemistry (TC 229 Nanotechnologies and TC 201 Surface chemical analysis, respectively).

Nanoparticles in nanomaterials are usually present as clusters of aggregates and/or agglomerates. While primary nanoparticles within aggregates are bound by strong chemical bonds (i.e. covalent or ionic bonds), binding between agglomerates is caused by van der Waals forces, which are much weaker. However, for inhalation studies nano-objects may be dispersed in air (aerosols) and for instillations in liquids. The aggregation status differs between liquid suspensions and aerosols, and is affected by many factors, such as the dispersion technique, dispersion aids, age of dispersion and experimental conditions. Further details see BAuA research report F2133 (Schaudien et al., 2011).

The size of the nano-objects or aggregates is considered to be relevant for their deposition behaviour in the lung. The deposition efficiency in the parts pharynx, bronchi and alveoli differs and depends on the particle size inhaled. Oberdörster et al illustrates these differences (see Fig 10), based on to the predictive mathematical model (International Commission on Radiological Protection 1994). Despite the deposition, also the disposition is considered to be fundamentally different from larger particles (Oberdörster et al., 2005).

Summarising the costs for the several characterisation tests, it is clear why none of the currently available studies fulfil the ISO guideline recommendations. On the other hand, the degree of characterisation is an important part for the quality of a publication / study. An addition it might be possible that some characterisation data are available to later time point, when identity parameters are consolidated.

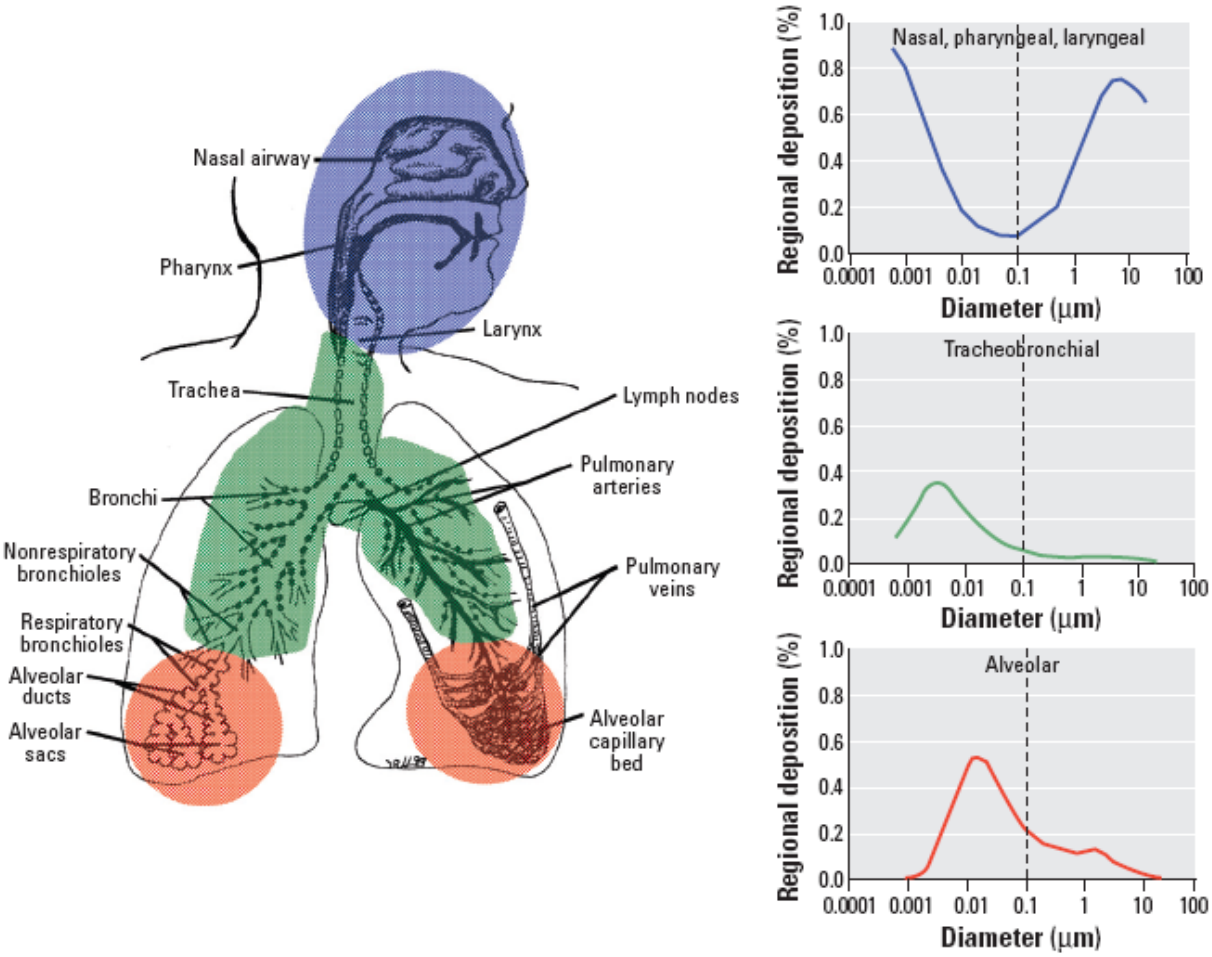

Fig 10: Predicted fractional deposition of inhaled particles (Oberdörster et al., 2005)

4.1.4 Application routes

Different application routes are used in experimental studies to assess potential inhalation hazard. In principal, four exposure techniques can be distinguished, the inhalation techniques nose / head only and whole body and the instillation types intratracheal and pharyngeal. They differ with respect to exposure condition, to suitability for regulatory purposes, and to costs (Tab. 32). Due to several reasons, these application techniques could be compared only to a limited extent.

1. While in inhalation studies, the (nano-)objects are actively inhaled, the exposure in instillation studies occurs passively. Due to these different exposure techniques, the deposition rate is completely different. In inhalation studies it depends on several physical parameters (Heyder, 2004; International Commission on Radiological Protection, 1994) and in instillation studies a full deposition can be assumed.
2. The continuity of exposure differs. In inhalation studies the exposure occurs continuously, at least for certain duration. In last years, exposure durations of 6 h/d and 5 d/wk are commonly used. In contrast, instillation studies are always event related exposure studies, also if the instillations are repeated daily. This difference results in different kinetics in the body, e.g. efficacy of protection mechanism, clearance, transport or distribution kinetic (Oberdörster et al., 2005; Sturm and Hofmann, 2003, 2006).

3. Whole body inhalation studies are technically much easier than nose only and traditionally often used. However, rats do protect themselves by hiding their nose in the fur. In that case the derived LOEL would be too high. The second issue is that the particles are deposited on the fur and taken up orally during their grooming (Oberdörster et al., 2002). In that case the systemic distribution of the particles is biased and possible effects could not be assigned correctly.
4. Instillation studies have some limitations, like the non-physiological rapid delivery of the particles or fibres, the possible delivery of non-respirable aggregates, and the bypassing of the nose (Driscoll et al., 2000). Additionally, especially studies with low instillation number, often apply high doses to achieve similar doses as in inhalation studies. This leads to acute overload situations. However, when particle deposition was comparable, pulmonary responses to bolus instillation tended to reflect the pulmonary response to inhalation (Henderson et al., 1995). Nevertheless, intratracheal studies are very useful for mechanistic considerations or identification of hazard potential.
5. Pharyngeal studies are considered to better simulate the deposition behaviour than intratracheal studies and avoid the trauma associated with intratracheal instillation (Rao et al., 2003). Impaction of nanoparticle in the pharynx and subsequently diffusion into the lung is considered as possible mechanism for the deposition of airborne nano-objects. Pharyngeal application has the same limitations as other instillation techniques (Shvedova et al., 2005).

Tab. 32: Comparison of application routes

Application route	Nose / head only	Whole body	Intratracheal	Pharyngeal
Type	Inhalation	Inhalation	Instillation	Instillation
Costs	+++	++	+	+
Feasibility (number of institutes)	+	++	+++	+++
Exposure type	Active inhalation of aerosols	Active inhalation of aerosols	Passive infusion of suspensions	Passive infusion of suspensions
Exposure regime	Continuous exposure several h/d	Continuous exposure several h/d	Event related exposure	Event related exposure
Feature	State of art method	Rodents protect themselves (nose in the fur) Particle on the fur could be taken up orally	Reflects artificial situations Insertion of needle or cannula through laryngeal opening	Is considered to reflect the impaction of nano-object in the pharynx
Animal stress by exposure technique	-	-	+++	++

No complete list

As described above intratracheal studies can generally be seen only as tool for hazard identification. As demonstrated in Tab. 16, Tab. 20 and Tab. 21 the toxicity ranking for different particles is similar in intratracheal studies compared to inhalation studies, e.g. effect levels for silicon dioxide are lower than for Carbon Black or for titanium dioxide. Therefore intratracheal studies may be useful at a screening level for identifying the general rank of toxicity for different particles or fibres, while inhalation studies then serve for risk assessment.

4.1.5 Dose measure

An adequate dose is necessary to derive reliable dose response curves. Instead of particle mass as dose measure, the particle number or particle surface were proposed and discussed (Donaldson and Poland, 2013; Oberdörster et al., 2005, 2007; Wittmaack, 2007). As particle number is currently seldom available, a corresponding analysis could not be performed. Therefore, after an initial comparison of dose measures mass and surface (Fig 7), surface was used as dose measure in our analyses on neutrophils/PMNs (Fig 7), total protein (Fig 8) and LDH (Fig 9). Particle surface is calculated by multiplying the specific surface of the particles with the corresponding mass dose and thus combines the normal dose measure mass with the property specific surface of the primary particle. The figures clearly demonstrate different effect levels for the different particles, but no consistent dose response was detected for one particle type in most of the studies. An exception is LDH at day 1 (Fig 9). There regression lines have been inserted for different particle types. However, the relevance of this finding only for one parameter and one time point is questionable. In interpreting this lack of dose response, one has to be aware that several studies are combined in these figures, where particles may differ from study to study as well as treatment of the particles before exposure. Furthermore, there are studies with rats or mice, which may give different results.

The specific surface used for the calculation of surface in Fig 7, Fig 8 and Fig 9 is based on the data for characterising the primary particles. However, nano-objects tend to aggregate or agglomerate in the exposure atmosphere or instillation medium and are therefore seldom applied as real nano-object (Fig 3). Furthermore, several studies have shown that aggregates/agglomerates stay stable in the lung. On the other side, our analyses have shown that the toxicity of nanoparticles tend to be higher than the toxicity of larger particles. Therefore it may be possible that the surface of the individual particles is still available for inducing reactions in the airways. Therefore it might be helpful to identify a relevant property of the secondary particles (aggregates/agglomerates) and to include this into the dose measure (e.g. MMAD, specific surface area or volume, biologically available surface area, (Han et al., 2012)).

An additional drawback is that not for all particles specific surfaces are available. For this reason e.g. silver, the most toxic particle in our LOEL analyses (Tab. 16, Tab. 20, Tab. 21) is missing in the three figures. On the other side, this presentation of the data allows an easy comparison of effect sizes.

Overall our analyses of dose response have to be considered as a first tier that was possible within the scope of this pilot project. More detailed analyses are desirable involving all time points, routes, measures of dose and also other endpoints. Also according to our analyses particle surface seems to be a better dose measure than particle mass.

4.1.6 Target organs and important effects

As expected, the respiratory tract with lung, nose, trachea, larynx, pharynx, bronchi, parameters in BALF and lymph nodes are important targets in studies with nano-objects (Fig 4, Tab. 12).

Many different effects are found in histopathological examinations of the respiratory tract, including infiltration (Tab. 12, Tab. 27, Tab. 28), fibrosis, inflammation, hyperplasia and different types of tumours (Tab. 12). In addition lung weight turned out to be a sensitive parameter for particle toxicity (Tab. 29 and Tab. 30). Increased lung weight may be caused by several processes in the lung such as invasion of inflammatory cells, haemorrhage, proteinosis, cell proliferation, fibrosis, collagen, oedema, granuloma, hyperplasia or tumours.

An important effect in BALF is increased numbers of neutrophils/PMNs and macrophages. This parameter has been further investigated in Fig 7. As described in section 3.5.1. Other frequent effects in BALF are total protein and LDH (Tab. 12), which also have been further explored (Fig 8, Fig 9).

Many studies investigated only effects in the respiratory tract. Therefore effects at other locations than the respiratory tract are not detected in a query for frequent effects. To detect also studies, where other target organs are studied we made a specific query (Tab. 13). Indeed, indications for migration of the nano-objects were found: In several studies besides the lymph nodes also effects in the haematological system were found. Some studies reveal effects in the pleura, known to be primarily affected by fibres. Effects in the liver probably come from oral uptake of the nano-objects, either as a result of clearance or from grooming the fur in whole body inhalation studies (see 4.1.4).

4.1.7 New parameters

In addition to the parameters mentioned above, different cytokines have been investigated. As Tab. 17 and Tab. 19 show for the example of heavy metals and nanotubes, there is a large variety and few have been studied in more than 1 study. Cytokines are often determined in in vitro studies, thus they may build a link to in vitro studies. However, actually no conclusions can be drawn on the relevance of these investigations.

It may be interesting to find out, which of the many new endpoints beyond the scope of OECD guideline studies in studies with particles or fibres are sensitive and also relevant for investigating the mode of action of particles and fibres.

4.1.8 LOELs

Effect LOELs are suitable tools for comparing endpoints/effects in toxicological studies. They have already been successfully used in our analyses with RepDose (Batke et al., 2011; Bitsch et al., 2006; Escher et al., 2010). Therefore we performed corresponding analyses with the PaFtox database.

At first we have compared the toxicity of different nanomaterials. The most toxic compound was silver (Tab. 16, Tab. 20, Tab. 21). High toxicity was also found for MWCNTs confirming the concern for the toxicity of nanotubes.

Another important question is, if – in general – nanoparticles are more toxic than larger particles with the same composition. Our analyses with the PaFtox database (Tab. 20, Tab. 21) have indeed shown that based on LOELs the toxicity of nanoparticles is generally higher than for the corresponding fine particles. Lower LOELs have been found for different time points for nanosized Carbon Black, silicon dioxide, titanium dioxide and Nickel oxide in inhalation and in intratracheal studies than for particles with diameters above 250 nm. The ratio of LOELs was calculated if pairs of LOELs for fine and nanoparticles of the same substance were available (whole body inhalation and intratracheal instillation). The median ratio of LOELs of nanoparticles (< 56 nm) and larger particles in Tab. 20 is about 18 (n=31). A similar pattern is observed, when LOELs are compared for different surface categories (Tab. 21). The median ratio here is 5 for LOELs of nanoparticles (>70 m^2/g) and larger particles (n=26).

Although the general trend is obvious, these results have to be treated with care, because some of the LOELs are not true LOELs, i.e. dose levels, where only marginal effects occur. In the case of particle or fibre studies, often only one dose level has been used, that had been selected to cause a significant effect. This is different from OECD guidelines, where three dose levels are, and the lowest dose is intended to provide the NOEL, the next dose the “real” LOEL. Thus it is not clear, whether these LOELs are real LOELs, as often the corresponding NOEL is missing (in 47 of 55 with more than 1 dose level), which would allow to identify the real LOEL. NOELs are available for subacute or subchronic inhalation studies with Silicon dioxide, Aluminium oxyhydroxide, Titan dioxide and Carbon Black.

In a similar approach Gebel (2012) has analysed tumour rates in carcinogenicity studies with GBS including talc, toner, coal dust, titanium dioxide, Carbon Black and diesel exhaust particulates. Gebel found, when comparing carcinogenic potency of GBP of fine particles and nanoparticles a median ratio of 2.26 if the dose measure was based on particle mass and a ratio of 0.91 when the dose measure was based on surface area of the particles. While we made a pairwise comparison i.e. compared fine particles with nanoparticles for each substance, e.g. TiO_2, Gebel has pooled potency indices for all different particles.

For a better comparison, for studies where the data basis consists of 3 dose levels and more, dose response could be modelled, as performed for TiO_2 by Dankovic et al. (2007). Suitable parameters would be parameters that are measured in many studies and are rather sensitive, such as neutrophils/PMNs, total protein in BALF, or lung weight. For these parameters one may compare the dose levels of nanoparticles and larger particles for a given effect size and derive a more reliable ratio.

4.1.9 Exposure duration versus time point

Currently, the PaFtox database contains long term studies i.e. the exposure duration in inhalation studies or observation duration in instillation studies is longer than 20 days. Shorter time points or categories thereof

analysed in this database are interim sacrifices. However, there are many other studies available with shorter study duration and valuable information on effects as already entered but also information on kinetics and fate of nano-objects in the body. Therefore these studies could supplement the current database, e.g. the study of Kreyling (2010).

4.1.10 Overload

If one looks at the definition of lung overload with a particle mass of 1-2 mg/g lung = 0.1 – 0.2% of (Oberdörster et al., 1990), inhalation studies with titanium dioxide and aluminium oxyhydroxide have been performed under severe overload conditions (Tab. 29). Also with Carbon Black studies overload was achieved, while studies with MWCNT just reach the border for overload. Studies with silicon dioxide were mostly below the threshold. In general it is questionable, whether studies with overload conditions are reasonable for characterizing the toxicity of a nano-object. It is assumed, that under overload situation unspecific responses occur that have not much to do with realistic exposure situations. However the latter have to be evaluated for the purpose of risk assessment.

4.1.11 Reversibility of effects

It was analysed if effects appear or disappear during the exposure or post exposure duration (3.4.2). Interestingly, no effect disappears during the postexposure duration indicating that no effect was in general reversible. However, it can be assumed, that at least for some effects the grading/scoring may be lower after postexposure. This has still to be analysed. Furthermore some effects appear firstly in the postexposure duration. The later appearing effects are mainly associated with chronic inflammation or follow up reaction (worst case tumour) to long term irritations by the nano-objects. This general effect pattern is different from studies with chemicals.

4.1.12 Genotoxicity

Genotoxicity is discussed as one possible mode of action for particles (Schins, 2002). However, *in vitro* genotoxicity may not be relevant in vivo due to defence mechanisms (Donaldson et al., 2010). In addition recent analyses have shown, that carcinogenicity is not predicted very well (Roller, 2011). In the PaFtox database genotoxicity *in vivo* was only included as endpoint, when it was analysed in corresponding long term studies (Tab. 22). For the different nano-objects positive as well as negative results were obtained for endpoints like 8-OHdG, 8-oxoGua, HPRT mutations and p53 mutations. For analysis of genotoxicity in vivo in more detail, it would be necessary to include specific genotoxicity studies in vivo into the database.

4.2 Concepts for Grouping of nanomaterials

4.2.1 Groups proposed in the literature

Based on the presumption that there are common modes of action for several types of nanomaterials, groups for different types of nano-objects have been proposed. These groups will be presented in the following including the compounds assigned to these groups and the rationale for grouping. Based on our analyses of the data in the PaFtox database these groupings will be evaluated.

Nano GBP

GBP means **G**ranular **B**iopersistent **P**articles with no or little intrinsic chemical toxicity (inert particles (DFG, 2013)). This grouping of nanoparticles is consistent with the grouping of granular micromaterials (Greim and Ziegler-Skylakakis, 2007). Roller and Pott (2006) originally introduced the term GBP with a slightly different definition, i.e. granular biodurable particles without known significant specific toxicity. According to Gebel (2012) there are also other abbreviations in the literature, i.e. PSP for poorly soluble particles and PSLT for poorly soluble, low toxicity particles that are specifying the same group of particles.

(Nano)particles assigned to this group are e.g.:

TiO_2, $BaSO_4$ (Dankovic et al., 2007), talc, toner, coal dust, Carbon Black, diesel exhaust emissions (Dankovic et al., 2007; Gebel, 2012; Roller, 2009), zirconium oxide (Packroff, 2011b).

GBP (nano)particles included in the PaFtox Database are Carbon Black, aluminium oxide, aluminium trioxide, aluminium oxyhydroxide, titanium dioxide and toner (Tab. 11).

These particles are called biodurable or persistent due to the fact that these particles stay in the lung in inhalation studies and after intratracheal administration and are not readily removed or dissolved. The PaFtox database contains data on lung burden of the GBP TiO_2, Carbon Black and aluminium oxyhydroxide in inhalation studies. Particle load in the lung is high, demonstrating the biopersistence of these particles.

Typical effects caused by GBP are inflammation, oxidative stress and secondary genotoxicity at the lung as the target organ after inhalation (DFG, 2013; Greim and Ziegler-Skylakakis, 2007). Accordingly analyses of the PaFtox database indicate the following important effects: in BALF neutrophils/PMNs, total cells, macrophages as well as LDH and total protein are increased, the lung weight is increased. Findings in histopathological examinations include infiltration of macrophages, hyperplasia of alveolar type II cells, fibrosis and finally tumours.

