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Evaluation of chronic toxicity/carcinogenicity of nanomaterials using nano-CeO2 (Bewertung der chronischen Toxizität/Kanzerogenität ausgewählter Nanomaterialien – hier nano-CeO2)

by

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Abstract

To fully reveal relevant human health hazards of nano-CeO2, putative lung carcinogenicity and putative systemic effects of low-dose life-time inhalation exposure, BASF Experimental Toxicology performed a long-term inhalation study. This study was co-financed by and in cooperation with the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, the German Environment Agency, the Federal Institute for Occupational Safety and Health, the German Federal Institute for Risk Assessment and the EU commission (Project NanoReg, FP 7/2007-2013, grant agreement number 310584). This whole-body inhalation study was performed according to OECD test guideline no. 453 with several protocol extensions. Female rats (100/group) were exposed to nano-CeO2 (NM212, 0.1; 0.3; 1; 3 mg/m^3) for two years and for two years plus a 6-month recovery period; a control group was exposed to clean air. The in-life part of the study was performed at BASF, Ludwigshafen. An extended histological examination of 60 and more step sections per lung was performed for all animals at Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover. Summarizing the results of this study part, no significant tumor induction was observed. Only 3 benign bronchiolo-alveolar adenomas were detected in the CeO2 groups after 24 months exposure (one animal in dose group 0.1, 0.3, and 1 mg/m3, respectively) and another 3 bronchiolo-alveolar adenomas after 30 months (one animal in dose group 0.3, 1, and 3 mg/m3, respectively). There were no significant numbers of pre-neoplasias (bronchiolo-alveolar hyperplasia with atypia) in the CeO2-exposed lungs at both examination time points. Dose-related significant findings in the cerium groups such as accumulation of particle-laden macrophages (alveolar/interstitial, bronchus-associated lymphoid tissue), (micro)granulomatous inflammation and inflammatory cell infiltration (mainly mononuclear cells, few granulocytes), interstitial fibrosis, alveolar lipoproteinosis, bronchiolo-alveolar hyperplasia of the alveolar or mixed type and alveolar bronchiolization were seen. A pronounced individual variation in the grade of the inflammatory response could be observed in all test groups, especially in the low doses. An inflammatory reaction to CeO2 which persisted until 30 months was already observed in the lowest dose group (0.1 mg/m3). Though a dose-dependent increase of the genotoxicity marker γ-H2AX was observed after 12 months of exposure, no consecutive tumor induction was found.

Kurzbeschreibung

Unter der Schirmherrschaft von und mittels Kofinanzierung durch das Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit mit Beteiligung der Bundesoberbehörden Umweltbundesamt, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin sowie Bundesinstitut für Risikobewertung und der Europäischen Kommission (NanoReg, FP 7/2007-2013, Nr. 310584) wurde bei der BASF eine über 24 Monate angelegte Inhalationsstudie mit zusätzlicher 6-monatiger Erholungsphase (Recoveryphase) an 500 weiblichen Wistar (Han: WIST) Ratten zur Ermittlung der chronischen Toxizität und evtl. Kanzerogenität von nanoskaligem CeO2 nach der OECD Prüfrichtlinie Nr. 453 durchgeführt. Die Studie beinhaltete eine Reinluftkontrolle und 4 CeO2 Behandlungsgruppen (Expositionskonzentration: 0,1; 0,3; 1 und 3 mg/m3 CeO2) mit jeweils 100 Tieren pro Gruppe aufgeteilt auf zwei Untersuchungszeitpunkte. Die beiden Zeitpunkte der Untersuchung waren erstens nach 2-jähriger Inhalation und zweitens nach 2-jähriger Inhalation mit einer anschließenden 6-monatigen Erholungsphase. Eine erweiterte histopathologische Untersuchung von je 60 und mehr Stufenschnitten der Lunge aller Tiere wurde im Fraunhofer Institut für Toxikologie und Experimentelle Medizin ITEM durchgeführt. Zusammenfassend betrachtet, konnte für die Lunge keine durch die Inhalation von CeO2 hervorgerufene erhöhte Tumorrate festgestellt werden. Es wurden jeweils 3 gutartige Tumore (bronchiolo-alveoläre Adenome) nach 24-monatiger Inhalation bzw. nach 30 Monaten detektiert, die sich um die verschiedenen Gruppen verteilten (je ein Tier in der Dosisgruppe 0,1; 0,3 und 1mg/m3 nach 24 Monaten und je ein Tier in der Dosisgruppe 0,3; 1 und 3mg/m3 nach 30 Monaten). Außerdem fand sich keine signifikante Anzahl von Tumorvorstufen (bronchiolo-alveoläre Hyperplasien mit Atypien) in den CeO2-exponierten Gruppen an beiden Untersuchungszeitpunkten. Es wurden Dosis-abhängige Veränderungen gefunden, die partikel-beladene Makrophagen (alveolar, interstitiell und im Bronchus-assoziierten lymphatischen Gewebe), (micro)granulomatöse Entzündung und Entzündungszellinfiltration (haupt-