Generally no NOELs are provided in these proposals for grouping, that would separate particles with little intrinsic toxicity from particles with higher toxicity. Based on our data the limit for the LOEL in inhalation studies could be about 0.1 mg/m^3 (Tab. 20). Usually LOELs decrease with increasing exposure duration. This was not evident from our analyses (Tab. 20), which include also short study durations. Therefore, it may be possible to use this preliminary threshold irrespective of study duration. When considering this value one has to be aware that the exposure concentration was normalized with respect to exposure duration to 24 hours/day and 7 days/week. This would include aluminium oxyhydroxide, Carbon Black and amorphous silicon dioxide, while titanium dioxide would be just below the limit. For intratracheal studies, the data are too inconsistent to derive such a value.

(Nano)particles with specific toxicity

In contrast to the “inert” particles described above, there are particles with specific toxicity, which have a higher toxicity than GBS.

Crystalline silica usually is considered to belong to this group. An interesting finding in the analyses of specific effects of silica (Tab. 18) is that nano amorphous or micro crystalline silica cause necrosis in the nose, bronchi, and lung reflecting the potential of silica to cause severe effects as a consequence of inflammation.

Another specific subgroup with high toxicity according to DFG (2013) are metal-based particles (presumably poorly soluble metal compounds). We propose, however, to use the more specific term “heavy metal-based particles”, as the light metals titanium and aluminium are also metals.

Examples for heavy metals belonging to this group are copper (DFG, 2013), cadmium, nickel, cobalt (Packroff, 2011b).

In the PaFtox database the following heavy metal compounds are included; iron(II,III) oxide, manganese(IV) oxide, nickel, nickel hydroxide, nickel oxide and silver.

According to DFG (2013), the "toxicity depends largely on the actual compound, i e. type of metal, metal species and surface characteristics; relevant endpoints are again oxidative stress and inflammation as well as metal-specific cellular interactions. "

Analyses of the PaFtox database correspondingly show that heavy metal based particles cause effects similar to GBS, but in addition, some metal specific effects were found (Tab. 17), e.g.

- increased weight of the adrenal gland and functional disorders of the brain and nervous system, increased liver weight for manganese oxide,
- liver hyperplasia, necrosis, vacuolization and weight increase for silver.

In this group of nanoparticles there may be differences in toxicity depending on solubility as stated by DFG (2013): "Interestingly, some nanoparticles, as shown in the case of copper, appear to be more toxic and stimulate more intense inflammatory responses than do their water soluble or microscale particle counterparts, based on the same metal content. This appears not to be due to increased extracellular solubility, but may be explained by higher intracellular bioavailability after endocytosis and lysosomal dissolution of the particles, although this still has to be confirmed experimentally". Similarly, soluble nickel sulphate nanoparticles were less toxic than less soluble nickel hydroxide nanoparticles in an inhalation study (Kang et al., 2011).

Analyses of the influence of solubility on the toxicity of nanoparticles were not possible with the PaFtox database, because data on solubility were not available. However, instead of water solubility, data on solubility in artificial body fluids, i.e. alveolar fluid, interstitial fluid and stomach fluid are considered as more relevant and should be measured for particles that have been investigated in toxicological studies in order to derive correlations.

Soluble (Nano)particles without significant toxicity

Another group that has been proposed are "soluble nanoparticles without significant toxicity" (Packroff, 2011b). Amorphous silicon dioxide belongs to that group.

This is consistent with our analyses showing low toxicity of silicon dioxide nanoparticles in subchronic to chronic inhalation studies (Tab. 20, Tab. 21) and a recent evaluation indicating low genotoxic/carcinogenic potential (Rittinghausen et al., 2013).

In contrast, in intratracheal studies low effect concentrations were found especially for acute effects, e.g. neutrophil count is a sensitive parameter (Fig 7) and serious effects are found also for amorphous silica, e.g. inflammation, necrosis, spongiosis, histiocytosis or changes in organ structure (Tab. 18). The acute toxicity is consistent with recent findings of Pavan et al. (2013), where fully amorphous silica showed more haemolytic activity than crystalline silica. The authors found that particle size and silanols or siloxane bridges at the surface are the main actors for the haemolytic activity, while crystallinity or free radical production are no strict predictors. In other in vitro tests (cytotoxic activity, oxidative stress, and inflammatory response) all amorphous silicas where less toxic than crystalline, but among the amorphous silicas pyrogenic silicas, irrespective of their size and agglomeration/ aggregation pattern, appeared more reactive towards cells than the precipitated ones (Gazzano et al., 2012).

Thus, it may be justified only for long-term studies to speak about low toxicity of amorphous silicon dioxide. The low toxicity presumably is mainly triggered by lack of accumulation and adaptation in the body in inhalation studies or dissolution in intratracheal studies.

We are not aware about clear criteria to be categorized as “soluble” nanoparticle. However, measuring stability of nanoparticles in artificial body fluids (as indicated above) could provide information on the dissolution behaviour of nanoparticles and borders should be defined to distinguish between high and low solubility.

Nanotubes (Nanofibres)

Fibre-like nanomaterials are also called HARN, which refers to High Aspect Ratio Nano-objects with the following specifications according to Tran et al. (2008):

- Length to diameter aspect ratio greater than 10 to 1
- Diameter thin enough to pass ciliated airways
- Length long enough to initiate the onset of e.g. frustrated phagocytosis and other inflammatory pathways,
- The nanomaterial must be biopersistent

From experience with asbestos and synthetic mineral fibres there is concern that the shape of the materials is an important parameter determining toxicity, especially carcinogenicity. Health effects of asbestos comprise several types of pleural and parenchymal lung disease associated with inhalation of asbestos fibres. Nanomaterials with fibrous shape therefore have been separated as a group. In fact several studies have confirmed the concern for carcinogenicity for fibrous nanomaterials, e.g. Takagi et al. (2008).

In the PaFtox database intratracheal and inhalation studies with SWCNT and MWCNT are included. Their toxicity ranges from low to high, indicating that in addition to shape as parameter determining toxicity additional factors are necessary.

When analysing the effects in studies with nanotubes in the PaFtox database, effects typical for asbestos-like fibres could be found such as thickening of the pleura indicating migration of fibres to the pleura and potential for induction of mesotheliomas, a tumour in the pleura typical for fibre exposure.

It is important to note that the chemical composition and size may be not sufficient to characterise a particle sufficiently. For silicon dioxide, crystalline and amorphous silicon dioxide are distinguished. Crystalline silicon dioxide is more toxic and more stable, while amorphous silicon dioxide has high acute toxicity and low long term toxicity and is soluble to some degree (Rittinghausen et al., 2013). Similarly for titanium dioxide two crystalline modifications, rutile and anatase are known that differ with respect to their crystal structure and with respect to toxicity. In addition to crystal structure, surface modifications are discussed to change the toxicity (Rossi et al., 2010) and are also subject of an ongoing BAuA research project at Fraunhofer ITEM (F2246).

4.2.2 Grouping of nanomaterials for optimized testing strategies

Nanomaterials fall under the legislation of REACH, where testing requirements are still discussed. It will be impossible to perform a full testing program for all nanomaterials with all modifications and in all use scenarios, notwithstanding that modification or use/release scenarios may alter their biological effects.

Alternatively, it should be evaluated, if it is possible to assign certain biological effects to specific material properties (physical properties and chemical and crystal structure) and group nanomaterials based on these material properties.

According to Nel et al. (2013), a high throughput-platform is required in analogy to the US ToxCast Project to investigate the bio-physico-chemical interactions at the nano/bio interface in order to make predictions about the physico-chemical properties of nanomaterials that may lead to pathology or disease outcome in vivo. In vivo results are used to validate and improve the in vitro high throughput screening and to establish structure-activity relationships that allow hazard ranking and modelling by an appropriate combination of in vitro and in vivo testing.

In the following parameters are briefly specified which may be part of a grouping strategy.

Toxicity in vivo

Performing studies in vivo would be the most reliable and toxicologically sound approach for assessing the risk of nanomaterials. The question is, whether there may be surrogates for chronic inhalation studies which render testing of nanomaterials more effective:

For in vivo studies study duration, application route, scope of examination may be analysed.

Concerning study duration a short term inhalation test has been proposed by Ma-Hock et al. (2009) based on comparison of a 5 day inhalation study with a 90 day inhalation study. Rats shall be exposed for 5 days, 6h/day. Examinations shall be performed at day 3 and 21 postexposure. Sensitive parameters that should be measured in BALF are cell counts, total protein levels, enzyme activities and mediators of inflammatory cell infiltration (MCP-1, MCP-3, MDC, IL-8, MIP-2) and cell proliferation (M-CSF, osteopontin) as well as immune modulation (IL-1, IL-6, TNF-α).

Our preliminary analyses support the view that short-term tests with appropriate postexposure periods may be sufficient for identification of risks of nano-objects. LOELs for short term exposure of nano-objects (i.e. 10 days) are in a similar order of magnitude as LOELs for longer durations up to chronic (Tab. 20, Tab. 21). Sensitive parameters are number of neutrophils and total protein in BALF as well as lung weight.

Concerning exposure route, intratracheal studies are frequently performed instead of inhalation studies, which are much more expensive. For risk assessment however, inhalation studies are clearly preferable. For hazard ranking or forming of groups, intratracheal studies seem to be sufficient. As discussed under “GBS” dose levels may be provided that allow distinguishing GBS from particles with specific toxicity.

Toxicity in vitro

In in vitro studies genotoxicity and generation of reactive oxygen species are frequently investigated with respect to their general predictive power for in vivo situations.

In vitro DNA damage tests (especially the Comet assay) and in vitro chromosome mutation assays are the most frequently used genotoxicity tests on nano-objects. According to Roller (2011) nearly all types of nanomaterials and control dusts used in the in vitro assays showed genotoxic effects in cell cultures (e.g. CoCr particles, diesel soot, crystalline and amorphous SiO_2,TiO_2, Carbon Black) but not consistently in all studies. Roller concluded that there is no clear correlation of the probability of a positive in vitro test with particle properties. We came to a similar conclusion in an evaluation of the literature in a research project from the German BAUA (Ziemann et al., 2011). Unfortunately, in most studies nano-objects used were not well characterized with respect to chemical composition and physicochemical properties. Therefore more

research is necessary with well characterized nanomaterial to identify suitable genotoxicity tests and dose measures for grouping of nano-objects.

According to Rushton et al. (2010) in vitro tests on ROS generation correlate well with inflammation in vivo (increase of neutrophils after intratracheal instillation), provided that particles are compared based on particle surface and the steepest slope of the dose response curve as dose metric.

Toxicokinetics

Fate and kinetics may also give a measure for grouping nanomaterials, for example, if the distribution and mode of action are the same, but the bioavailability is different (due to differences in size). Identical distribution patterns in tissues could be a criterion for grouping different nanomaterials.

An example would be grouping of nanomaterials in a separate group that can be found in other locations than the respiratory tract, raising concern for nano-objects specific systemic effects.

Biopersistence is another important criterion for particle and fibre toxicity, because it is assumed that it has impact on the mode of action and reversibility of effects. If a nanomaterial rapidly dissolves, the substance could be treated as a conventional chemical. We are not aware about criteria for defining nanomaterials as persistent or not.

Physicochemical properties

Several physicochemical properties of nanoparticles are currently proposed to be good predictors of toxicity of nanoparticles. In the following some examples are given from recent studies:

Crystal structure is an important determinant for reactivity of the surface of a nanomaterial. For silicon dioxide, crystalline and amorphous silicon dioxide are distinguished. Crystalline silicon dioxide is more toxic and more stable, while amorphous silicon dioxide has high acute toxicity and low long term toxicity and is soluble to some degree (Rittinghausen et al., 2013). Similarly for titanium dioxide two crystalline modifications, rutile and anatase are known that differ with respect to their crystal structure and with respect to toxicity. Jiang et al. (2008) showed that the in vitro-reactivity of different types of TiO_2 nanoparticles depends on the defect site density in the crystal structure. The number of defects per unit surface area was translated into the activity in producing ROS in vitro. Amorphous TiO_2 has much higher surface defect density compared to anatase and rutile TiO_2 since its structure is not periodic. These analyses correlate with generation of ROS in in vitro systems as well as pulmonary inflammatory response of nanoparticles determined by number of neutrophils in lung lavage 24 hous after intratracheal instillation of different nanomaterials (Rushton et al., 2010).

In addition to crystal structure, surface modifications are discussed to change the toxicity (Rossi et al., 2010) and are also subject of an ongoing BAuA research project at Fraunhofer ITEM (F2246).

The zeta potential provides information concerning the particles surface charge. It is the electrical potential created between the surface of a particle with its associated ions, and it’s medium. According toCho et al. (2012) the acute pulmonary inflammogenicity of 15 nanoparticles showed a significant correlation for low solubility nanoparticles. This correlation found for zeta potential was not applicable for soluble nanoparticles.

Conduction band energy levels have recently been proposed as promising approach for future screening of nanomaterials (Zhang et al., 2012). For 24 different metal oxide nanoparticles of the same size it was shown that the toxicity of nanoparticles was high when the conduction band energy levels of the metal oxides was

close to the redox potential in cells which ranges from -4.12 to -4.84eV. Metals with conduction band energy outside this range were less toxic. Soluble nanoparticles had a higher toxicity than predicted.

Both analyses support the general view that soluble and insoluble nanomaterials have to be distinguished.

Dose measure for grouping of nanoparticles

In the conventional chemical toxicology, researchers generally use the mass as the metric to describe dose. In the case of nanoparticles, the particle number or the surface area have been discussed. Several recent studies have confirmed the use of particle surface as generally usable dose measure for insoluble particles (Han et al., 2012; Jiang et al., 2008; Rushton et al., 2010).

Concerning effect size, the reference values ED10 or ED50 (dose with 10 or 50 % effect) have been used, LOELs and NOELs (Roller, 2011), or the steeped slope of the dose response curve (Rushton et al., 2010).

Combination of parameters

As already obvious from groupings of nanoparticles currently proposed (see 4.2.1) several parameters are necessary for specifying a group, i.e. shape, biopersistence and toxicity.

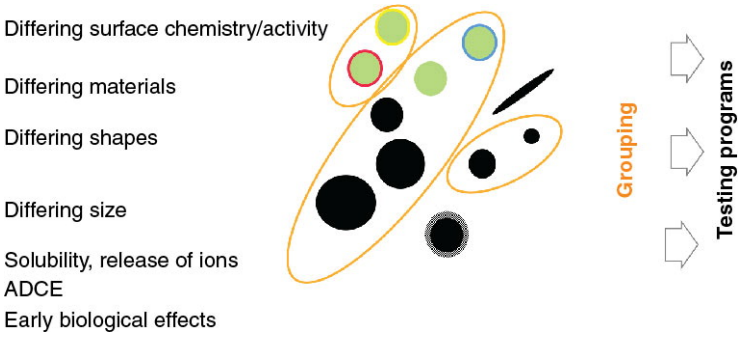

Fig 11: Illustration of grouping of nanoparticles based on material properties and/or biological effects (from Oomen et al. (2013)).

Fig 11 shows nanomaterials grouped according to surface chemistry/activity, material, shape, size, solubility, release of ions, ADCE (absorption, distribution, corona formation, and elimination), early biological effects and also nanomaterials not assignable to a group. A given nanomaterial could also belong to more than one group.

A large number of property combinations should be considered in order to assess the overall hazard of a single material type.

The PaFtox database would be a useful tool in the future for collecting data on the parameters described above, selecting suitable properties and validate the groupings.

5 Conclusion and outlook

The amount and quality of cancer studies as well as studies on genotoxicity of nanomaterials are insufficient to assess the carcinogenicity of nanomaterials. On the other side cell proliferation in the lung as a consequence of inflammation may be an important precursor of cancer. Therefore repeated dose inhalation studies or studies with intratracheal instillation were analysed with the purpose to identify effects that may be

precursors of carcinogenicity and to analyse dose response for these effects as basis for regulation of nanomaterials. For getting a better overview and as basis for statistical analyses and analyses of dose response the data were collected in the relational database PaFtox.

Despite the heterogeneous data available, some general conclusions can be drawn.

Inflammation was detected with all nanomaterials investigated, however, at different dose levels. Sensitive parameters of nano-object toxicity are lung weight, numbers of neutrophils/PMNs in BALF, as well as total protein and LDH in BALF. Further histopathological examinations of the overall respiratory tract reveal numerous effects that are related to inflammation and subsequent cell proliferation.

Nano-objects have consistently a higher toxicity than non-nano-objects. On the other side there are considerable differences in the toxicity of different materials. From the particles selected for this project, silver had the highest toxicity.

Although we did not detect a reliable dose response for effects as a function of particle surface, this dose measure gave a good graphical representation of the toxicity of different particles. While inhalation studies are preferable for risk assessment, intratracheal studies are suitable for ranking toxicity of different nano-objects.

We have used our data also to reflect on proposals for grouping of nanomaterials made by different institutions. Although groups are proposed, the criteria for assigning nanomaterials to these groups are not well defined, this refers to toxicity as well as solubility. Concerning toxicity a preliminary LOEL of 0.1 mg/m^3 (exposure 24 hours/day, 7 days/week) is proposed to distinguish so called “inert particles” (carbon black, titanium dioxide, silicon dioxide) from particles with specific toxicity, i.e. particles with specific toxicity have lower LOELs. Our data further support to have nanotubes in a separate group. Currently interesting new parameters are identified that influence the toxicity of (nano)particles that may help in the future for better separation of groups.

Currently, only a selection of particles and fibres was analysed by means of the database. In the last years a huge amount of short term in vivo and in vitro studies with various nanomaterials was performed. At least some studies are publicly available and the nanomaterial investigated fulfil more criteria of nano-object characterisation. It would therefore be desirable to analyse all these data along the lines of this project to identify more criteria for grouping approaches and /or to identify patterns of nanomaterial behaviour. Also recent studies with a more guideline-like design as undertaken under the OECD sponsorship program should be included. By specific searches more information for the physico-chemical characterization of the particles and fibres should be integrated, such as BET surface or solubility. Based on a broader database, additional and more specific queries should be performed addressing the question of dose response, particle specific effects, prediction of long term effects (including cancer) from short term studies, influence of surface changes on particle toxicity etc. Based on such data, criteria for read across and QSAR-like approaches for nanoparticles could be developed as well as final criteria for grouping of nanomaterials.

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Zhang H, Ji Z, Xia T, Meng H, Low-Kam C, Liu R, Pokhrel S, Lin S, Wang X, Liao YP, Wang M, Li L, Rallo R, Damoiseaux R, Telesca D, Madler L, Cohen Y, Zink JI and Nel AE, (2012): Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. Acs Nano, 6, 4349-4368.

Ziemann C, Rittinghausen S, Ernst H, Kolling A, Mangelsdorf I and Creutzenberg O, (2011): Genotoxic mode of action of fine and ultrafine dusts in lungs. Federal Institute for Occupational Safety and Health, Dortmund, Berlin, Dresden, pp.

7 Appendix

7.1 Publications in PaFtox database (October 2012)

Author	Year	Publication	Pages
Bai R et al.	2010	Toxicology Letters	288–300
Belinsky SA, et al.	1995	Report HEI-RR-68 Part III	1-25
Bermudez E, et al.	2002	Toxicological Sciences	86-97
Bermudez E, et al.	2004	Toxicological Sciences	347-357
Borm PJA, et al.	2005	Toxicol Appl Pharmacol	157-167
Borm PJA, et al.	2005	Toxicology Applied Pharmacology	157-167
Carter JM, et al.	2006	J Occup Environ Med	1265-1278
Chen H-W, et al.	2006	FASEB Journal	2393-2395
Chen H-W, et al.	2006	FASEB Journal	E1732-E1741
Chen Y, et al.	2004	Toxicology Industrial Health	21-27
Chen Z, et al.	2008	Environ Sci Technol	8985-8992, Support Info
Cho W-S, et al.	2007	Toxicology Letters	24-33
Choi M, et al.	2008	Toxicology Letters	97-101
Creutzenberg O, et al.	1990	J Aerosol Science	S455-S458
Dasenbrock C, et al.	1996	Toxicology Letters	15-21
Driscoll KE, et al.	1996	Toxicology Applied Pharmacology	372 - 380
Driscoll KE, et al.	1997	Carcinogenesis	423-430
Elder A, et al.	2005	Toxicological Sciences	614-629
Endoh S, Uchida K	2008	Int Symp risk assessment manufactured nanoparticles	21-30
Ernst H, et al.	2002	Exp Toxic Pathol	109-126
Ferin J, et al.	1991	J Aerosol Medicine	57-68
Ferin J, et al.	1992	Am J Resp Cell Mol Biol	535-542
Gallagher J, et al.	2003	Toxicology Applied Pharmacology	224-231
Gillespie PA, et al.	2010	Nanotoxicology	106-119
Heinrich U, and Fuhst, R	1992	"Abschlussbericht: Untersuchungen zur tumorinduzierenden Wirkung von inhalierten Dieselmotorabgasen und anderen Teststäuben in der Mäuselunge. in German"	
Heinrich U, and Fuhst, R	1992	"Abschlussbericht: Vergleichende Untersuchungen zur Frage der tumorinduzierenden Wirkung von Diese1motorabgasen in der Rattenlunge. in German"	
Heinrich U, et al.	1995	Inhalation Toxicology	533-556
Hext PM, et al.	2002	Ann Occup Hyg	191-196
Hyun J-S, et al.	2008	Toxicology Letters	24-28
Inoue K, et al.	2005	Respiratory Research	1-12
Inoue K, et al.	2010	Free Rad Biol Med	924-934

Author	Year	Publication	Pages
Ji JH, et al.	2007	Inhalation Toxicology	857-871
Johnston CJ, et al.	2000	Toxicological Sciences	405-413
Kaewamatawong T, et al.	2006	Toxicologic Pathology	958-965
Kim JS, et al.	2011	Safe Health Work	34-38
Kobayashi N, et al.	2009	Toxicology	110-118
Kobayashi N, et al.	2010	Toxicology	143-153
Koike E, et al.	2008	Int J Immunopathol Pharmacol	35-42
Kolling A, et al.	2011	Inhalation Toxicology	544-554
Lee KP, & Kelly, DP	1992	Fund Appl Toxicol	399-410
Lee KP, & Kelly, DP	1993	Toxicology	205-222
Lee KP, et al.	1985	Exp Mol Pathol	331-343
Lee KP, et al.	1985	Toxicol Appl Pharmacol	179-192
Lee KP, et al.	1986	Environ Research	144-167
Li J et al.	2007	Environ Toxicol	415-421
Ma JY, et al.	2011	Nanotoxicology	312-325
Ma-Hock L, et al.	2009	Toxicological Sciences	468-481
Mauderly JL, et al.	1995	Report HEI-RR-68 Part I	1-106
Morimoto Y, et al.	2010	Nanotoxicology	161-176
Muhle H, et al.	1994	In: Mohr U, et al. (Ed.) Toxic and carcinogenic effects of solid particles in the respiratory tract	29-41
Nikula KJ, et al.	1995	Fund Appl Toxicol	80-94
Nishi K, et al.	2009	Inhalation Toxicology	1030-1039
Niwa Y, et al.	2008	Circulation Journal	144-149
Oberdörster G, et al.	1990	J Aerosol Science	384-387
Oberdörster G, et al.	1994	Ann Occup Hyg	295-302
Oberdörster G, et al.	1994	Environ Health Perspect	173-179
Ogami A, et al.	2009	Inhalation Toxicology	812-818
Oszlánczi G, et al.	2010	Ecotox Environ Safety	2004-2009
Park EJ, et al.	2010	Toxicology	65-71
Pauluhn J	2009	Toxicological Sciences	152-167
Pauluhn J	2009	Toxicology	140-148
Pauluhn J	2010	Regulatory Toxicology Pharmacology	78-89
Pauluhn J	2010	Toxicological Sciences	226-242
Pott F, and Roller M	2005	Eur J Oncol	249-281
Pott F, and Roller M	2005	Eur J Oncol	3606
Randerath K, et al.	1995	Report HEI-RR-68 Part II, NTIS PB96-138623	
Rehn B, et al.	2003	Toxicology Applied Pharmacology	84-95

Author	Year	Publication	Pages
Reuzel PGJ, et al.	1991	Food Chem Toxicol	341-354
Roller M	2008	Forschungsprojekt F 2083	
Roller M, and Pott F	2006	Ann NY Acad Sci	266-280
Rossi EM, et al.	2010	Particle Fibre Toxicology	1-14
Sager TM	2008	Dissertation	1-290
Sager TM and Castranova V	2009	Particle Fibre Toxicology	1-12
Sager TM, et al.	2008	Particle Fibre Toxicology	1-15
Santhanam P, et al.	2008	Int J Nanotechnol	30-54
Seiler F, et al.	2001	Arch Toxicol	716-719
Shvedova AA, et al.	2005	Am J Physiol Lung Cell Mol Physiol	L698–L708
Shvedova AA, et al.	2008	Am J Physiol Lung Cell Mol Physiol	L552-L565
Sung J, et al.	2008	Inhalation Toxicology	567-574
Sung J, et al.	2009	Toxicological Sciences	452-461
Warheit DB, et al.	1991	Fund Appl Toxicol	590-601
Warheit DB, et al.	1995	Scand J Environ Health	19-21
Warheit DB, et al.	2004	Toxicological Sciences	117-125
Sung J, et al.	2008	Inhalation Toxicology	567-574
Warheit DB, et al.	2007	Toxicological Sciences	270-280
Warheit DB, et al.	2007	Toxicology	90-104
Zhang QW, et al.	1998	J Occup Health	171-176
Zhang QW, et al.	1998	J Toxicol Environ Health A	423-438

7.2 Data analysis by databases

In this project, a database has been used for the analysis of repeated dose toxicity studies with nano-objects. It may be questioned, why the PaFtox Database has been used although several databases on nanomaterials are available already. Therefore in the following the already existing databases are described briefly and then the special features of the PaFtox Database are discussed.

7.2.1 NanoSafety Cluster

The EU projects addressing all aspects of nanosafety including toxicology, ecotoxicology, exposure assessment, mechanisms of interaction, risk assessment and standardisation can be found on the corresponding website - NanoSafety Cluster[1]. This initiative helps to maximise the synergies between the existing FP6 and FP7 projects. Participation in the NanoSafety cluster is voluntary for projects that commenced prior to April 2009, and is compulsory for nano-EHS projects that have started since April 2009. The goal is to provide industrial stakeholders and the general public with appropriate knowledge on the risks of nanomaterials for human health and the environment.

7.2.2 DaNa

DaNa[2] is an umbrella project aiming at collecting and evaluating scientific results in an interdisciplinary approach with scientists from different research areas, such as human and environmental toxicology, biology, physics, chemistry, and sociology. Research findings from the field of human and environmental nanotoxicology which fulfil the criteria[3] are summarised and presented together with material properties and possible applications for interested laymen and stakeholders. The DaNa project team wishes to provide for more transparency and to process results of research on nanomaterials and their influence on humans and the environment in an understandable way.

7.2.3 Database on Research into the Safety of Manufactured Nanomaterials

The OECD Working Party on Manufactured Nanomaterials (WPMN) developed the "Database on Research into the Safety of Manufactured Nanomaterials[4]", which is an inventory of safety research information on manufactured (engineered) nanomaterials. It is designed as a global resource (Database), which details research projects, helps to identify research needs, provides opportunities to identify the similar fields, and may lead to create new collaboration and networks.

The following information is stored in distinct fields:

- Project Title; Start date; End date;
- Project Status (Current; planned; or completed);

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[1] http://www.nanosafetycluster.eu/

[2] http://nanopartikel.info/cms/lang/en/Projekte/dana;jsessionid=E81E1110A9100B389E0F010A2294E942

[3] http://nanopartikel.info/cms/lang/en/Wissensbasis/kriterienkatalog

[4] http://www.oecd.org/env/ehs/nanosafety/oecddatabaseonresearchintothesafetyofmanufacturednanomaterials.htm

- Country or organisation;
- Funding information (where available, on approximate total funding; approximate annual funding; and funding source);
- Project Summary; Project URL; Related web links;
- Investigator information: name, research affiliation, contact details;
- Categorisation by material name, relevance to the safety, research themes, test methods;
- Overall outcomes and outputs.

NHECD

NHECD[5] is a free access, robust and sustainable web based information system including a knowledge repository on the impact of nanoparticles on health, safety and the environment. It includes a robust content management system (CMS) as its backbone, to hold unstructured data (e.g., scientific papers and other relevant publications). It also includes a mechanism for automatically updating its knowledge repository, thus enabling the creation of a large and developing collection of published data on environmental and health effects following exposure to nanoparticles.

NHECD is based on text mining methods and algorithms that make possible the transition from metadata (such as author names, journals, keywords) to more sophisticated metadata and to additional information extracted from the scientific papers themselves. These methods and algorithms were implemented to specifically extract pertinent information from large amount of documents. NHECD created a systematic domain model of concepts and terms (i.e., a wide set of domain taxonomies) to support the categorization of published papers and the information extraction process within this project. Up to now around 10,000 open source articles have been gathered. NHECD is intended for - academics, industry, public institutions and the general public.

Besides the databases on research projects or databases / information platforms containing different level of toxicological information, databases on products containing nanomaterial are available. The Danish Consumer Council and the Danish Ecological Council have created a nano database[6] that will help consumers to identify more than 1,200 products that may contain nanomaterials. A German database[7] was developed by BUND containing more than 1000 products available in Germany.

[5] http://nhecd.jrc.ec.europa.eu/

[6] http://nano.taenk.dk/ (Posted on December 25, 2012)

[7] http://www.bund.net/themen_und_projekte/nanotechnologie/nanoprodukt datenbank/

7.2.4 Special features of the PaFtox database

As can be seen from the description of the databases above, they are rather general, either related to collections of research projects or publications. The PaFTox Database in contrast contains all details for repeated dose toxicity studies (up to livelong exposure) in a very structured way.

There are several advantages in using such a database for toxicological analyses: the structure of the database forces the user to collect data systematically. Deficiencies in study description and design become obvious and can be accounted for in the analyses. Different queries allow systematic data mining of large datasets. As a result effect patterns, LOELs, dose response can be compared for different types of (nano-)objects, for different studies and sensitive parameters can be identified. Further, the results of the database queries can be further analysed by statistics and these results can be easily visualized (e.g. Fig 7, Fig 8, Fig 9), which improves understanding compared with lengthy descriptions in the text or huge tables.

Based on our experiences with RepDose we made several improvements:

We included the scope of examination. This was especially important for studies with particles and fibres, because currently available studies with (nano-)objects are mostly not according to guidelines and differ widely in scope investigated; this relates to organs as well as to endpoints investigated. With the scope of examination, it is possible to query, how often effects have been examined and how often they were positive. The scope of examination has already been taken into consideration in our analyses in Tab. 24 and Tab. 25. As the scope of examination is differing from studies according to OECD guidelines the Klimisch code is not useful for this database and the criteria used in the database RepDose were adapted for the PaFtox database (see 2.2.3).

In addition for each effect the effect levels or incidences were entered into the database, including significance. This rendered data entry very time consuming (see example data sheets in the Appendix), but allowed queries on effect levels e.g. for neutrophils/PMN levels (Fig 7), total protein (Fig 8), LDH (Fig 9), lung burden (Tab. 29), lung weight (Tab. 30) and allows future analyses of dose response with the benchmark dose approach.

7.3 Study reports from PaFtox database (October 2012)

Carbon Black

molecular weight 12 **g / mol**

study pk 4108 **Study Data**

Specification by Producer / Supplier

object Primary particle

synonym Printex 90TM

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean	14			
SD				
min				
max				

medium

distribution type no data

cristal structure

specific surface in m²/g

solubility in mg/l

particle density in g/cm³

determ. method

surface property

shape

specific volume in m³/g

at in °C

additional High-purity furnace carbon black

reference Heinrich & Fuhst (1992)

producer Degussa, Frankfurt, Germany

Specification by Authors

object Primary particle

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean				
SD				
min				
max				

medium

distribution type no data

cristal structure

specific surface in m²/g 227

solubility in mg/l

particle density in g/cm³ 1.85

determ. method

surface property

shape

specific volume in m³/g

at in °C

additional coarse particles removed by a cyclone with 50% cut-off diameter ca. 1 µm for a flow rate of 100 m3/h; spec. surface area by BETg, 0,04% extractable organic matter

reference Heinrich & Fuhst (1992)

Hydrodynamic diameter

median 640 **nm**

GSD 2.6

min **nm**

distibution type no data

bulk density **mg/ml**

isoelectric point **in**

max	**nm**	**zeta potential**	**mV**	**in**			
medium		**peak SPR**	**nm**	**in**			
determ. method	10-stage Berner impactor	**conductivity**	**µS/cm**	**in**			
sample treatment		**solubility**	**mg/L**	**in**	**at**	**°C**	
applic. medium							
dispersant							
additional							

Study Design

species	rat	**strain**	Wistar (Crl:(WI) BR)
sex	female	**animal/group**	100
route	whole-body	**age of animal**	7 w
purity			
exposure in h/d	18	**exposure in d/w**	5
study dur. in d	730	**postexp. dur. in d**	182
no. of instillation		**frequency**	

exposure (additional) whole-body in 6 or 12 m3 horizontal flow type chambers; nominal concentration of 11,6 mg/m3 as time-weighted average of 7,4 mg/m³ for 4 months + 12,2 mg/m³ for 20 months, cumulative exposure 102,2 g/m³ x h

dose / concentration

0

11.63

Unit

mg/m³

1 dose study ☑

reliability B

confidential ☐

additional interim sacrifices at d 91, 182, 365, 547, 670, 730 and 910 d
number of animals (control/exposed):
carcinogenicity: 220/100, additionally
histology (serial sacrifice): 80/80
DNA-adducts (d 14, 60, 730): 14/14
lung burden: 66/66
lung clearance: 28/28, measured as clearance of radiolabeled Fe2O3 tracer particles (MMAD 0,35 µm)
BALF only after 730 d
CB burden after digestion of tissue measured by turbidity of particles suspended in 0,01 N NaOH

Reference

author	Heinrich U, and Fuhst, R	**source**	Abschlussbericht: Vergleichend Untersuchungen zur Frage der tumorinduzierenden Wirkung von Dieselmotorabgasen in der Rattenlunge. Final Report in German		
volume	07VAG06	**year**	1992	**page**	1-56 plus Append
institution	Fraunhofer ITEM				
author	Muhle H, et al.	**source**	In: Mohr U, et al. (Ed.) Toxic and carcinogenic effects of solid particles in the respiratory tract		
volume		**year**	1994	**page**	29-41
institution	Fraunhofer ITEM				
author	Creutzenberg O, et al.	**source**	J Aerosol Science		
volume	21, Suppl 1	**year**	1990	**page**	S455-S458
institution	Fraunhofer ITEM				
author	Heinrich U, et al.	**source**	Inhalation Toxicology		
volume	7	**year**	1995	**page**	533-556
institution	Fraunhofer ITEM				

Scope

organ	animal/group	necropsy	organ weight	histopathology
guideline				
body weight	100	☐	☐	☐
lung	100	☐	☑	☑
additional	total lung burden (no lavage); histopatho: H&E stain; alveolar lung clearance of radiolabeled tracer particles; PAH-DNA adducts by 32P-postlabeling (total lung)			
nose	100	☐	☐	☑
additional	nasal and paranasal cavities			
larynx	100	☐	☐	☑
trachea	100	☐	☐	☑
BALF		☐	☐	☐

alveolar macrophages, granulocytes, lymphocytes
lactate dehydrogenase (LDH), hydroxyproline, total protein
β-glucuronidase

additional: both lobes, 5x4ml

Effect data

BALF

effect	sex	LOEL study unit	LOEL mg/kg	transient
lactate dehydrogenase (LDH)	female	11.63	8.3736	☐

% dose	sex	timepoint	level	score	significance	%
0	female	730	100		n.g.	100
11.63	female	730	1700		<0.01	1700

additional: sign. increase of LDH activity (% of control) after 24 mo exposure (Fig.25), effect lower than after 18 exposure + 6 mo p-e (see additional data in Study number 3999)

effect	sex	LOEL study unit	LOEL mg/kg	transient
β-glucuronidase	female	11.63	8.3736	☐

% dose	sex	timepoint	level	score	significance	%
0	female	730	100		n.g.	100
11.63	female	730	7200		<0.01	7200

additional: sign. increase of β-glucuronidase activity (% of control) after 24 mo exposure (Fig. 26), effect lower after 18 mo exposure + 6 mo p-e (see additional data in Study number 3999)

effect	sex	LOEL study unit	LOEL mg/kg	transient
total protein	female	11.63	8.3736	☐

% dose	sex	timepoint	level	score	significance	%
0	female	730	100		n.g.	100
11.63	female	730	1100		<0.01	1100

additional: sign. increase of totasl protein (% of control) after 24 mo exposure (Fig. 27), effect comparable to 1 exposure + 6 mo p-e (see additional data in Study number 3999)

effect		sex	LOEL study unit	LOEL mg/kg	transient		
hydroxyproline		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	730	100		n.g.	100
	11.63	female	730	310		<0.01	310
	additional	sign. increase of free hydroxyproline (% of control) after 24 mo exposure (Fig.28), effect higher than after 18 mo exposure + 6 mo p-e (see additional data in Study number 3999)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
alveolar macrophages total		female	11.63	2.64318	☐		
x10E6/ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	670	0.29		n.g.	100
	0	female	730	0.95		n.g.	100
	11.63	female	670	2.29		n.g.	789.65
	11.63	female	730	3.05		n.g.	321.05
	additional	increase of number of AM after 22 and 24 mo exposure, effects comparable to 18 mo exposure + 4 mo p-e (see additional data in Study number 3999)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
PMN total		female	11.63	8.3736	☐		
x10E6/ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	670	0		n.g.	100
	0	female	730	0		n.g.	100
	11.63	female	670	2.19		n.g.	
	11.63	female	730	3.14		n.g.	
	additional	increase of number of PMNs after 22 and 24 mo exposure, effects higher than after 18 mo exposur or 6 mo p-e (see additional data in Study number 3999)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
lymphocytes total		female			☐
x10E6/ml					
	additional	no effect: number of lymphocytes (Fig.29)			

body weight

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight decreased		female	11.63	8.3736	☐		
gram	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	730	417		n.g.	100
	0	female	910	390		n.g.	100
	11.63	female	730	325		<0.05	77.93
	11.63	female	910	323		<0.05	82.82
	additional	sign. decrease of bw from ca. d 400 of exposure throughout d 910					

clinical symptoms

effect		sex	LOEL study unit	LOEL mg/kg	transient		
mortality		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	730	42		n.g.	100
	0	female	912	85		n.g.	100
	11.63	female	730	56		<0.05	133.33
	11.63	female	912	92		<0.05	108.23
	additional	mean lifetime sign. decreased; increase of mortality (% of rats) at termination of exposure and after p-e					

lung

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight		female	11.63	8.3736	☐		
gram	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	91	1.28		n.g.	100
	0	female	182	1.55		n.g.	100
	0	female	365	1.33		n.g.	100
	0	female	547	1.54		n.g.	100
	0	female	670	1.34		n.g.	100
	0	female	730	1.44		n.g.	100
	11.63	female	91	2.24		<0.001	175
	11.63	female	182	3.64		<0.001	234.83
	11.63	female	365	6.46		<0.001	485.71
	11.63	female	547	7.64		<0.001	496.1
	11.63	female	670	6.93		<0.001	517.16
	11.63	female	730	6.83		<0.001	474.3
	additional	sign. increases of lung wet wt. with maximum after 18 mo exposure, slightly reversible within 24 mo exposure (no data for p-e)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
burden		female	11.63	8.3736	☐		
mg/total lung	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	91	0		n.g.	100
	0	female	182	0		n.g.	100
	0	female	365	0		n.g.	100
	0	female	547	0		n.g.	100
	0	female	670	0		n.g.	100
	0	female	730	0		n.g.	100
	11.63	female	91	7.56		n.g.	
	11.63	female	182	19.9		n.g.	
	11.63	female	365	38		n.g.	
	11.63	female	547	50.2		n.g.	
	11.63	female	670	45.2		n.g.	
	11.63	female	730	43.9		n.g.	
	additional	increase of total lung burden (no lavage) up to 18 mo exposure, slight increase within 24 mo exposu (no data for p-e), half-time for clearance of TiO2 burden 550 d; further data for 18 mo exposure + p-Study Number 3999					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
alveolar clearance		female	11.63	8.3736	☐		
days	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	91	61		n.g.	100
	0	female	365	72		n.g.	100
	0	female	547	96		n.g.	100
	11.63	female	91	244		<0.01	400
	11.63	female	365	368		<0.01	511.11
	11.63	female	547	363		<0.01	378.12
	additional	sign. increase of clearance time for radiolabeled tracer particles (Fe-59-oxid) from 3 mo exposure up further increase up to 12-18 mo exposure; further data for 18 mo exposure + p-e in Study Number 3					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
hyperplasia bronchiolo-alveolar		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	182	0		n.g.	100
	0	female	910	0		n.g.	100
	11.63	female	182	100	minimal	<0.01	
	11.63	female	910	96	severe	<0.01	
	additional	sign. increase of bronchiolo-alveolar hyperplasia in 20/20 rats at 6 mo exposure, and 96/100 rats aft 24 mo expsoure + 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
fibrosis		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	182	0		n.g.	100
	0	female	365	10	minimal	n.g.	
	0	female	547	5.6	minimal	n.g.	
	0	female	730	0		n.g.	100
	0	female	910	4.1	mild	n.g.	
	0	female	910	4.1	minimal	n.g.	
	11.63	female	182	100	minimal	n.g.	
	11.63	female	365	52.6	mild	n.g.	
	11.63	female	365	5.3	medium	n.g.	
	11.63	female	547	66.7	medium	n.g.	
	11.63	female	547	33.3	mild	n.g.	
	11.63	female	730	11.1	minimal	n.g.	
	11.63	female	730	11.1	severe	n.g.	
	11.63	female	730	77.8	medium	n.g.	
	11.63	female	910	30	medium	n.g.	
	11.63	female	910	2	minimal	n.g.	
	11.63	female	910	57	mild	n.g.	
	additional	time-dependent increase of incidences and severity of interstitial fibrosis at >= 6 mo exposure; effec reversible in severity at 24 mo exposure + 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
bronchiolo-alveolar adenoma		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	910	0		n.g.	100
	11.63	female	910	13		<0.05	
	additional	increased incidence of bronchiolo-alveolar adenoma 13/100 vs. 0/217 in controls					

effect	sex	LOEL study unit	LOEL mg/kg	transient
bronchiolo-alveolar adenocarcinoma	female	11.63	8.3736	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	910	0.46		n.g.	100
	11.63	female	910	13		<0.05	2826.1
	additional	increased incidence of bronchiolo-alveolar adenocarcinoma 13/100 vs. 1/217 in controls					

effect	sex	LOEL study unit	LOEL mg/kg	transient
squamous cell carcinoma	female	11.63	8.3736	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	910	0		n.g.	100
	11.63	female	910	4		n.g.	
	additional	increased incidence of squamous cell carcinoma 4/100 vs. 0/217 in controls					

effect	sex	LOEL study unit	LOEL mg/kg	transient
benign cystic keratinizing squamous-cell tumor	female	11.63	8.3736	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	910	0		n.g.	100
	11.63	female	910	20		n.g.	
	additional	increased incidence of benign cystic keratinizing squamous-cell tumour 20/100 vs. 0/217 in controls					

effect	sex	LOEL study unit	LOEL mg/kg	transient
clearance	female	11.63	8.3736	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	730	0		n.g.	100
	11.63	female	730	100		n.g.	
	additional	particle-laden macrophages in lungs of all CB-exposed rats					

effect	sex	LOEL study unit	LOEL mg/kg	transient
PAH-derived DNA adducts	female			☐

n.a.