sächlich mononukleäre Zellen und wenige Granulozyten), interstitielle Fibrose, alveoläre Lipoproteinose, bronchiolo-alveoläre Hyperplasien des alveolären und gemischten Typs sowie alveoläre Bronchiolisation umfassten. Es wurden ausgeprägte individuelle Unterschiede in der Schwere der Veränderungen innerhalb der verschiedenen Gruppen, besonders in den niedrigen Dosisgruppen, gefunden. Eine entzündliche Reaktion, welche bis zum Ende der Untersuchung, das heißt bis zum Ende der Recoveryphase, bestand, wurde auch bereits in der niedrigsten Dosisgruppe gefunden. Obwohl eine Dosis-abhängige Erhöhung des Gentoxizitätsmarkers γ-H2AX nach 12-monatiger Inhalation festgestellt wurde, wurde keine daraus folgende Tumorbildung gefunden.

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Glossary

Acronym	Meaning
8-OHdG	8-Hydroxy-2'-desoxyguanosine
amu	atomic mass unit
ANOVA	Analysis of variance
Asd	Absolute standard deviation
BALT	Bronchus-associated lymphoid tissue
BAuA	Federal Institute for Occupational Safety and Health
BCR	***BCR***® is a registered trademark of the European Commission
BfR	German Federal Institute for Risk Assessment
BMU	Federal Ministry for the Environment, Nature Conservation and Nuclear Safety
Bronchiolization	Population of the alveolar region with migrating bronchiolar cells as an attempt to increase the alveolar clearance
CeO_2	Cerium dioxide
Ce	Cerium (German: Cer)
DIN	German industry norm [Deutsche Industrienorm]
FG	Wet weight of organs [Feuchtgewicht]
Granulocyte	Inflammatory cell with a segmented / multi-lobed nucleus
Granulomatous inflammation	A distinct type of chronic inflammation in which mainly macrophages and maybe multinucleated giant cells are involved
Hyperplasia	Increase in cell number
ICP-MS	Inductively coupled plasma mass spectrometry
LALN	Lung-associated lymph nodes
Lipoproteinosis	Accumulation of acidophilic, acellular material
LOD	Limit of detection
LOQ	Limit of quantification
Mononuclear cell	Inflammatory cell with a round nucleus such as of lymphocytes and monocytes
OECD	Organization for Economic Co-operation and Development
Ppb	Parts per billion
PSP	Poorly soluble particle with low toxicity
QA	Quality assurance
Rsd	Relative standard deviation
Trim	Specific way to cut organs according to published guidelines: https://reni.item.fraunhofer.de/reni/trimming/
UBA	German Environment Agency

Summary

Though the amount of information about acute and subacute toxicity of nanomaterials is constantly growing, data about the long-term effects of nanomaterials is still fragmentary. Studies dealing with the chronic inhalation of nanomaterials were only performed using nano-titanium dioxide or carbon black. These studies showed that high doses lead to inflammation in the lung and tumor induction. Furthermore, DNA damages were detectable when using dosages, which resulted in tumor formation later on. However, the processes which are causative for the tumor induction are still not known. If nanomaterials cause effects when inhaled in low doses, is still not known. Furthermore, the systemic distribution and the excretion of nanomaterials following chronic inhalation is mostly unknown.