additional	no effect: no increased level of PAH-derived DNA adducts (32P postlabeling)

lymph node

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	female	11.63	8.3736	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	730	100		n.g.	100
	11.63	female	730	800		n.g.	800
	additional	8-fold increase at termination of exposure					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
burden		female	11.63	8.3736	☐		
mg/organ	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	670	0		n.g.	100
	11.63	female	670	6.72		n.g.	
	additional	increase of retained particle mass in LALN					

nose

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
squamous cell metaplasia		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	365	0		n.g.	100
	0	female	547	0		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	7.7		n.g.	100
	11.63	female	365	27.8		n.g.	
	11.63	female	547	68.8		n.g.	
	11.63	female	730	55.6		n.g.	
	11.63	female	910	61		n.g.	792.2
	additional	increased incidences of squamous cell metaplasia in epithelia of nasal cavity and paranasal sinuses after >= 12 mo exposure, effect not reversible after 24 mo exposure + 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
degeneration		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	182	0		n.g.	100
	0	female	365	9.5		n.g.	100
	0	female	547	33.3		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	34.4		n.g.	100
	11.63	female	182	30		n.g.	
	11.63	female	365	100		n.g.	1052.6
	11.63	female	547	100		n.g.	300.3
	11.63	female	730	88.9		n.g.	
	11.63	female	910	75		n.g.	218.02
	additional	sign. increase of incidences of degenerative changes in mucus membranes of nasal cavity and paranasal sinuses after >= 6 mo exposure, effect slightly reversible after 24 mo exposure + 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
inflammation		female	11.63	8.3736	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	365	4.8		n.g.	100
	0	female	547	0		n.g.	100
	0	female	730	10		n.g.	100
	0	female	910	5.9		n.g.	100
	11.63	female	365	22.2		n.g.	462.5
	11.63	female	547	50		n.g.	
	11.63	female	730	55.6		n.g.	556
	11.63	female	910	40		n.g.	677.96
	additional	increased incidences of inflammatory changes in mucus membranes of nasal cavity and paranasal sinuses after >= 12 mo exposure, effect slightly reversible after 24 mo exposure + 6 mo p-e					

Carbon Black

molecular weight 12 **g / mol**

study pk 4131 **Study Data**

Specification by Producer / Supplier

object Primary particle

synonym Elftex-12

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean	37			
SD				
min				
max				

medium

distribution type no data

cristal structure

specific surface in m²/g 43

solubility in mg/l

particle density in g/cm³

additional

reference Ash M, Ash I (Ed.) Handbook of Fillers, Extenders, and Diluents, 2007, p.

producer Cabot, Boston, MA

determ. method

surface property

shape

specific volume in m³/g

at in °C

Specification by Authors

object Primary particle

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean				
SD				
min				
max				

medium

distribution type no data

cristal structure

specific surface in m²/g

solubility in mg/l

particle density in g/cm³

additional

reference

determ. method

surface property

shape

specific volume in m³/g

at in °C

Hydrodynamic diameter

median **nm**

GSD

min **nm**

distibution type bidispers

bulk density **mg/ml**

isoelectric point **in**

max	**nm**	**zeta potential**	**mV**	**in**			
medium		**peak SPR**	**nm**	**in**			
determ. method		**conductivity**	**µS/cm**	**in**			
sample treatment		**solubility**	**mg/L**	**in**	**at**	**°C**	
applic. medium							
dispersant							
additional	bimodal particle size distribution: small-size mode (parallel-floe diffusion battery): mass median diffusion diameter 100 nm, GSD 2,16, 33% of particle mass; large-size mode (cascade impactor): MMAD 1950 nm, GSD 1,84, 67% of particle mass						

Study Design

species	rat	**strain**	F344/N
sex	male & female	**animal/group**	114-118
route	whole-body	**age of animal**	7.5 w
purity			
exposure in h/d	16	**exposure in d/w**	5
study dur. in d	730	**postexp. dur. in d**	42
no. of instillation		**frequency**	
exposure (additional)	whole-body, airflow 425 l/min; nominal conc. 2,5 and 6,5 mg/m3, analytical conc. 2,46+-0,03; 6,55 +-0,06 mg/m3		
dose / concentration	0 2.46 6.55	**1 dose study**	☐
		reliability	**B**
Unit		**confidential**	☐
mg/m³			

additional Serial sacrifices (N=3 per sex/group) at d 91, 182, 365, 547, 700, terminal sacrifice about d 772; histopatho all tp, BALF at d 365, 547 & 700;
nonneoplastic findings (sacrifice, euthanasia, death): 547 d (<= 18 mo), N=32-44 per sex/group; 780 d (18 mo - end of life), N=71-86 per sex/group; squamous cysts (incl. died animals): 730 d (18 - 24 mo), N=54-77 per sex/group; 780 d (24 mo - end of life), N=0-36
neoplastic findings (except sacrificed betw. mo 3 - 12): 780 d (18 mo - end of life), N=105-109 per sex/group
genotoxicity: PAH-DNA adducts adducts in left lung (all tp, HD, N=3/sex); PAH-DNA adducts in alveolar type II cells (d 91, HD, N=5/sex); mutations of K-ras or p53 in lung tumors (N=14 adenocarcinomas, N=3 squamous cell carcinomas, N=1 adenosquamous carcinomas); SCE and micronuclei in circulating lymphocytes ex vivo (only at d 91, N=3/sex); Hb-adducts in erythrocytes (only at d 91, N=3/sex)
lung burden (all tp): optical density at 620 nm after homogenization of left lung in saline; burden in LALN (all tp): digestion of tissue with acid, washed residue of inorganic carbon converted to carbon dioxide and measured by IR spectrometry
Clearance of radiolabeled carbon black [7Be]CB after single exposures on d 91 & 547 (N=8/sex): whole-body counting at d 0, 4, 7, 13, 28, 35, 42, 56, 73, 84, 98, 112 & 126 d after exposure
Translocation and sequestration of inhaled fluorescent latex microspheres applied on d 81 & 547: histopatho and morphometry on d 1, 4, 28 & 90 (N=2/sex) to identify microspheres in single alveolar macrophages, aggregated macrophages & other locations

Reference

author	Mauderly JL, et al.	**source**	Report HEI-RR-68 Part I		
volume		**year**	1995	**page**	1-106
institution	Lovelace Biomed. & Environm. Research Institute, Albuquerque				
author	Belinsky SA, et al.	**source**	Report HEI-RR-68 Part III		
volume		**year**	1995	**page**	1-25
institution	Lovelace Biomed. & Environm. Research Institute, Albuquerque				

author	Randerath K, et al.	**source**	Report HEI-RR-68 Part II, NTIS PB96-138623		
volume		**year**	1995	**page**	
institution	Lovelace Biomed. & Environm. Research Institute, Albuquerque				

author	Nikula KJ, et al.	**source**	Fund Appl Toxicol		
volume	25	**year**	1995	**page**	80-94
institution	Inhalation Toxicology Research Institute				

Scope

organ	**animal/group**	**necropsy**	**organ weight**	**histopathology**
guideline				
body weight	115	☐	☐	☐
lung	115	☑	☑	☑
additional	histopatho (right lobe): H&E stain; lung burden (left lobe after lavage, N=3); PAH-DNA adducts by 32P-postlabeling in total lung; PAH-DNA adducts in alveolar type II cells; immunohistochemistry of p53 protein in lung tumors; mutations of K-ras or p53 in lung tumors by PCR			
mortality	115	☐	☐	☐
clinical symptoms	115	☐	☐	☐
lymph node	3	☐	☑	☐
additional	particle burden			
BALF	3	☐	☐	☐
additional	left lobe, 2x2 ml for f, 2x2,5 ml for m			
haematology	3	☐	☐	☐
additional	genotoxicity: SCE and micronuclei in primary cultures of circulating lymphocytes ex vivo; Hb-adducts			

Effect data

BALF

effect	**sex**	**LOEL study unit**	**LOEL mg/kg**	**tran-sient**
total protein	male & female			☐
mg/ml				
additional	no effect (Mauderly: Tab.E1-E3)			

effect	**sex**	**LOEL study unit**	**LOEL mg/kg**	**tran-sient**
glutathione reductase	male & female	2.46	1.5744	☐

units/liter (U/l)	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	34		n.g.	100
	0	male & f	547	20		n.g.	100
	0	male & f	700	19		n.g.	100
	2.46	male & f	365	33		none	97.05
	2.46	male & f	547	32		none	160
	2.46	male & f	700	40		<0.05	210.52
	6.55	male & f	365	48		none	141.17
	6.55	male & f	547	52		none	260
	6.55	male & f	700	51		none	268.42
	additional	sign. increase at d 700 at LD only, increase at HD not sign. due to high SD (Mauderly: Tab.E1-E3)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
β-glucuronidase		male & female	2.46	1.5744	☐		
units/liter (U/l)	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	0.26		n.g.	100
	0	male & f	547	0.25		n.g.	100
	0	male & f	700	0.09		n.g.	100
	2.46	male & f	365	4.13		<0.05	1588.5
	2.46	male & f	547	5.25		<0.05	2100
	2.46	male & f	700	4.07		<0.05	4522.2
	6.55	male & f	365	11.94		<0.05	4592.3
	6.55	male & f	547	11.13		<0.05	4452
	6.55	male & f	700	9.63		none	10700
	additional	sign. increase at LD & HD, increase at HD at d 700 not sign. due to high SD, effects not reversible (Mauderly: Tab.E1-E3)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
lactate dehydrogenase (LDH)		male & female	2.46	1.5744	☐		
units/liter (U/l)	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	112		n.g.	100
	0	male & f	547	86		n.g.	100
	0	male & f	700	76		n.g.	100
	2.46	male & f	365	368		<0.05	328.57
	2.46	male & f	547	280		<0.05	325.58
	2.46	male & f	700	335		<0.05	440.78
	6.55	male & f	365	653		<0.05	583.03
	6.55	male & f	547	553		<0.05	643.02
	6.55	male & f	700	535		<0.05	703.94
	additional	sign. dose-related increase at all tp, not reversible (Mauderly: Tab.E1-E3)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
neutrophils total		male & female	2.46	1.5744	☐		
x10E3/ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	14		n.g.	100
	0	male & f	547	9		n.g.	100
	0	male & f	700	4		n.g.	100
	2.46	male & f	365	864		<0.05	6171.4
	2.46	male & f	547	865		none	9611.1
	2.46	male & f	700	854		none	21350
	6.55	male & f	365	1417		<0.05	10121
	6.55	male & f	547	1976		<0.05	21956
	6.55	male & f	700	943		<0.05	23575
	additional	sign. increase at LD at d 365, at HD at all tp, other increases at LD not sign. due to high SD, max. e at HD at d 547 (Mauderly: Tab.E1-E3)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
alveolar macrophages total		male & female	2.46	1.5744	☐		
x10E3/ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	850		n.g.	100
	0	male & f	547	575		n.g.	100
	0	male & f	700	660		n.g.	100
	2.46	male & f	365	1889		<0.05	222.23
	2.46	male & f	547	1259		<0.05	218.95
	2.46	male & f	700	726		none	110
	6.55	male & f	365	1192		none	
	6.55	male & f	547	1877		none	326.43
	6.55	male & f	700	855		none	129.54
	additional	sign. increase of alveolar macrophages at LD at 365 & 547 d, increases at HD not sign. due to high increases not dose-related, all doses reversible within d 700 (Mauderly: Tab.E1-E3)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
leukocytes		male & female	2.46	1.5744	☐		
x10E3/ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	874		n.g.	100
	0	male & f	547	591		n.g.	100
	0	male & f	700	664		n.g.	100
	2.46	male & f	365	2820		<0.05	322.65
	2.46	male & f	547	2149		none	363.62
	2.46	male & f	700	1583		<0.05	238.4
	6.55	male & f	365	2659		<0.05	304.23
	6.55	male & f	547	3899		<0.05	659.72
	6.55	male & f	700	1806		<0.05	271.98
	additional	sign. increase of total leukocytes, increase of LD at d 547 not sign. due to high SD, effect partly reversible (Mauderly: Tab.E1-E3)					

body weight

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight decreased		male & female	2.46	1.5744	☐		
gram	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	300	272		n.g.	100
	0	male	450	485		n.g.	100
	0	male	500	485		n.g.	100
	0	female	500	324		n.g.	100
	0	male	680	405		n.g.	100
	0	female	730	296		n.g.	100
	2.46	female	300	268		none	98.52
	2.46	male	450	480		none	98.96
	2.46	male	500	465		<0.05	95.87
	2.46	female	500	314		<0.05	96.91
	2.46	male	680	385		<0.05	95.06
	2.46	female	730	268		<0.05	90.54
	6.55	female	300	260		<0.05	95.58
	6.55	male	450	440		<0.05	90.72
	6.55	female	500	296		<0.05	91.35
	6.55	female	500	435		<0.05	134.25
	6.55	male	680	355		<0.05	87.65
	6.55	female	730	244		<0.05	82.43
	additional	sign. dose-dependent decrease of bw, p<0.05 at HD after ~d300(f)+450(m), at LD after d500 (f+m) (					

clinical symptoms

effect		sex	LOEL study unit	LOEL mg/kg	transient		
mortality		male & female	2.46	1.5744	☐		
median survival ti	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	780	639		n.g.	100
	0	female	780	696		n.g.	100
	2.46	male	780	605		<0.05	94.67
	2.46	female	780	707		none	101.58
	6.55	male	780	599		<0.05	93.74
	6.55	female	780	675		<0.05	96.98
	additional	sign. increase in LD m and HD m & f, m more sensitive, LOEL f = 6,55 mg/m3					

effect	sex	LOEL study unit	LOEL mg/kg	transient
mortality	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	547	16		n.g.	100
	0	female	547	8		n.g.	100
	0	male	700	92		n.g.	100
	0	female	700	48		n.g.	100
	2.46	male	547	30		n.g.	187.5
	2.46	female	547	8		n.g.	100
	2.46	male	700	92		n.g.	100
	2.46	female	700	42		n.g.	87.5
	6.55	male	547	28		n.g.	175
	6.55	female	547	14		n.g.	175
	6.55	male	700	98		n.g.	106.52
	6.55	female	700	62		n.g.	129.16
	additional	increased mortality in m at LD & HD after 500 d, and in f at HD after 700 d (Fig.1); m more sensitive LOEL f 6,55 mg/m3					

haematology

effect	sex	LOEL study unit	LOEL mg/kg	transient
SCE	male & female			☐

n.a.

additional	no effect: no increase of SCE in primary cultures of circulating lymphocytes ex vivo at d 91 (not investigated at later tp

effect	sex	LOEL study unit	LOEL mg/kg	transient
micronuclei	male & female			☐

n.a.

additional	no effect: no increase of micronuclei in in primary cultures of circulating lymphocytes ex vivo at d 91 investigated at later tp

lung

effect	sex	LOEL study unit	LOEL mg/kg	transient
tumor supressor protein p53	male & female	6.55	4.192	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	780		no change	n.a.	
	6.55	male & f	780			n.a.	
	additional	p53 protein in 1/3 squamous cell carcinomas in HD group (sex not specified) with 26-50% of nuclei showing immunoreactivity					

effect	sex	LOEL study unit	LOEL mg/kg	transient
fibrosis	male & female	2.46	1.5744	☐

%

dose	sex	timepoint	level	score	significance	%
0	male	547	3		n.g.	100
0	female	547	0		n.g.	100
0	male	772	1		n.g.	100
0	female	772	2		n.g.	100
2.46	male	547	61		n.g.	2033.3
2.46	female	547	52		n.g.	
2.46	male	772	92		n.g.	9200
2.46	female	772	96		n.g.	4800
6.55	male	547	75		n.g.	2500
6.55	female	547	78		n.g.	
6.55	male	772	99		n.g.	9900
6.55	female	772	100		n.g.	5000

additional increase of incidences of interstitial fibrosis in alveolar septa for rats died or euthanized < d 547 or > 547 with dose- and time-dependent increase of severity scores, prominent lesion >=365d, correlatin with increased interstitial aggregation of macrophages; n of control, LD, HD: <547d, m N=32, 44, 44 N=23, 21, 27 ; >547d, m N=86, 71, 71, f N=91, 95, 87 (tab.5,6)

effect	sex	LOEL study unit	LOEL mg/kg	transient
bronchiolo-alveolar adenocarcinoma	female	2.46	1.5744	☐

%

dose	sex	timepoint	level	score	significance	%
0	male	772	0.92		n.g.	100
0	female	772	0		n.g.	100
2.46	male	772	0.94		n.g.	102.17
2.46	female	772	5.6		n.g.	
6.55	male	772	0.94		n.g.	102.17
6.55	female	772	19		n.g.	

additional dose-dependent increase in f (tab.10), pathogenesis from a continuum that began with alveolar epith hyperplasia; susceptible rats (>365 d up to d 772; control, LD, HD): m N=109, 106, 106, f N=105, 1 105; biased by low survival rates of m >= 680 d

effect	sex	LOEL study unit	LOEL mg/kg	transient
p53 mutations	male & female			☐

n.a.

additional no effect: no increase of mutations of p53 in lung tumors

effect	sex	LOEL study unit	LOEL mg/kg	transient
K-ras mutations	male & female			☐

n.a.

additional no effect: no increase of mutations of K-ras in lung tumors

effect	sex	LOEL study unit	LOEL mg/kg	transient
PAH-derived DNA adducts	male & female			☐

n.a.

additional no effect: no increased level of PAH-derived DNA adducts in total lung tissue (32P postlabeling)

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
deposits		male & female	2.46	1.5744	☐		
n.a.	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365		no change	n.a.	
	0	male & f	547		no change	n.a.	
	0	male & f	772		no change	n.a.	
	2.46	male & f	365			n.a.	
	2.46	male & f	547			n.a.	
	2.46	male & f	772			n.a.	
	6.55	male & f	365			n.a.	
	6.55	male & f	547			n.a.	
	6.55	male & f	772			n.a.	
	additional	increased incidences of cholesterol clefts (by-products of necrosis) as part of inflammatory response 365d (see inflammation)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
infiltration		male & female	2.46	1.5744	☐		
n.a.	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365		no change	n.a.	
	0	male & f	547		no change	n.a.	
	0	male & f	772		no change	n.a.	
	2.46	male & f	365			n.a.	
	2.46	male & f	547			n.a.	
	2.46	male & f	772			n.a.	
	6.55	male & f	365			n.a.	
	6.55	male & f	547			n.a.	
	6.55	male & f	772			n.a.	
	additional	increased incidences of infiltration of neutrophils as part of inflammatory response >= 365d (see inflammation)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
adenosquamous carcinoma		male & female	6.55	4.192	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	772	0		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	772	0		n.g.	
	2.46	female	772	0		n.g.	
	6.55	male	772	0.94		n.g.	
	6.55	female	772	0.95		n.g.	
	additional	single cases at HD m and f (tab.10); susceptible rats (>365 d up to d 772; control, LD, HD): m N=10 106, 106, f N=105, 107, 105; biased by low survival rates of m >= 680 d					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
squamous cell carcinoma	male & female	6.55	4.192	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	772	0.92		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	772	0		n.g.	0
	2.46	female	772	0		n.g.	
	6.55	male	772	1.89		n.g.	205.43
	6.55	female	772	0.95		n.g.	
	additional	single cases at HD m & f and at control m (tab.10); susceptible rats (>365 d up to d 772; control, LD HD): m N=109, 106, 106, f N=105, 107, 105; biased by low survival rates of m >= 680 d					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
bronchiolo-alveolar adenoma	female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	772	0.92		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	772	0.94		n.g.	102.17
	2.46	female	772	1.87		n.g.	
	6.55	male	772	0		n.g.	0
	6.55	female	772	12.4		n.g.	
	additional	dose-dependent increase in f (tab.10); pathogenesis from a continuum that began with alveolar epith hyperplasia; n of susceptible rats (>365 d up to d 772; control, LD, HD): m N=109, 106, 106, f N=10 107, 105; biased by low survival rates of m >= 680 d					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
squamous cysts	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	0		n.g.	100
	0	female	730	0		n.g.	100
	0	male	772	0		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	730	0		n.g.	
	2.46	female	730	1.9		n.g.	
	2.46	male	772	100		n.g.	
	2.46	female	772	19.4		n.g.	
	6.55	male	730	5.4		n.g.	
	6.55	female	730	7.2		n.g.	
	6.55	female	772	44.4		n.g.	
	additional	f more sensitive, LOEL m = 6,55 mg/m3: increased incidences of squamous cysts (squamous epithelium with central keratin accumulation) at d 547-730 in LD & HD f with dose- & time-dependen increase, only HD in m; >730 d increased effect in f, low survival rate of m ; N of control, LD, HD: 54 730 d, m N=77, 72, 74, f N=56, 54, 69; >730d, m N=9, 1, 0, f N=35, 36, 18 (tab.7)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
squamous metaplasia		female	6.55	4.192	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	0		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	547	2		n.g.	
	2.46	female	547	0		n.g.	
	2.46	male	772	1		n.g.	
	2.46	female	772	6		n.g.	
	6.55	male	547	2		n.g.	
	6.55	female	547	0		n.g.	
	6.55	male	772	3		n.g.	
	6.55	female	772	24		n.g.	
	additional	sign. effect at HD f > 547 for rats died or euthanized < d 547 or > d 547 (tab.5,6); N of control, LD, H <547 d, m N=32, 44, 44, f N=23, 21, 27 ; >547 d, m N=86, 71, 71, f N=91, 95, 87					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
fibrosis		male & female	2.46	1.5744	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	0		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	547	0		n.g.	
	2.46	female	547	0		n.g.	
	2.46	male	772	6		n.g.	
	2.46	female	772	17		n.g.	
	6.55	male	547	2		n.g.	
	6.55	female	547	7		n.g.	
	6.55	male	772	25		n.g.	
	6.55	female	772	31		n.g.	
	additional	increase of incidences of focal fibrosis with epithelial hyperplasia (nodular focus of fibrosis compose dense collagen bundles and small alveolar structures, surreounded by hyperplastic epithelium and associated neutrophils) for rats died or euthanized < d 547 or > d 547 with dose- and time-depende increase of severity scores >= d485 in f and d516 in m; f more sensitive with higher incidences and severity scores; n of control, LD, HD: <547d, m N=32, 44, 44, f N=23, 21, 27 ; >547d, m N=86, 71, 7 N=91, 95, 87 (tab.5,6)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
hyperplasia alveolar type II cells	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	91	0		n.g.	100
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	12		n.g.	100
	2.46	male & f	91	67		n.g.	
	2.46	male & f	182	100		n.g.	
	2.46	male & f	365	100		n.g.	
	2.46	male & f	547	100		n.g.	
	2.46	male & f	700	100		n.g.	
	2.46	male & f	772	100		n.g.	833.33
	6.55	male & f	91	100		n.g.	
	6.55	male & f	182	100		n.g.	
	6.55	male & f	365	100		n.g.	
	6.55	male & f	547	100		n.g.	
	6.55	male & f	700	100		n.g.	
	6.55	male & f	772	100		n.g.	833.33
	additional	increase of incidences of alveolar epithelial hyperplasia (increased number of alveolar type II cells) (affected rats), all rats affected at LD >= d 182 & at HD all tp, not reversible during 42 d p-e with dos and time-dependent increase of grading (see hyperplasia: grading); serial sacrifices: N=3/sex and g terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
alveolar proteinosis	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	0		n.g.	100
	0	female	772	0		n.g.	100
	2.46	male	547	0		n.g.	
	2.46	female	547	14		n.g.	
	2.46	male	772	0		n.g.	
	2.46	female	772	27		n.g.	
	6.55	male	547	18		n.g.	
	6.55	female	547	56		n.g.	
	6.55	male	772	25		n.g.	
	6.55	female	772	99		n.g.	
	additional	f more sensitive, LOEL m 6,55 mg/m3; increase of incidences of alveolar proteinosis for rats died or euthanized < d 547 or > d 547, dose- and time-dependent increase of severity scores >=365d; effec seen in LD & HD f, only HD m and at lower incidence than in f; n of control, LD, HD: < 547d, m N=32 44, f N=23, 21, 27 ; >547d, m N=86, 71, 71, f N=91, 95, 87 (tab.5,6)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
fibrosis	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	700	0		n.g.	100
	0	male & f	772	0		n.g.	100
	2.46	male & f	700	0		n.g.	
	2.46	male & f	772	28		n.g.	
	6.55	male & f	700	67		n.g.	
	6.55	male & f	772	33		n.g.	
	additional	increase of incidences of focal fibrosis (% affected rats), from d 700 at HD & at d 772 at LD, partly reversible at HD, for grading see fibrosis: grading; serial sacrifices: N=3/sex and group; terminal sac control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
inflammation	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	1		n.g.	100
	0	female	772	5		n.g.	100
	2.46	male	547	9		n.g.	
	2.46	female	547	24		n.g.	
	2.46	male	772	14		n.g.	1400
	2.46	female	772	34		n.g.	680
	6.55	male	547	20		n.g.	
	6.55	female	547	37		n.g.	
	6.55	male	772	34		n.g.	3400
	6.55	female	772	63		n.g.	1260
	additional	increase of incidences of chronic-active inflammation, dose- and time-dependent increase of severit scores >= 365d, focal aggregates of neutrophils, degenerate inflammatory cells, cell debris and cholesterol clefts; n of control, LD, HD: <547d, m N=32, 44, 44, f N=23, 21, 27 ; <780d, m N=86, 71 f N=91, 95, 87 (tab.5,6)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
hyperplasia alveolar type II cells	male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	2		n.g.	100
	0	female	772	9		n.g.	100
	2.46	male	547	98		n.g.	
	2.46	female	547	90		n.g.	
	2.46	male	772	100		n.g.	5000
	2.46	female	772	100		n.g.	1111.1
	6.55	male	547	100		n.g.	
	6.55	female	547	93		n.g.	
	6.55	male	772	100		n.g.	5000
	6.55	female	772	100		n.g.	1111.1
	additional	increase of incidences of alveolar epithelial hyperplasia (increased number of alveolar type II cells) f rats died or euthanized < d 547 or > d 547, dose- and time-dependent increase of severity scores > d91, localization mainly in centriacinar region; n of control, LD, HD: <547 d, m N=32, 44, 44, f N=23 27 ; <780 d, m N=86, 71, 71, f N=91, 95, 87 (tab.5,6)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
macrophage infiltration		male & female	2.46	1.5744	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	547	3		n.g.	100
	0	female	547	0		n.g.	100
	0	male	772	0		n.g.	100
	0	female	772	4		n.g.	100
	2.46	male	547	100		n.g.	3333.3
	2.46	female	547	100		n.g.	2500
	2.46	male	772	100		n.g.	3333.3
	2.46	female	772	100		n.g.	2500
	6.55	male	547	10		n.g.	333.33
	6.55	female	547	96		n.g.	
	6.55	male	772	100		n.g.	
	6.55	female	772	100		n.g.	2500
	additional	increase of incidences of enlarged alveolar macrophages (alveolar macrophage hyperplasia) for rats died or euthanized < d 547 or > d 547; at later tps macrophage aggregation in centriacinar region; n control, LD, HD: <547 d, m N=32, 44, 44, f N=23, 21, 27 ; >547d, m N=86, 71, 71, f N=91, 95, 87					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
burden		male & female	2.46	1.5744	☐		
mg/total lung	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	0		n.g.	100
	0	female	91	0		n.g.	100
	0	male	182	0		n.g.	100
	0	female	182	0		n.g.	100
	0	male	365	0		n.g.	100
	0	female	365	0		n.g.	100
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	700	0		n.g.	100
	0	female	700	0		n.g.	100
	2.46	male	91	1.7		n.g.	100
	2.46	female	91	1.7		n.g.	
	2.46	male	182	5.6		n.g.	
	2.46	female	182	3.3		n.g.	
	2.46	male	365	7.9		n.g.	
	2.46	female	365	6.2		n.g.	
	2.46	male	547	16		n.g.	
	2.46	female	547	12.1		n.g.	
	2.46	male	700	24.7		n.g.	
	2.46	female	700	17.3		n.g.	
	6.55	male	91	5.9		n.g.	347.05
	6.55	female	91	4.9		n.g.	
	6.55	male	182	13.7		n.g.	
	6.55	female	182	11		n.g.	
	6.55	male	365	15.1		n.g.	
	6.55	female	365	12.2		n.g.	
	6.55	male	547	29.9		n.g.	
	6.55	female	547	22.7		n.g.	
	6.55	male	700	40.1		n.g.	
	6.55	female	700	36.9		n.g.	
	additional	dose & time-dependent increase of total lung burden (no lavage), progressive accumulation accelera after 365d control values assumed, tab.3)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
metaplasia		male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	780	0		n.g.	100
	0	female	780	1		n.g.	100
	2.46	male	547	2		n.g.	
	2.46	female	547	29		n.g.	
	2.46	male	780	15		n.g.	
	2.46	female	780	66		n.g.	6600
	6.55	male	547	25		n.g.	
	6.55	female	547	52		n.g.	
	6.55	male	780	66		n.g.	
	6.55	female	780	97		n.g.	9700
	additional	increase of incidences of bronchiolar-alveolar metaplasia, dose- and time-dependent increase of severity scores >= 365d for rats died or euthanized < d 547 or > d 547, f more sensitive with higher incidences and severity scores; n of control, LD, HD: <547d, m N=32, 44, 44, f N=23, 21, 27 ; <780d N=86, 71, 71, f N=91, 95, 87 (tab.5,6)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
metaplasia		male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	3		n.g.	100
	2.46	male & f	365	0		n.g.	
	2.46	male & f	547	50		n.g.	
	2.46	male & f	700	83		n.g.	
	2.46	male & f	772	94		n.g.	3133.3
	6.55	male & f	365	33		n.g.	
	6.55	male & f	547	100		n.g.	
	6.55	male & f	700	100		n.g.	
	6.55	male & f	772	100		n.g.	3333.3
	additional	dose- and time-dependent increase of incidences of bronchiolar-alveolar metaplasia (% affected rat from d 365 at HD % d 547 at LD, not reversible, for grading see bronchiolar-alveolar metaplasia: grading; serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
alveolar clearance		male & female	2.46	1.5744	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	217	79		n.g.	100
	0	male & f	673	69		n.g.	100
	2.46	male & f	217	41		n.g.	51.89
	2.46	male & f	673	18		n.g.	26.08
	6.55	male & f	217	24		n.g.	30.37
	6.55	male & f	673	14		n.g.	20.28
	additional	decrease of alveolar clearance rate indicated from decreased clearance of radiolabeled tracer partic ([7Be]CB) on d 126 each after inhalative uptake on d 91 or d 547 = tps of d 217 & d 673 (100% - %retained, calculated from Tab.11)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
clearance		male & female	2.46	1.5744	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	91		no change	n.a.	
	0	male & f	182		no change	n.a.	
	0	male & f	365		no change	n.a.	
	0	male & f	547		no change	n.a.	
	0	male & f	772		no change	n.a.	
	2.46	male & f	91			n.a.	
	2.46	male & f	182			n.a.	
	2.46	male & f	365			n.a.	
	2.46	male & f	547			n.a.	
	2.46	male & f	772			n.a.	
	6.55	male & f	91			n.a.	
	6.55	male & f	182			n.a.	
	6.55	male & f	365			n.a.	
	6.55	male & f	547			n.a.	
	6.55	male & f	772			n.a.	
	additional	particle-laden macrophages seen at all tps, however, at later tps free particles increased due to cellu damage of macrophages					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
macrophage damage		male & female	2.46	1.5744	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	547		no change	n.a.	
	0	male & f	772		no change	n.a.	
	2.46	male & f	547			n.a.	
	2.46	male & f	772			n.a.	
	6.55	male & f	547			n.a.	
	6.55	male & f	772			n.a.	
	additional	increase of cellular debris from macrophages at >= 547 d					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient
macrophages interstitial		male & female	2.46	1.5744	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	91		no change	n.a.	
	0	male & f	182		no change	n.a.	
	0	male & f	365		no change	n.a.	
	0	male & f	547		no change	n.a.	
	0	male & f	772		no change	n.a.	
	2.46	male & f	365			n.a.	
	2.46	male & f	547			n.a.	
	2.46	male & f	772			n.a.	
	6.55	male & f	91			n.a.	
	6.55	male & f	182			n.a.	
	6.55	male & f	365			n.a.	
	6.55	male & f	547			n.a.	
	6.55	male & f	772			n.a.	
	additional	few particle-containing interstitial macrophages at d 91 at HD, marked increase at >=d 365 at both d including both single and aggregated macrophages					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
foci		male & female	2.46	1.5744	☐		
n.a.	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	547		no change	n.a.	
	2.46	male & f	547			n.a.	
	6.55	male & f	547			n.a.	
	additional	foci with increased amount of epithelial hyperplasia including some foci with papillary projections of hyperplasitc epithelium or multilayering of hyperplastic epithelial cells at d 547					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
PAH-derived DNA adducts		male & female	6.55	4.192	☐		
adducts/10E9 bas	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	91	6.1		n.g.	100
	6.55	male & f	91	20.1		<0.05	329.5
	additional	sign. increase of PAH-derived DNA adducts in alveolar type II cells at HD at d 91 (no data for LD or other tp)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
squamous cysts		male & female	2.46	1.5744	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	700	0		n.g.	100
	0	male & f	772	0		n.g.	100
	2.46	male & f	700	0		n.g.	
	2.46	male & f	772	28		n.g.	
	6.55	male & f	700	33		n.g.	
	6.55	male & f	772	42		n.g.	
	additional	increase of incidences of squamous cysts (% affected rats) from d 700 at HD & at d 772 at LD, not reversible; serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
squamous metaplasia		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	772	0		n.g.	100
	2.46	male & f	772	0.11		n.g.	
	6.55	male & f	772	0.75		n.g.	
	additional	increase of grading (% affected lung) of alveolar squamous metaplasia at d 772 (for incidences see: squamous metaplasia: %), grading scale (% of affected lung): 1 (<10%), 2 (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
macrophage infiltration		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	91	0		n.g.	100
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0.06		n.g.	100
	2.46	male & f	91	1		n.g.	
	2.46	male & f	182	1.33		n.g.	
	2.46	male & f	365	2		n.g.	
	2.46	male & f	547	2.33		n.g.	
	2.46	male & f	700	3		n.g.	
	2.46	male & f	772	2.69		n.g.	4483.3
	6.55	male & f	91	2		n.g.	
	6.55	male & f	182	2.17		n.g.	
	6.55	male & f	365	3		n.g.	
	6.55	male & f	547	3		n.g.	
	6.55	male & f	700	3.5		n.g.	
	6.55	male & f	772	3.42		n.g.	5700
	additional	dose- and time-dependent increase of grading of enlarged alveolar macrophages (alveolar macroph hyperplasia), for incidences see macrophage infiltration: %; serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
metaplasia		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0.06		n.g.	100
	2.46	male & f	365	0		n.g.	
	2.46	male & f	547	1		n.g.	
	2.46	male & f	700	1.33		n.g.	
	2.46	male & f	772	2		n.g.	3333.3
	6.55	male & f	365	0.5		n.g.	
	6.55	male & f	547	1.83		n.g.	
	6.55	male & f	700	2.5		n.g.	
	6.55	male & f	772	3.08		n.g.	5133.3
	additional	dose- and time-dependent increase of grading (% affected lung) of bronchiolar-alveolar metaplasia f d 365 at HD & d 547 at LD, not reversible (for incidences see: bronchiolar-alveolar metaplasia: %), grading scale (% of affected lung): 1 (<10%), 2 (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7 F9)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
hyperplasia alveolar type II cells	male & female	2.46	1.5744	☐

grading	dose	sex	timepoint	level	score	significance	%
	0	male & f	91	0		n.g.	100
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0.18		n.g.	100
	2.46	male & f	182	1.33		n.g.	
	2.46	male & f	365	2		n.g.	
	2.46	male & f	547	2.17		n.g.	
	2.46	male & f	700	3		n.g.	
	2.46	male & f	772	3.06		n.g.	1700
	6.55	male & f	91	2		n.g.	
	6.55	male & f	182	2		n.g.	
	6.55	male & f	365	2.83		n.g.	
	6.55	male & f	547	2.83		n.g.	
	6.55	male & f	700	3.17		n.g.	
	6.55	male & f	772	3.67		n.g.	2038.9
	additional	dose- and time-dependent increase of grading (% affected lung) of alveolar epithelial hyperplasia; no reversible during 42 d p-e (for incidences see: hyperplasia: %), grading scale (% of affected lung): 1 (<10%), 2 (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect	sex	LOEL study unit	LOEL mg/kg	tran-sient
alveolar proteinosis	male & female	2.46	1.5744	☐

grading	dose	sex	timepoint	level	score	significance	%
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0		n.g.	100
	2.46	male & f	365	0.17		n.g.	
	2.46	male & f	547	0.33		n.g.	
	2.46	male & f	700	0.33		n.g.	
	2.46	male & f	772	0.78		n.g.	
	6.55	male & f	365	1.33		n.g.	
	6.55	male & f	547	1.33		n.g.	
	6.55	male & f	700	1.83		n.g.	
	6.55	male & f	772	3.5		n.g.	
	additional	dose- and time-dependent increase of grading (% affected lung) of alveolar proteinosis from d 365, reversible (for incidences see: alveolar proteinosis: %), grading scale (% of affected lung): 1 (<10%) (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
alveolar proteinosis		male & female	2.46	1.5744	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0		n.g.	100
	2.46	male & f	365	17		n.g.	
	2.46	male & f	547	33		n.g.	
	2.46	male & f	700	33		n.g.	
	2.46	male & f	772	56		n.g.	
	6.55	male & f	365	100		n.g.	
	6.55	male & f	547	67		n.g.	
	6.55	male & f	700	67		n.g.	
	6.55	male & f	772	100		n.g.	
	additional	dose- and time-dependent increase of incidences of alveolar proteinosis (% affected rats) from d 36 not reversible, for grading see alveolar proteinosis: grading; serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
fibrosis		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	700	0		n.g.	100
	0	male & f	772	0		n.g.	100
	2.46	male & f	700	0		n.g.	
	2.46	male & f	772	0.5		n.g.	
	6.55	male & f	700	1.33		n.g.	
	6.55	male & f	772	0.75		n.g.	
	additional	increase of grading (% affected lung) of focal fibrosis from d 700 at HD & at d 772 at LD, partly reve at HD (for incidences see: fibrosis: %), grading scale (% of affected lung): 1 (<10%), 2 (10-25%), 3 (50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
fibrosis		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0.03		n.g.	100
	2.46	male & f	182	0		n.g.	
	2.46	male & f	365	0.33		n.g.	
	2.46	male & f	547	2		n.g.	
	2.46	male & f	700	2.83		n.g.	
	2.46	male & f	772	2.67		n.g.	8900
	6.55	male & f	182	0.33		n.g.	
	6.55	male & f	365	1.17		n.g.	
	6.55	male & f	547	2.5		n.g.	
	6.55	male & f	700	3		n.g.	
	6.55	male & f	772	3		n.g.	10000
	additional	dose- and time-dependent increase of grading (% affected lung) of alveolar septal fibrosis; from d 18 HD & d 365 at LD, not reversible (for incidences see: fibrosis: %), grading scale (% of affected lung) (<10%), 2 (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
weight		male & female	2.46	1.5744	☐		
gram	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	1.32		n.g.	100
	0	female	91	0.94		n.g.	100
	0	male	182	1.4		n.g.	100
	0	female	182	1.03		n.g.	100
	0	male	365	1.58		n.g.	100
	0	female	365	1.09		n.g.	100
	0	male	547	1.77		n.g.	100
	0	female	547	1.45		n.g.	100
	0	male	700	1.99		n.g.	100
	0	female	700	1.25		n.g.	100
	2.46	male	91	1.35		none	102.27
	2.46	female	91	1.06		none	112.76
	2.46	male	182	1.71		none	122.14
	2.46	female	182	1.17		<0.05	113.59
	2.46	male	365	2.17		<0.05	137.34
	2.46	female	365	1.59		<0.05	145.87
	2.46	male	547	2.69		none	151.97
	2.46	female	547	2.01		none	138.62
	2.46	male	700	3.12		none	156.78
	2.46	female	700	2.42		<0.05	193.6
	6.55	male	91	1.56		none	118.18
	6.55	female	91	1.21		<0.05	128.72
	6.55	male	182	2.12		none	151.42
	6.55	female	182	1.68		<0.05	163.1
	6.55	male	365	3.31		<0.05	209.49
	6.55	female	365	2.56		<0.05	234.86
	6.55	male	547	3.5		<0.05	197.74
	6.55	female	547	3.71		<0.05	255.86
	6.55	male	700	4.48		none	225.12
	6.55	female	700	4.95		<0.05	396
	additional	dose- & time-dependent increase of lung wt, sign. in f HD, f more sensitive: sign. effect seen earlier and at more tp at LD (tab.4)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
macrophage infiltration		male & female	2.46	1.5744	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male & f	91	0		n.g.	100
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	6		n.g.	100
	2.46	male & f	91	100		n.g.	
	2.46	male & f	182	100		n.g.	
	2.46	male & f	365	100		n.g.	
	2.46	male & f	547	100		n.g.	
	2.46	male & f	700	100		n.g.	
	2.46	male & f	772	100		n.g.	1666.7
	6.55	male & f	91	100		n.g.	
	6.55	male & f	182	100		n.g.	
	6.55	male & f	365	100		n.g.	
	6.55	male & f	547	100		n.g.	
	6.55	male & f	700	100		n.g.	
	6.55	male & f	772	100		n.g.	1666.7
	additional	increase of incidences of enlarged alveolar macrophages (alveolar macrophage hyperplasia), all rat affected from d 91-772; for grading see macrophage infiltration: grading; serial sacrifices: N=3/sex a group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
fibrosis		male & female	2.46	1.5744	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	3		n.g.	100
	2.46	male & f	182	0		n.g.	
	2.46	male & f	365	33		n.g.	
	2.46	male & f	547	100		n.g.	
	2.46	male & f	700	100		n.g.	
	2.46	male & f	772	100		n.g.	3333.3
	6.55	male & f	182	33		n.g.	
	6.55	male & f	365	67		n.g.	
	6.55	male & f	547	100		n.g.	
	6.55	male & f	700	100		n.g.	
	6.55	male & f	772	100		n.g.	3333.3
	additional	increase of incidences of alveolar septal fibrosis (% affected rats), from d 182 at HD & d 365 at LD, rats affected at >= d 547, not reversible, for grading see fibrosis: grading; serial sacrifices: N=3/sex group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
inflammation		male & female	2.46	1.5744	☐		
grading	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	0.09		n.g.	100
	2.46	male & f	182	0.17		n.g.	
	2.46	male & f	365	0.17		n.g.	
	2.46	male & f	547	1.17		n.g.	
	2.46	male & f	700	0.67		n.g.	
	2.46	male & f	772	1		n.g.	1111.1
	6.55	male & f	182	0		n.g.	
	6.55	male & f	365	0.17		n.g.	
	6.55	male & f	547	2		n.g.	
	6.55	male & f	700	1.83		n.g.	
	6.55	male & f	772	1.75		n.g.	1944.4
	additional	dose- and time-dependent increase of grading (% affected lung) of chronic active inflammation; max d 547, partially reversible thereafter (for incidences see: inflammation: %), grading scale (% of affec lung): 1 (<10%), 2 (10-25%), 3 (25-50%), 4 (>50%); serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
inflammation		male & female	2.46	1.5744	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	182	0		n.g.	100
	0	male & f	365	0		n.g.	100
	0	male & f	547	0		n.g.	100
	0	male & f	700	0		n.g.	100
	0	male & f	772	9		n.g.	100
	2.46	male & f	182	17		n.g.	
	2.46	male & f	365	17		n.g.	
	2.46	male & f	547	100		n.g.	
	2.46	male & f	700	100		n.g.	
	2.46	male & f	772	72		n.g.	800
	6.55	male & f	182	0		n.g.	
	6.55	male & f	365	17		n.g.	
	6.55	male & f	547	100		n.g.	
	6.55	male & f	700	100		n.g.	
	6.55	male & f	772	100		n.g.	1111.1
	additional	increase of incidences of chronic active inflammation (% affected rats), single rats on d 182 & 365, a rats affected at >= d 547, partially reversible at LD after 42 d p-e, for grading see inflammation: grad serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, HD: N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
squamous metaplasia		male & female	2.46	1.5744	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	772	0		n.g.	100
	2.46	male & f	772	6		n.g.	
	6.55	male & f	772	33		n.g.	
	additional	increase of incidences of alveolar squamous metaplasia (% affected rats) at d 772, for grading see squamous metaplasia: grading; serial sacrifices: N=3/sex and group; terminal sacrifice control, LD, H N=34, 18, 12 (Mauderley: Tab.F1, F3, F5, F7, F8, F9)					

lymph node

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
burden		male & female	2.46	1.5744	☐		
mg/organ	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	0		n.g.	100
	0	female	91	0		n.g.	100
	0	male	182	0		n.g.	100
	0	female	182	0		n.g.	100
	0	male	365	0		n.g.	100
	0	female	365	0		n.g.	100
	0	male	547	0		n.g.	100
	0	female	547	0		n.g.	100
	0	male	700	0		n.g.	100
	0	female	700	0		n.g.	100
	2.46	male	91	0.026		n.g.	
	2.46	female	91	0.004		n.g.	
	2.46	male	182	0.37		n.g.	
	2.46	female	182	0.233		n.g.	
	2.46	male	365	0.949		n.g.	
	2.46	female	365	0.763		n.g.	
	2.46	male	547	2.461		n.g.	
	2.46	female	547	1.478		n.g.	
	2.46	male	700	3.124		n.g.	
	2.46	female	700	1.782		n.g.	
	6.55	male	91	0.188		n.g.	
	6.55	female	91	0.173		n.g.	
	6.55	male	182	0.978		n.g.	
	6.55	female	182	0.766		n.g.	
	6.55	male	365	2.934		n.g.	
	6.55	female	365	1.471		n.g.	
	6.55	male	547	4.318		n.g.	
	6.55	female	547	1.997		n.g.	
	6.55	male	700	4.448		n.g.	
	6.55	female	700	2.076		n.g.	
	additional	time-related increase of LALN burden, m > f, control values assumed (Mauderly: Tab.D1-D5)					

Titanium dioxide

molecular weight 79.9 **g / mol**

study pk 4099 **Study Data**

Specification by Producer / Supplier

object Primary particle

synonym TiO2 P25

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean	21			
SD				
min	15			
max	40			

medium **determ. method**

distribution type no data **surface property** hydrophilic

cristal structure Anatase **shape**

specific surface in m²/g **specific volume in m³/g**

solubility in mg/l **at in °C**

particle density in g/cm³ 3.8

additional hydrophil pyrogen

reference MSDS Aug.2011, Degussa A.G., Frankfurt am Main, Germany

producer Degussa A.G., Frankfurt am Main, Germany

Specification by Authors

object Primary particle

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean				
SD				
min				
max				

medium **determ. method**

distribution type no data **surface property**

cristal structure ~80 % anatase and ~20 % rutile **shape**

specific surface in m²/g 48 **specific volume in m³/g**

solubility in mg/l **at in °C**

particle density in g/cm³

additional Titanium dioxide P25, Degussa, Germany; coarse particles removed by a cyclone (shift of MMAD from 1500 nm to 800 nm)

reference Heinrich et al. (1995)

Hydrodynamic diameter

median 800 **nm** **distibution type** no data

GSD 1.8 **bulk density** **mg/ml**

min		**nm**	**isoelectric point**		**in**		
max		**nm**	**zeta potential**	**mV**	**in**		
medium			**peak SPR**	**nm**	**in**		
determ. method	10-stage Berner impactor		**conductivity**	**µS/cm**	**in**		
sample treatment			**solubility**	**mg/L**	**in**	**at**	**ºC**
applic. medium							
dispersant							
additional							

Study Design

species	rat	**strain**	Wistar (Crl:(WI) BR)
sex	female	**animal/group**	100
route	whole-body	**age of animal**	7 w
purity			
exposure in h/d	18	**exposure in d/w**	5
study dur. in d	730	**postexp. dur. in d**	180
no. of instillation		**frequency**	

exposure (additional)
dose / concentration

whole-body in 6 or 12 m3 horizontal flow type chambers; nominal concentration of 10 mg/m3 as time-weighted average of 7,2 mg/m³ for 4 months + 14,8 mg/m³ for 9,5 months + 9,4 mg/m³ for 5,5 months
cumulative exposure 88,1 g/m³ x h

0
10

Unit
mg/m³

1 dose study ☑
reliability B
confidential ☐

additional

interim sacrifices at d 91, 182, 365, 547, 670, 730 and 910 d
number of animals (control/exposed):
carcinogenicity: 220/100, additionally
histology (serial sacrifice): 80/80
DNA-adducts: 14/14
lung burden: 66/66
lung clearance: 28/28, measured as clearance of radiolabeled Fe2O3 tracer particles (MMAD 0,35 µm)
Ti burden via AAS after ashing of tissue

Reference

author	Heinrich U, and Fuhst, R	**source**	Abschlussbericht: Vergleichend Untersuchungen zur Frage der tumorinduzierenden Wirkung von Dieselmotorabgasen in der Rattenlunge. Final Report in German		
volume	07VAG06	**year**	1992	**page**	1-56 plus Append
institution	Fraunhofer ITEM				
author	Muhle H, et al.	**source**	In: Mohr U, et al. (Ed.) Toxic and carcinogenic effects of solid particles in the respiratory tract		
volume		**year**	1994	**page**	29-41
institution	Fraunhofer ITEM				
author	Creutzenberg O, et al.	**source**	J Aerosol Science		
volume	21 Suppl 1	**year**	1990	**page**	S455-S458
institution	Fraunhofer ITEM				

Titanium dioxide

author	Heinrich U, et al.	**source**	Inhalation Toxicology		
volume	7	**year**	1995	**page**	533-556
institution	Fraunhofer ITEM				

Scope

organ	animal/group	necropsy	organ weight	histopathology	
guideline					
lung	10	☐	☐	☑	
additional	total lung burden (no lavage), alveolar lung clearance of radiolabeled tracer particles; histopatho: H&E stain				
trachea	10	☐	☐	☑	
larynx	10	☐	☐	☐	
nose	10	☐	☐	☑	
additional	nasal and paranasal cavities				
body weight	100	☐	☐	☐	
BALF	10	☐	☐	☐	
	lymphocytes	granulocytes		alveolar macrophages	
	hydroxyproline	total protein		β-glucuronidase	
	lactate dehydrogenase (LDH)				
additional	both lobes, 5x4ml				
mortality	100	☐	☐	☐	
lymph node	10	☐	☐	☐	
additional	burden in LALN				

Effect data

BALF

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
alveolar macrophages total		female	10	7.2	☐		
x10E6	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	670	0.29		n.g.	100
	0	female	730	0.95		n.g.	100
	10	female	670	1.52		n.g.	524.13
	10	female	730	1.02		n.g.	107.36
	additional	increase of number of AM at 22 and 24 mo of exposure, effect slightly lower than after 18 mo expos 6 mo p-e (addtional BALF data in Study Number 4002)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
hydroxyproline		female	10	7.2	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	730	100		n.g.	100
	10	female	730	260		<0.01	260
	additional	sign. increase of free hydroxyproline at termination of 24 mo exposure, effect higher than after 18 m exposure + 6 mo p-e (addtional BALF data in Study Number 4002)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
total protein		female	10	7.2	☐		
	dose	sex	timepoint	level	score	significance	%
	0	female	730	100		n.g.	100
	10	female	730	890		<0.01	890
	additional	sign. increase of total protein at termination of 24 mo exposure, effect lower than after 18 mo exposu 6 mo p-e (addtional BALF data in Study Number 4002)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
β-glucuronidase		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	730	100		n.g.	100
	10	female	730	6750		<0.01	6750
	additional	sign. increase of β-glucuronidase activity at termination of 24 mo exposure, effect lower than after 1 exposure + 6 mo p-e (addtional BALF data in Study Number 4002)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
lactate dehydrogenase (LDH)		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	730	100		n.g.	100
	10	female	730	1300		<0.01	1300
	additional	sign. increase of LDH activity at termination of 24 mo exposure, effect lower than after 18 mo expos 6 mo p-e (addtional BALF data in Study Number 4002)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
granulocytes		female	10	7.2	☐		
x10E6	dose	sex	timepoint	level	score	significance	%
	0	female	670	0		n.g.	100
	0	female	730	0		n.g.	100
	10	female	670	1.42		n.g.	
	10	female	730	2.67		n.g.	
	additional	increase of number of granulocytes (10E6/ml) at 22 and 24 mo of exposure, effect higher than after mo exposure + 6 mo p-e (addtional BALF data in Study Number 4002)					

body weight

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight decreased		female	10	7.2	☐		
gram	dose	sex	timepoint	level	score	significance	%
	0	female	730	417		n.g.	100
	10	female	730	365		<0.05	87.52
	additional	sign. decrease of bw from d 400 until termination of exposure					

clinical symptoms

effect		sex	LOEL study unit	LOEL mg/kg	transient		
mortality		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	730	42		n.g.	100
	0	female	910	85		n.g.	100
	10	female	730	60		<0.05	142.85
	10	female	910	90		<0.05	105.88
	additional	sign. decease of mean lifetime; mortality increased at termination of exposure an after 6 mo p-e					

lung

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight		female	10	7.2	☐		
gram	dose	sex	timepoint	level	score	significance	%
	0	female	91	1.28		n.g.	100
	0	female	182	1.55		n.g.	100
	0	female	365	1.33		n.g.	100
	0	female	547	1.54		n.g.	100
	0	female	670	1.34		n.g.	100
	0	female	730	1.44		n.g.	100
	10	female	91	1.93		<0.001	150.78
	10	female	182	2.96		<0.001	190.96
	10	female	365	4.48		<0.001	336.84
	10	female	547	6.16		<0.001	400
	10	female	670	5.72		<0.001	426.86
	10	female	730	5.29		<0.001	367.36
	additional	sign. increase of lung burden from 3 mo throughout 24 mo of exposure, maximum level at 18 mo exposure, partly reversible up to 24 mo exposure (no data for p-e)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
burden		female	10	7.2	☐		
mg/organ	dose	sex	timepoint	level	score	significance	%
	0	female	91	0		n.g.	100
	0	female	182	0		n.g.	100
	0	female	365	0		n.g.	100
	0	female	547	0		n.g.	100
	0	female	670	0		n.g.	100
	0	female	730	0		n.g.	100
	10	female	91	5.2		n.g.	
	10	female	182	23.2		n.g.	
	10	female	365	34.8		n.g.	
	10	female	547	40		n.g.	
	10	female	670	37.7		n.g.	
	10	female	730	39.3		n.g.	
	additional	increase of total lung burden (no lavage) from 3 mo throughout 24 mo exposure, maximum level at 1 mo exposure, practically not reversible up to 24 mo exposure (no data for p-e); clearance half-time d; further data for 18 mo exposure + p-e in Study Number 4002					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
alveolar clearance		female	10	7.2	☐		
days	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	91	61		n.g.	100
	0	female	365	72		n.g.	100
	0	female	547	96		n.g.	100
	10	female	91	208		<0.01	340.98
	10	female	365	403		<0.01	559.72
	10	female	547	357		<0.01	371.87
	additional	sign. increase of alveolar clearance half- time of radiolabeled tracer particles, maximum at 12 mo exposure (no data for p-e); further data for 18 mo exposure + p-e in Study Number 4002					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
hyperplasia bronchiolo-alveolar		female	10	7.2	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	182	0		n.g.	100
	0	female	910	0		n.g.	100
	10	female	182	100	medium	n.g.	
	10	female	910	99	severe	n.g.	
	additional	increased incidences of bronchiolo-alveolar hyperplasia in all rats of interim sacrifices, and in 99/100 at 24 mo exposure + 6 mo p-e with increase in severity					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
fibrosis		female	10	7.2	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	182	0		n.g.	100
	0	female	265	10	minimal	n.g.	
	0	female	547	5.6	minimal	n.g.	
	0	female	730	0		n.g.	100
	0	female	910	4.1	minimal	n.g.	
	0	female	910	4.1	minimal	n.g.	
	10	female	182	100	minimal	n.g.	
	10	female	365	15	medium	n.g.	
	10	female	365	75	mild	n.g.	
	10	female	547	15	severe	n.g.	
	10	female	547	75	medium	n.g.	
	10	female	547	10	mild	n.g.	
	10	female	730	100	medium	n.g.	
	10	female	910	60	medium	n.g.	
	10	female	910	36	mild	n.g.	
	additional	sign. increase of incidences and severity of interstitial fibrosis after 6 mo throughout 24 mo exposure mo p-e, effect partly reversible during p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
bronchiolo-alveolar adenoma		female	10	7.2	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	female	910	0		n.g.	100
	10	female	910	4		none	
	additional	increased incidence of bronchiolo-alveolar adenoma at 24 mo exposure + 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
bronchiolo-alveolar adenocarcinoma		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	547	0		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	0.46		n.g.	100
	10	female	547	10		none	
	10	female	730	11		none	
	10	female	910	13		<0.05	2826.1
	additional	increased incidences of bronchiolo-alveolar adenocarcinoma at 18 mo and 24 of exposure (2/20 and 1/9) throughout 6 mo p-e (13/100), sign. at termination of study					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
macrophage infiltration		female	10	7.2	☐		
n.a.	dose	sex	timepoint	level	score	significance	%
	0	female	91		no change	n.a.	
	0	female	182		no change	n.a.	
	0	female	365		no change	n.a.	
	0	female	547		no change	n.a.	
	0	female	670		no change	n.a.	
	0	female	730		no change	n.a.	
	10	female	91			n.a.	
	10	female	182			n.a.	
	10	female	365			n.a.	
	10	female	547			n.a.	
	10	female	670			n.a.	
	10	female	730			n.a.	
	additional	accumulation of particle-laden macrophages					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
squamous cell carcinoma		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	547	0		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	0		n.g.	100
	10	female	547	15		none	
	10	female	730	22		none	
	10	female	910	3		none	
	additional	increased incidences of squamous cell carcinoma at 18 mo and 24 of exposure (3/20 and 2/9), lowe incidence at 6 mo p-e (3/100)					

effect	sex	LOEL study unit	LOEL mg/kg	transient
benign cystic keratinizing squamous-cell tumor	female	10	7.2	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	547	0		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	0		n.g.	100
	10	female	547	10		none	
	10	female	730	22		none	
	10	female	910	20		<0.05	
	additional	increased incidences of benign cystic keratinizing squamous-cell tumours at 18 mo and 24 of expos (2/20 and2/9) throughout 6 mo p-e (20/100), sign. at termination of study					

lymph node

effect	sex	LOEL study unit	LOEL mg/kg	transient
burden	female	10	7.2	☐

mg/organ	dose	sex	timepoint	level	score	significance	%
	0	female	670	0		n.g.	100
	10	female	670	5.75		n.g.	
	additional	burden in LALN					

nose

effect	sex	LOEL study unit	LOEL mg/kg	transient
atrophy	female	10	7.2	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	730	0		n.g.	100
	10	female	730	22.2		n.g.	
	additional	increased incidence of atrophy of respiratory epithelium					

effect	sex	LOEL study unit	LOEL mg/kg	transient
squamous cell metaplasia	female	10	7.2	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	365	0		n.g.	100
	0	female	547	0		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	7.7		n.g.	100
	10	female	365	5		n.g.	
	10	female	547	35		n.g.	
	10	female	730	77.8		n.g.	
	10	female	910	56		n.g.	727.27
	additional	sign. increase of incidences of squamous cell metaplasia in mucus membranes of nasal cavity and paranasal sinuses at >= 12 mo exposure, effect partly reversible after 24 mo exposure and 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
inflammation		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	365	4.8		n.g.	100
	0	female	547	0		n.g.	100
	0	female	730	10		n.g.	100
	0	female	910	5.9		n.g.	100
	10	female	365	10		n.g.	208.33
	10	female	547	35		n.g.	
	10	female	730	44.4		n.g.	444
	10	female	910	30		n.g.	508.47
	additional	sign. increase of incidences of inflammative changes in mucus membranes of nasal cavity and paranasal sinuses at >= 12 mo exposure, effect partly reversible after 24 mo exposure and 6 mo p-e					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
degeneration		female	10	7.2	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	female	182	0		n.g.	100
	0	female	365	9.5		n.g.	100
	0	female	547	33.3		n.g.	100
	0	female	730	0		n.g.	100
	0	female	910	34.4		n.g.	100
	10	female	182	30		n.g.	
	10	female	365	85		n.g.	894.73
	10	female	547	95		n.g.	285.28
	10	female	730	100		n.g.	
	10	female	910	59		n.g.	171.51
	additional	sign. increase of incidences of degenerative changes in mucus membranes of nasal cavity and paranasal sinuses at >= 6 mo exposure, effect partly reversible after 24 mo exposure and 6 mo p-e					

Titanium dioxide

molecular weight 79.9 **g / mol**

study pk 4116 **Study Data**

Specification by Producer / Supplier

object Primary particle

synonym TiO2 particle grade

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean				
SD				
min				
max				

medium

distribution type no data

cristal structure rutile

specific surface in m²/g

solubility in mg/l

particle density in g/cm³

determ. method

surface property

shape spherical

specific volume in m³/g

at in °C

additional Titanium dioxide particle grade

reference Lee et al., 1985, 1986

producer du Pont Co., Inc.

Specification by Authors

object Primary particle

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean	250			
SD				
min				
max				

medium

distribution type no data

cristal structure

specific surface in m²/g

solubility in mg/l

particle density in g/cm³

determ. method assumed

surface property

shape spherical

specific volume in m³/g

at in °C

additional refer to pigment-TiO2 --> usually 200-300 nm

reference

Hydrodynamic diameter

median 1600 **nm**

GSD

min **nm**

distibution type no data

bulk density **mg/ml**

isoelectric point **in**

max	**nm**	**zeta potential**	**mV**	**in**			
medium		**peak SPR**	**nm**	**in**			
determ. method	no data	**conductivity**	**µS/cm**	**in**			
sample treatment		**solubility**	**mg/L**	**in**	**at**	**°C**	
applic. medium							
dispersant							
additional	Ranges of MMAD 1500, 1700 and 1600 nm at LD, MD and HD with respirable fractions (< 13000 nm MMAD) of 78, 89 and 84%, respectively						

Study Design

species	rat	**strain**	CD
sex	male & female	**animal/group**	71-79
route	whole-body	**age of animal**	5 w
purity	= 0.99		
exposure in h/d	6	**exposure in d/w**	5
study dur. in d	730	**postexp. dur. in d**	0
no. of instillation		**frequency**	

exposure (additional) whole-body exposure chamber 3,85 qm, nominal conc. 0, 10, 50, and 250 mg/m3; analytical conc. 10,6+- 2,1; 50,3+-8,8; 250,1+- 24,7 mg/m3

dose / concentration	0 10.6 50.3 250.1	**1 dose study**	☐
		reliability	**B**
Unit		**confidential**	☐
mg/m³			

additional additional groups for interim sacrifices after 91 & 182 d (5/sex and group) and 365 d (10/sex and group) corresponding to timepoints of 91, 182, 365, 730 d
Ti burden in lung by ICPS after digestion of tissue; burden in other tissues as particle deposition by microscopy

Reference

author	Lee KP, et al.	**source**	Environ Research		
volume	41	**year**	1986	**page**	144-167
institution	Du Pont Company, Haskell Laboratory Toxicology & Industrial Medicine				

author	Lee KP, et al.	**source**	Exp Mol Pathol		
volume	42	**year**	1985	**page**	331-343
institution	Du Pont Company, Haskell Laboratory Toxicology & Industrial Medicine				

author	Lee KP, et al.	**source**	Toxicol Appl Pharmacol		
volume	79	**year**	1985	**page**	179-192
institution	Du Pont Company, Haskell Laboratory Toxicology & Industrial Medicine				

Scope

organ	**animal/group**	**necropsy**	**organ weight**	**histopathology**
guideline				
lung	75	☐	☑	☑
additional	total lung (no lavage); histopatho: H&E stain, PAS, trichrome and silver stain; light microscopy, TEM and SEM; Ti burden by ICPS			
trachea	75	☐	☐	☑

nose	75	☐	☐	☑
body weight	100	☐	☐	☐
lymph node	75	☐	☐	☑
additional	burden: dust deposition in tracheobronchial lymph nodes, cervical lymph nodes, and in mesenteric lymph nodes by light microscopy			
liver	75	☐	☐	☑
additional	burden: dust deposition by light microscopy			
spleen	75	☐	☐	☑
additional	burden: dust deposition by light microscopy			

Effect data

body weight

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
	additional	no effect			

liver

effect		sex	LOEL study unit	LOEL mg/kg	transient
burden		male & female	10.6	2.544	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	730	0		n.g.	100
	10.6	male & f	730	5.3		n.g.	
	50.3	male & f	730	20.4		n.g.	
	250.1	male & f	730	56.3		n.g.	
	additional	increased incidences of particle deposition (microspcopy) with particle accumulation in Kupffer cells macrophages (no tissue response or damage of hepatocytes)					

lung

effect		sex	LOEL study unit	LOEL mg/kg	transient
inflammation		male & female	10.6	2.544	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	1.3		n.g.	100
	0	female	730	1.3		n.g.	100
	10.6	male	730	10		n.g.	769.23
	10.6	female	730	15		n.g.	1153.8
	50.3	male	730	11		n.g.	846.15
	50.3	female	730	14		n.g.	1076.9
	250.1	male	730	9.1		n.g.	700
	250.1	female	730	7		n.g.	538.46
	additional	increased incidences (%) of broncho/bronchiolar pneumonia					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
weight		male & female	50.3	12.072	☐		
gram	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	2.4		n.g.	100
	0	female	91	1.9		n.g.	100
	0	male	182	2.5		n.g.	100
	0	female	182	1.8		n.g.	100
	0	male	365	2.6		n.g.	100
	0	female	365	2.2		n.g.	100
	0	male	730	3.25		n.g.	100
	0	female	730	2.35		n.g.	100
	10.6	male	91	2.3		none	95.83
	10.6	female	91	1.7		none	89.47
	10.6	male	182	2.5		none	100
	10.6	female	182	2.2		none	122.22
	10.6	male	365	2.7		none	103.84
	10.6	female	365	2.2		none	100
	10.6	male	730	3.56		none	109.53
	10.6	female	730	2.76		none	117.44
	50.3	male	91	2.4		none	100
	50.3	female	91	2		none	105.26
	50.3	male	182	3		<0.05	120
	50.3	female	182	2.6		<0.05	144.44
	50.3	male	365	3.5		<0.05	134.61
	50.3	female	365	3.3		<0.05	150
	50.3	male	730	4.47		<0.05	137.53
	50.3	female	730	3.1		<0.05	131.91
	250.1	male	91	3.6		n.g.	150
	250.1	female	91	2.8		n.g.	147.36
	250.1	male	182	4.4		<0.05	176
	250.1	female	182	4.3		<0.05	238.88
	250.1	male	365	6.3		<0.05	242.3
	250.1	female	365	5.7		<0.05	259.09
	250.1	male	730	7.84		<0.05	241.23
	250.1	female	730	7.21		<0.05	306.8
	additional	sign. dose and time-dependent increases of abs. and rel. wt.: at MD from 6-24 mo of exposure, at H marked increase at 3 mo, sign. from 6-24 mo					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
macrophages foamy		male & female	50.3	12.072	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	730	18		n.g.	100
	0	female	730	10		n.g.	100
	10.6	male	730	27		n.g.	150
	10.6	female	730	20		n.g.	200
	50.3	male	730	71		n.g.	394.44
	50.3	female	730	95		n.g.	950
	250.1	male	730	99		n.g.	550
	250.1	female	730	100		n.g.	1000
	additional	increased incidences (%) of aggregates of foamy alveolar macrophages with densely packed myelin figures after >= 12 mo at MD and after >= 6 mo at HD indicating particle overload (Tab.3)					

effect	sex	LOEL study unit	LOEL mg/kg	transient
hyperplasia alveolar type II cells	female	10.6	2.544	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	0		n.g.	100
	0	female	730	0		n.g.	100
	10.6	male	730	94		n.g.	
	10.6	female	730	96		n.g.	
	50.3	male	730	100		n.g.	
	50.3	female	730	100		n.g.	
	250.1	male	730	100		n.g.	
	250.1	female	730	100		n.g.	
	additional	increased incidences (%) of alveolar type II cell hyperplasia, minimal effect also seen after 6 and 12 of exposure at >= LD					

effect	sex	LOEL study unit	LOEL mg/kg	transient
alveolar bronchiolization	male & female	50.3	12.072	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	1.3		n.g.	100
	0	female	730	1.3		n.g.	100
	10.6	male	730	0		none	0
	10.6	female	730	4		n.g.	307.69
	50.3	male	730	32		n.g.	2461.5
	50.3	female	730	77		n.g.	5923.1
	250.1	male	730	82		n.g.	6307.7
	250.1	female	730	99		n.g.	7615.4
	additional	increased incidences (%) of alveolar bronchiolization					

effect	sex	LOEL study unit	LOEL mg/kg	transient
deposits	male & female	50.3	12.072	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	9		n.g.	100
	0	female	730	2.6		n.g.	100
	10.6	male	730	13		n.g.	144.44
	10.6	female	730	8		n.g.	307.69
	50.3	male	730	75		n.g.	833.33
	50.3	female	730	72		n.g.	2769.2
	250.1	male	730	97		n.g.	1077.8
	250.1	female	730	96		n.g.	3692.3
	additional	increased incidences of cholesterol granuloma and increased incidences of deposits of cholesterol, granular or fibronous materials, cellular debris					

effect		sex	LOEL study unit	LOEL mg/kg	transient
fibrosis		male & female	50.3	12.072	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	14		n.g.	100
	0	female	730	3.9		n.g.	100
	1.6	female	730	5		none	128.2
	10.6	male	730	10		none	71.42
	50.3	male	730	65		n.g.	464.28
	50.3	female	730	55		n.g.	1410.3
	250.1	male	730	99		n.g.	707.14
	250.1	female	730	99		n.g.	2538.5
	additional	increased incidences (%) of collagenized fibrosis, effect also seen at MD after 12 mo exposure, and HD after >= 3 mo exposure					

effect		sex	LOEL study unit	LOEL mg/kg	transient
macrophage damage		male & female	50.3	12.072	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	730		no change	n.a.	
	50.3	male & f	730			n.a.	
	250.1	male & f	730			n.a.	
	additional	degenerative and disintegrated foamy macrophages					

effect		sex	LOEL study unit	LOEL mg/kg	transient
alveolar proteinosis		male & female	50.3	12.072	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	0		n.g.	100
	0	female	730	0		n.g.	100
	10.6	male	730	0		none	
	10.6	female	730	0		none	
	50.3	male	730	51		n.g.	
	50.3	female	730	61		n.g.	
	250.1	male	730	97		n.g.	
	250.1	female	730	96		n.g.	
	additional	increased incidences (%) of alveolar proteinosis, effect also seen at MD after 12 mo exposure, and HD after >= 3 mo exposure					

effect		sex	LOEL study unit	LOEL mg/kg	transient
changes in organ structure		male & female	250.1	60.024	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	730		no change	n.a.	
	250.1	male & f	730			n.a.	
	additional	alveoli lined with ciliated columnar cells dilated and coalesced to form large multiple cystic spaces w mucinous secretions and dust cells					

effect	sex	LOEL study unit	LOEL mg/kg	transient
bronchiolo-alveolar adenoma	male & female	250.1	60.024	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	2.5		n.g.	100
	0	female	730	0		n.g.	100
	10.6	male	730	1.4		none	56
	10.6	female	730	0		n.g.	
	50.3	male	730	1.4		none	56
	50.3	female	730	0		n.g.	
	250.1	male	730	15.6		n.g.	624
	250.1	female	730	17.6		n.g.	
	additional	increased incidence (%) at HD					

effect	sex	LOEL study unit	LOEL mg/kg	transient
clearance	male & female	10.6	2.544	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	91		no change	n.a.	
	0	male & f	182		no change	n.a.	
	0	male & f	365		no change	n.a.	
	0	male & f	730		no change	n.a.	
	10.6	male & f	91			n.a.	
	10.6	male & f	182			n.a.	
	10.6	male & f	365			n.a.	
	10.6	male & f	730			n.a.	
	50.3	male & f	91			n.a.	
	50.3	male & f	182			n.a.	
	50.3	male & f	365			n.a.	
	50.3	male & f	730			n.a.	
	250.1	male & f	91			n.a.	
	250.1	male & f	182			n.a.	
	250.1	male & f	365			n.a.	
	250.1	male & f	730			n.a.	
	additional	particle-laden macrophages in alveoli seen at all doses after 3 mo of exposure, effect with dose- and time-response-relationship					

effect	sex	LOEL study unit	LOEL mg/kg	transient
collagen	male & female	50.3	12.072	☐

n.a.	dose	sex	timepoint	level	score	significance	%
	0	male & f	730		no change	n.a.	
	50.3	male & f	730			n.a.	
	250.1	male & f	730			n.a.	
	additional	minute collagen fiber deposition in alveolar walls at MD and HD, sign. collagen fiber deposition in cholesterol granulomas at HD at termination of study; effect not seen at LD					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
burden		male & female	10.6	2.544	☐		
mg/organ	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	0.001		n.g.	100
	0	female	91	0.001		n.g.	100
	0	male	182	0.001		n.g.	100
	0	female	182	0.001		n.g.	100
	0	male	365	0.001		n.g.	100
	0	female	365	0.001		n.g.	100
	0	male	730	0.001		n.g.	100
	0	female	730	0.001		n.g.	100
	10.1	female	365	8.7		n.g.	870000
	10.6	male	91	2.5		n.g.	250000
	10.6	female	91	2.8		n.g.	280000
	10.6	male	182	4.8		n.g.	480000
	10.6	female	182	4.4		n.g.	440000
	10.6	male	365	10.1		n.g.	1E+06
	10.6	male	730	20.7		n.g.	2E+06
	10.6	female	730	32.3		n.g.	3E+06
	50.3	male	91	21.7		n.g.	2E+06
	50.3	female	91	16.6		n.g.	2E+06
	50.3	male	182	57.3		n.g.	6E+06
	50.3	female	182	54		n.g.	5E+06
	50.3	male	365	75.6		n.g.	8E+06
	50.3	female	365	59.7		n.g.	6E+06
	50.3	male	730	118.3		n.g.	1E+07
	50.3	female	730	130		n.g.	1E+07
	150.1	female	365	381.5		n.g.	4E+07
	250.1	male	91	180.8		n.g.	2E+07
	250.1	female	91	136.8		n.g.	1E+07
	250.1	male	182	275.3		n.g.	3E+07
	250.1	female	182	238.6		n.g.	2E+07
	250.1	male	365	361.7		n.g.	4E+07
	250.1	male	730	784.8		n.g.	8E+07
	250.1	female	730	545.8		n.g.	5E+07
	additional	dose- and time-dependent increase of lung burden (mg/lung, detection limit < 0,002 mg/g dry lung); particle overload at HD based on impaired lung clearance after >= 12 mo exposure					

effect		sex	LOEL study unit	LOEL mg/kg	transient
tumor					☐
%					
	additional	no effect: single case of large cell anaplastic carcinoma in 1/71 LD m, effect not seen at higher dose in f; effect probably not treatment-dependent			

effect	sex	LOEL study unit	LOEL mg/kg	transient
squamous cell carcinoma	male & female	250.1	60.024	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	730	0		n.g.	100
	0	female	730	0		n.g.	100
	10.6	male	730	0		n.g.	
	10.6	female	730	1.3		n.g.	
	50.3	male	730	0		n.g.	
	50.3	female	730	0		n.g.	
	250.1	male	730	1.3		n.g.	
	250.1	female	730	17.6		n.g.	
	additional	increased incidence (%) in HD f (13/74), single cases in HD m (1/75) and LD f (1/75)					

lymph node

effect	sex	LOEL study unit	LOEL mg/kg	transient
burden	male & female	50.3	12.072	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	730	0		n.g.	100
	10.6	male & f	730	0		none	
	50.3	male & f	730	4.5		n.g.	
	250.1	male & f	730	61.2		n.g.	
	additional	Increased incidenceof particle deposition (microspcopy) in mesenteric lymph nodes with particle-lad cell accumulation: slight increase at MD and distinct effect at HD					

effect	sex	LOEL study unit	LOEL mg/kg	transient
burden	male & female	10	2.4	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	730	0		n.g.	100
	10.6	male & f	730	31.5		n.g.	
	50.3	male & f	730	78		n.g.	
	250.1	male & f	730	93.3		n.g.	
	additional	Increased incidence of particle deposition (microspcopy) in cervical lymph nodes with particle-laden accumulation					

effect	sex	LOEL study unit	LOEL mg/kg	transient
burden	male & female	10.6	2.544	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	730	0		n.g.	100
	10.6	male & f	730	87.2		n.g.	
	50.3	male & f	730	96.6		n.g.	
	250.1	male & f	730	100		n.g.	
	additional	Increased incidence of particle deposition (microspcopy) in tracheobronchial lymph nodes with partic laden cell accumulation					

nose

effect		sex	LOEL study unit	LOEL mg/kg	transient		
metaplasia		male & female	10.6	2.544	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male	730	10		n.g.	100
	0	female	730	9		n.g.	100
	10.6	male	730	37		n.g.	370
	10.6	female	730	19		n.g.	211.11
	50.3	male	730	27		n.g.	270
	50.3	female	730	28		n.g.	311.11
	250.1	male	730	58		n.g.	580
	250.1	female	730	55		n.g.	611.11
	additional	increased incidences of anterior squamous metaplasia (%); no increase of posterior squamous metaplasia					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
rhinitis		male & female	10.6	2.544	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male	730	32		n.g.	100
	0	female	730	24		n.g.	100
	10.6	male	730	80		n.g.	250
	10.6	female	730	49		n.g.	204.16
	50.3	male	730	66		n.g.	206.25
	50.3	female	730	46		n.g.	191.66
	250.1	male	730	92		n.g.	287.5
	250.1	female	730	86		n.g.	358.33
	additional	increased incidences of anterior rhinitis (%), no clear dose-response; incidences of posterior rhinitis were not increased in m, increases in f only at LD and HD					

pleura

effect		sex	LOEL study unit	LOEL mg/kg	transient		
inflammation		male & female	10.6	2.544	☐		
%	dose	sex	timepoint	level	score	significance	%
	0	male	730	5.1		n.g.	100
	0	female	730	2.6		n.g.	100
	10.6	male	730	10		n.g.	196.07
	10.6	female	730	9		n.g.	346.15
	50.3	male	730	37		n.g.	725.49
	50.3	female	730	35		n.g.	1346.2
	89	female	730	89		n.g.	3423.1
	250.1	male	730	71		n.g.	1392.2
	additional	increased incidences of pleuritis					

spleen

effect		sex	LOEL study unit	LOEL mg/kg	transient		
burden		male & female	10.6	2.544	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male & f	730	0		n.g.	100
	10.6	male & f	730	10.6		n.g.	
	50.3	male & f	730	19.3		n.g.	
	250.1	male & f	730	67.8		n.g.	
	additional	increased particle deposition (microspcopy) with particle accumulation mostly in white pulp with occasional aggregates of foamy macrophages (no cellular changes in spleen)					

trachea

effect		sex	LOEL study unit	LOEL mg/kg	transient		
tracheitis		male & female	10.6	2.544	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	730	3		n.g.	100
	0	female	730	1.3		n.g.	100
	10.6	male	730	76		n.g.	2533.3
	10.6	female	730	46		n.g.	3538.5
	50.3	male	730	72		n.g.	2400
	50.3	female	730	50		n.g.	3846.2
	250.1	male	730	79		n.g.	2633.3
	250.1	female	730	43		n.g.	3307.7
	additional	sign. increase of incidences (%) to similar levels in all dosed m or f, m more sensitive (higher incidences) than f					

Silver

molecular weight 107.9 **g / mol**

study pk4114 **Study Data**

Specification by Producer / Supplier

object Primary particle

synonym Silver nanoparticles (self-made)

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean	18.48			
SD	1.45			
min	6			
max	55			

medium

determ. method TEM (count median diameter)

distribution type no data

surface property

cristal structure

shape spherical

specific surface in m²/g

specific volume in m³/g

solubility in mg/l

at in °C

particle density in g/cm³

additional Self made: freshly prepared nanoparticles by means of evaporation/condensation with ceramic heater; no-aggregated particles (same technique as for 28 d study of Ji et al., 2007)

reference Sung et al., 2008, 2009

producer Self made: Korea Environment & Merchandise Testing Institute, Korea

Specification by Authors

object Primary particle

size	diameter in nm	inner diameter in nm	outer diameter in nm	length in nm
mean				
SD				
min				
max				

medium

determ. method

distribution type no data

surface property

cristal structure

shape

specific surface in m²/g

specific volume in m³/g

solubility in mg/l

at in °C

particle density in g/cm³

additional

reference

Hydrodynamic diameter

median **nm**

distibution type monodispers

GSD

bulk density **mg/ml**

min **nm**

isoelectric point **in**

Silver

max	**nm**	**zeta potential**	**mV**	**in**	
medium		**peak SPR**	**nm**	**in**	
determ. method	differential mobility analyzer and ultrafine condensation particle counter	**conductivity**	**µS/cm**	**in**	
sample treatment		**solubility**	**mg/L**	**in**	**at** **ºC**
applic. medium					
dispersant					
additional	geometric mean diameter and GSD in aerosol (LD; MD; HD): 18,12 +-1,31 nm; 18,33 +- 1,12 nm; 18,93 +- 1,59 nm				

Study Design

species	rat	**strain**	Sprague-Dawley
sex	male & female	**animal/group**	10
route	whole-body	**age of animal**	8 w
purity			
exposure in h/d	6	**exposure in d/w**	5
study dur. in d	91	**postexp. dur. in d**	0
no. of instillation		**frequency**	
exposure (additional)	whole-body 1,3 qm exposure chamber; analytical conc.: 48,94+-0,47; 133,19+-1,05; 514,78+-3,74 µg/m³; particle conc.: 66400; 1430000; 2850000 particles/cm³; surface area: 1,08; 2,37; 6,61 x109 nm²/cm³; bw m 253 g, f 162 g		
dose / concentratio	0 0.048 0.133 0.515	**1 dose study**	☐
		reliability	**A**
Unit	mg/m³	**confidential**	☐
additional	Lung function weekly by whole-body plethysmography 40 min after termination of daily exposure; other endpoints on d 90; Study according to OECD guideline 413 (1995) Ag burden by AAS after tissue digestion		

Reference

author	Kim JS, et al.	**source**	Safe Health Work		
volume	2	**year**	2011	**page**	34-38
institution	Toxicological Research Center, Hoseo University, Korea				

author	Sung J, et al.	**source**	Toxicological Sciences		
volume	108	**year**	2009	**page**	452-461
institution	Korea Environment & Merchandise Testing Institute, Korea				

author	Sung J, et al.	**source**	Inhalation Toxicology		
volume	20	**year**	2008	**page**	567-574
institution	Korea Environment & Merchandise Testing Institute, Korea				

Scope

organ	**animal/group**	**necropsy**	**organ weight**	**histopathology**
guideline				
BALF	4	☑	☐	☐

total protein	alveolar macrophages	PMN
albumin	lactate dehydrogenase (LDH)	lymphocytes

additional 14x3 ml PBS, total lung

Silver

clinical symptoms	10	☐	☐	☐
additional	including food consumption and signs of irritancy			
body weight	10	☐	☐	☐
lung	6	☐	☑	☑
additional	organ weight for left and right lung lobe, no lavage (N=6); lung burden (total lung, no lavage, N=4-5); Histopatho: H&E stain; lung function parameters: tidal and minute volume, respiratory frequency, inspiration and expiration time, peak inspiration and expiration flow			
testes	10	☐	☑	☑
additional	organ weight for left and right testis			
kidney	10	☐	☑	☑
additional	weight of left and right kidney; organ burden (N=4-5			
spleen	10	☐	☑	☑
liver	10	☐	☑	☑
additional	organ burden (N=3-5)			
adrenal gland	10	☐	☑	☑
additional	organ weight for left and right adrenal			
heart	10	☐	☑	☑
thymus	10	☐	☑	☑
brain	10	☐	☑	☑
additional	organ burden (2-5 m and 4-5 f per dose)			
nose	10	☐	☑	☑
additional	weight and burden of olfactory bulb (N=3-5)			
ovary	10	☐	☑	☑
additional	organ weight for left and right ovary			
haematology	10	☐	☐	☐

hematocrit	haemoglobin	erythrocyte count
erythrocyte aggregation	partial thromboplastin time	Prothrombin time
Monocytes	basophils	eosinophils
Lymphocytes	Red cell distribution width	Neutrophils
Reticulocytes	Total leukocyte count	Platelet count
Mean corpuscular haemoglobin conc	Mean corpuscular volume	Mean corpuscular haemoglobin
Platelet volume	differential leukocyte count	

clinical chemistry	10	☐	☐	☐
thyroid gland	10	☐	☐	☑
bladder	10	☐	☐	☑
uterus	10	☐	☐	☑
epididymis	10	☐	☐	☑
seminal vesicle	10	☐	☐	☑
trachea	10	☐	☐	☑

oesophagus	10	☐	☐	☑
tongue	10	☐	☐	☑
prostate	10	☐	☐	☑
pancreas	10	☐	☐	☑
urine analysis	5	☐	☐	☐
Total protein		ß-NAG		
bone marrow		☐	☐	☐

additional femurs: in vivo micronucleus test (OECD GL 474)

Effect data

adrenal gland

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	male & female			☐
additional	no effect			

BALF

effect	sex	LOEL study unit	LOEL mg/kg	transient
albumin	female	0.515	0.11705	☐

µg/ml

dose	sex	timepoint	level	score	significance	%
0	male	91	8.3		n.g.	100
0	female	91	9.3		n.g.	100
0.048	male	91	16.5		none	198.79
0.048	female	91	11.3		none	121.5
0.133	male	91	11.8		none	142.16
0.133	female	91	11.8		none	126.88
0.515	male	91	1.8		none	21.68
0.515	female	91	37.75		<0.05	405.91

additional sign. increase in HD f (mean with high SD: 37,75 +- 20,1), however, in HD m a decrease was seen t was sign. compared to LD and MD values

effect	sex	LOEL study unit	LOEL mg/kg	transient
lymphocytes total	male & female			☐

x10E6

additional no effect: number of lymphocytes

effect	sex	LOEL study unit	LOEL mg/kg	transient
PMN total	male & female			☐
x10E6				
additional	no effect: number of PMN			

effect	sex	LOEL study unit	LOEL mg/kg	transient
alveolar macrophages total	male & female			☐
x10E6				
additional	no effect: number of AM			

effect	sex	LOEL study unit	LOEL mg/kg	transient
total cells	male & female			☐
x10E6				
additional	no effect: total cells			

effect	sex	LOEL study unit	LOEL mg/kg	transient
total protein	female	0.515	0.11705	☐

µg/ml	dose	sex	timepoint	level	score	significance	%
	0	male	91	13.3		n.g.	100
	0	female	91	13.9		n.g.	100
	0.048	male	91	16.5		none	124.06
	0.048	female	91	10.4		none	74.82
	0.133	male	91	15.1		none	113.53
	0.133	female	91	10.9		none	78.41
	0.515	male	91	13.8		none	103.75
	0.515	female	91	20.33		<0.05	146.25
additional	sign. increase in HD f; no increase in m						

effect	sex	LOEL study unit	LOEL mg/kg	transient
lactate dehydrogenase (LDH)	female	0.515	0.11705	☐

units/liter (U/l)	dose	sex	timepoint	level	score
	0	male	91	55.3	
	0	female	91	57.3	
	0.048	male	91	73.4	
	0.048	female	91	41.8	
	0.133	male	91	69.1	
	0.133	female	91	41.8	
	0.515	male	91	73	
	0.515	female	91	116.7	
additional	sign. increase of LDH activity in HD f (2fold); no increase in m				

bladder

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

body weight

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
	additional	no effect			

bone

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

bone marrow

effect		sex	LOEL study unit	LOEL mg/kg	transient
micronuclei		male & female			☐
	additional	no effect: no increased frequencies of micronucleated polychromatic erythrocytes			

brain

effect		sex	LOEL study unit	LOEL mg/kg	transient
burden		male & female	0.133	0.03023	☐

µg/g wet organ	dose	sex	timepoint	level	score	significance	%
	0	male	91	0.00112		n.g.	100
	0	female	91	0.00066		n.g.	100
	0.048	male	91	0.00345		none	308.03
	0.048	female	91	0.00409		none	619.69
	0.133	male	91	0.00789		<0.01	704.46
	0.133	female	91	0.0102		<0.01	1545.5
	0.515	male	91	0.0186		<0.01	1660.7
	0.515	female	91	0.02		<0.01	3030.3
	additional	sign. increase of brain burden (Tab.7, 8)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	male & female			☐
additional	no effect			

clinical chemistry

effect	sex	LOEL study unit	LOEL mg/kg	transient
parameters acc. to picklist	male & female			☐
additional	no effect			

clinical symptoms

effect	sex	LOEL study unit	LOEL mg/kg	transient
food consumption	male & female			☐
additional	no effect: including food consumption			

epididymis

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male			☐
additional	no effect			

haematology

effect	sex	LOEL study unit	LOEL mg/kg	transient
erythrocyte aggregation	female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	91	8		n.g.	100
	0.048	female	91	4		none	50
	0.133	female	91	19		none	237.5
	0.515	female	91	28		<0.01	350
	additional	sign. increase of % aggregated erythrocytes in HD f					

heart

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	male & female			☐
additional	no effect			

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

kidney

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

effect		sex	LOEL study unit	LOEL mg/kg	transient
burden		male & female	0.133	0.03023	☐

µg/g wet organ	dose	sex	timepoint	level	score	significance	%
	0	male	91	0.00085		n.g.	100
	0	female	91	0.00094		n.g.	100
	0.048	male	91	0.00163		none	191.76
	0.048	female	91	0.00261		none	277.65
	0.133	male	91	0.00358		<0.01	421.17
	0.133	female	91	0.0118		none	1255.3
	0.515	male	91	0.00949		<0.01	1116.5
	0.515	female	91	0.0377		<0.01	4010.6
	additional	m more sensitive, sign. increase of kidney burden in f at HD only; however, at MD and HD absolute kidney burdens of f were 3-4 fold higher than in m (Tab.7, 8)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
	additional	no effect			

liver

effect		sex	LOEL study unit	LOEL mg/kg	transient
burden		male & female	0.515	0.11705	☐

µg/g wet organ	dose	sex	timepoint	level	score	significance	%
	0	male	91	0.0007		n.g.	100
	0	female	91	0.0009		n.g.	100
	0.048	male	91	0.00352		none	502.85
	0.048	female	91	0.00455		none	505.55
	0.133	male	91	0.0138		none	1971.4
	0.133	female	91	0.0121		none	1344.4
	0.515	male	91	0.133		<0.01	19000
	0.515	female	91	0.071		<0.01	7888.9
	additional	sign. increase at HD only (Tab.7, 8)					

Silver

effect	sex	LOEL study unit	LOEL mg/kg	transient
hyperplasia	male & female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	91	0	minimal	n.g.	100
	0	female	91	30	minimal	n.g.	100
	0.048	male	91	0	minimal	none	0
	0.048	female	91	20	minimal	none	66.66
	0.133	male	91	10	minimal	none	33.33
	0.133	female	91	40	minimal	none	133.33
	0.515	male	91	44.4	minimal	<0.05	148
	0.515	female	91	10	medium	<0.05	33.33
	0.515	female	91	80	minimal	<0.05	266.66
	additional	sign. increase of incidence of minimal bile-duct hyperplasia at HD					

effect	sex	LOEL study unit	LOEL mg/kg	transient
necrosis	female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	91	0		n.g.	100
	0.048	female	91	0		none	
	0.133	female	91	0		none	
	0.515	female	91	30		<0.05	
	additional	sign. increase of incidence of single-cell hepatocellular necrosis in HD f (effect not seen in m); multif hyperplasia of minimal grade seen in HD m (11.1%) and control f (20%) and of moderate grade in 1 HD f, effect not seen in other groups					

effect	sex	LOEL study unit	LOEL mg/kg	transient
fibrosis	female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	91	0		n.g.	100
	0.515	female	91	10	medium	none	
	additional	single HD f with moderate centrilobular fibrosis together with moderate bile-duct hyperplasia, minima single-cell hepatocyte necrosis, mild pigment accumulation and moderat multifocal necrosis					

effect	sex	LOEL study unit	LOEL mg/kg	transient
pigmentation	female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	91	0		n.g.	100
	0.515	female	91	10	medium	none	
	additional	single HD f with mild pigment accumulation together with moderate bile-duct hyperplasia, minimal si cell hepatocyte necrosis, moderate centrilobular fibrosis and moderate multifocal necrosis					

effect	sex	LOEL study unit	LOEL mg/kg	transient
vacuolization	male	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	91	0		n.g.	100
	0	female	91	0		n.g.	100
	0.048	female	91	10	minimal	none	
	0.515	male	91	11.1	minimal	none	
	additional	single HD m with hepatocellular vacuolation; effect also seen in single LD f but not at other doses, e in f no considered to be substance-dependent					

effect		sex	LOEL study unit	LOEL mg/kg	transient
mineralization		male	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	91	0		n.g.	100
	0.515	male	91	11.1	minimal	none	
	additional	single HD m with minimal portal mineralization together with minimal bile-duct hyperplasia					

effect		sex	LOEL study unit	LOEL mg/kg	transient
granuloma		male & female			☐

%	dose	sex	timepoint	level	score	significance	%
	0	female	91	0		n.g.	100
	0.048	female	91	20	minimal	none	
	additional	no effect: 2 LD f with minimal multifocal granuloma, effect not seen at higher doses and not conside to be substance-dependent					

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
gram					
	additional	no effect			

lung

effect		sex	LOEL study unit	LOEL mg/kg	transient
thickening		male & female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	91		no change	n.a.	
	0.515	male & f	91			n.a.	
	additional	sign. increased incidence of alveolar wall thickening (Tab.9, 10; see inflammation)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
gram					
	additional	no effect: total wt. (no lavage)			

Silver

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
inflammation		male & female	0.515	0.11705	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	20	minimal	n.g.	100
	0	female	91	30	minimal	n.g.	100
	0.048	male	91	30	minimal	n.g.	150
	0.048	female	91	20	minimal	n.g.	66.66
	0.133	male	91	20	minimal	n.g.	100
	0.133	female	91	0	minimal	n.g.	0
	0.515	male	91	89	minimal	n.g.	350
	0.515	female	91	80	minimal	n.g.	266.66
	additional	increased incidence of minimal chronic alveolar inflammation including alveolitis, granulomatous lesions, and alveolar wall thickening (Tab.9, 10)					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
infiltration		male & female	0.133	0.03023	☐		
%	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	30	minimal	n.g.	100
	0	female	91	0	minimal	n.g.	100
	0.048	male	91	40	minimal	n.g.	133.33
	0.048	female	91	0	minimal	n.g.	
	0.133	male	91	60	minimal	n.g.	200
	0.133	female	91	10	minimal	n.g.	
	0.515	male	91	78	minimal	n.g.	266.66
	0.515	female	91	70	minimal	n.g.	
	additional	Minimal perivascular infiltration of mixed cells: m more sensitive, LOEL in f at 0,515 mg/m3 (Tab.9, 1					

effect		sex	LOEL study unit	LOEL mg/kg	tran-sient		
tidal volume		male & female	0.048	0.01091	☐		
ml	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	21	0.265		n.g.	
	0	female	70	0.24		n.g.	100
	0	male	91	0.305		n.g.	100
	0.048	male	21	0.225		<0.01	0.97
	0.048	female	70	0.22		none	91.66
	0.048	male	91	0.275		<0.01	90.16
	0.133	male	21	0.21		<0.01	0.91
	0.133	female	70	0.21		<0.01	87.5
	0.133	male	91	0.265		<0.01	86.88
	0.515	male	21	0.195		<0.01	0.84
	0.515	female	70	0.195		<0.01	81.25
	0.515	male	91	0.26		<0.01	85.24
	additional	dose-dependent decrease of tidal volume: sign. at single tps, effect more pronounced in m (Fig.3A,					

Silver

effect		sex	LOEL study unit	LOEL mg/kg	transient		
burden		male & female	0.133	0.03023	☐		
µg/g wet organ	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	91	0.00077		n.g.	100
	0	female	91	0.00101		n.g.	100
	0.048	male	91	0.614		none	79740
	0.048	female	91	0.296		none	29307
	0.133	male	91	5.45		<0.01	707792
	0.133	female	91	4.241		<0.01	419901
	0.515	male	91	14.65		<0.01	2E+06
	0.515	female	91	20.59		<0.01	2E+06
	additional	sign. dose-dependent increase of total lung burden (no lavage): effect at LD not statistically sign. (Tab.7,8)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
peak inspiration flow		male & female	0.048	0.01091	☐		
ml/s	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	14	1.25		n.g.	100
	0	female	14		no change	n.a.	
	0.048	male	14	0.9		<0.01	72
	0.048	female	14			n.a.	
	0.133	male	14	1.08		<0.01	86.4
	0.133	female	14			n.a.	
	0.515	male	14	0.8		<0.01	64
	0.515	female	14			n.a.	
	additional	dose-dependent decrease of peak inspiration flows in m & f of all groups (partly sign.) (Fig.3C, f not shown)					

effect		sex	LOEL study unit	LOEL mg/kg	transient		
minute volume		male & female	0.048	0.01091	☐		
ml/min	**dose**	**sex**	**timepoint**	**level**	**score**	**significance**	**%**
	0	male	14	27.5		n.g.	100
	0	female	28	23		n.g.	100
	0	male	49	28		n.g.	100
	0.048	male	14	18.5		<0.05	67.27
	0.048	female	28	18.5		<0.01	80.43
	0.048	male	49	24		<0.01	85.71
	0.133	male	14	24		<0.05	87.27
	0.133	female	28	18		<0.01	78.26
	0.133	male	49	23		<0.01	82.14
	0.515	male	14	18		<0.05	65.45
	0.515	female	28	15.7		<0.01	68.26
	0.515	male	49	20		<0.01	71.42
	additional	dose-dependent decrease of minute volume in m & f of all groups (partly sign.) (Fig.3B, 4B)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
granuloma		male & female	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male & f	91		no change	n.a.	
	0.048	male & f	91		no change	n.a.	
	0.133	male & f	91		no change	n.a.	
	0.515	male & f	91			n.a.	
	additional	sign. increased incidence of granulomatous lesions (Tab.9, 10; see inflammation)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
macrophage infiltration		male	0.515	0.11705	☐

%	dose	sex	timepoint	level	score	significance	%
	0	male	91	30	minimal	n.g.	100
	0	female	91	70	minimal	n.g.	100
	0.048	male	91	50	minimal	n.g.	166.66
	0.048	female	91	40	minimal	n.g.	57.14
	0.113	male	91	50	minimal	n.g.	166.66
	0.133	female	91	40	minimal	n.g.	57.14
	0.515	male	91	89	minimal	n.g.	266.66
	0.515	female	91	60	minimal	n.g.	85.71
	additional	minimal accumulation of alveolar macrophages: increased incidence in HD m; no increase in f due t high background incidence (Tab.9, 10)					

nose

effect		sex	LOEL study unit	LOEL mg/kg	transient
burden		male & female	0.515	0.11705	☐

µg/g wet organ	dose	sex	timepoint	level	score	significance	%
	0	male	91	0.00051		n.g.	100
	0	female	91	0.00226		n.g.	100
	0.048	male	91	0.00644		none	1262.7
	0.048	female	91	0.00743		none	328.76
	0.133	male	91	0.0171		none	3352.9
	0.133	female	91	0.0138		none	610.61
	0.515	male	91	0.0305		<0.01	5980.4
	0.515	female	91	0.00328		<0.01	145.13
	additional	sign. dose-dependent increase in olfactory bulb (Tab.7, 8)					

effect		sex	LOEL study unit	LOEL mg/kg	transient
weight		male & female			☐
	additional	no effect: wt. of olfactory bulb			

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		male & female			☐
	additional	no effect			

oesophagus

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

ovary

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	female			☐
additional	no effect			

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	female			☐
additional	no effect			

pancreas

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

prostate

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male			☐
additional	no effect			

seminal vesicle

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male			☐
additional	no effect			

testes

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	male			☐
additional	no effect			

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male			☐
additional	no effect			

thymus

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

effect	sex	LOEL study unit	LOEL mg/kg	transient
weight	male & female			☐
additional	no effect			

thyroid gland

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

trachea

effect	sex	LOEL study unit	LOEL mg/kg	transient
histopathology	male & female			☐
additional	no effect			

urine analysis

effect	sex	LOEL study unit	LOEL mg/kg	transient
protein	male	0.515	0.11705	☐

g/g creatinine

dose	sex	timepoint	level	score	significance	%
0	male	91	1.89		n.g.	100
0	female	91	3.88		n.g.	100
0.048	male	91	1.58		none	83.59
0.048	female	91	0.9		none	23.19
0.133	male	91	2.39		none	126.45
0.133	female	91	2.93		none	75.51
0.515	male	91	2.57		<0.05	135.97
0.515	female	91	2.26		none	58.24

additional sign. increase of protein in urine in HD m, effect not seen in f due to high mean control value with hig SD

uterus

Silver

effect		sex	LOEL study unit	LOEL mg/kg	transient
histopathology		female			☐
	additional	no effect			