To fully reveal relevant human health hazards of nano-CeO_2, putative lung carcinogenicity and putative systemic effects of low-dose life-time inhalation exposure, BASF Experimental Toxicology performed a long-term inhalation study co-financed by and in cooperation with the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, the German Environment Agency, the Federal Institute for Occupational Safety and Health, the German Federal Institute for Risk Assessment and the EU commission (Project NanoReg, FP 7/2007-2013, grant agreement number 310584). The goal of this study is to draw conclusions about the long-term effects of this selected nanomaterial after inhalation. A specific focus was laid on the investigation of low dose effects, which may gain environmental and work place relevance in case of exposure. Nano-cerium dioxide (CeO_2) was chosen as test material, since CeO_2 has a commercial relevance. It is used as ultraviolet absorber in dyes and plastic, as part of polishing and grinding material for silicon wafers, which are required by the electronical industry for ultra modern chip systems and solar cells, as well as as fuel additive. However, it has only been tested in short-term toxicity studies. The test material, NM212, was sourced – due to the large quantities which were required for the Long-term study - directly from the respective producer. The characterization of the test material NM212 is available (link EU SCIENCE HUB)

This whole-body inhalation study was performed according to OECD test guideline no. 453 with several protocol extensions. Female rats from the strain Han: WIST (100/group) were exposed to nano-CeO_2 (NM212) at four different dosages (0.1; 0.3; 1; 3 mg/m^3) for two years and for two years plus a 6-month recovery period. A control group was exposed to clean air. The in-life part of the study was performed at BASF, Ludwigshafen. The German Federal Institute for Risk Assessment investigated the lung burden at different time points. Furthermore, an extended histopathological investigation of the lungs after two years as well as two years plus a 6-month recovery was done at the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover. In addition, a histochemical investigation of two different genotoxicity markers within the lung was performed after 12 months of exposure at ITEM. Initially, a pre-study as dose range finding study was performed with higher doses (low dose: 0.5 mg/m^3, mid dose: 5 mg/m^3, high dose: 25 mg/m^3). The institute was also involved in the work of the pre-study in preparation of the main study, so that the work has been divided into three different work packages.

Work package 1:

Within this work package the cerium content within rat lungs and the lung-associated lymph nodes (LALN) of the pre-study were measured using the inductively coupled plasma mass spectrometry (ICP-MS). The pre-study with 28-days CeO_2 inhalation was performed as dose range finding study.

The preparation of the specimens for this analysis involved freeze drying, plasma combustion and decomposition using microwaves and finally dilution of the digestion solution. The lungs of group 1 (low dose, 0.5 mg/m^3) revealed an obvious clearance within the time period investigated. In contrast group 2 (mid dose 5 mg/m^3) and 3 (high dose, 25 mg/m^3) did not show a significant reduction of the initial cerium content within the lung. There was a linear correlation of the exposed dosage and the cerium content in the lungs. Furthermore, there was evidence of a particle overload beginning at a concentration of > 0.5 mg/m^3. Within the LALN cerium was detectable in all cerium exposed groups (group 1, 2 and 3). Furthermore, group 2 and 3 showed a significant dose- and time-dependent increase of cerium within the LALN.

Within the control group 0 (clean air control) no cerium was detectable within the lung or LALN at any time investigated. The measured values were below the defined limit of detection in this group.

Work package 2:

A histochemical investigation of the lung tissue was performed using the genotoxicity markers 8-OHdG and γ-H2AX. Three µm thin lung slices were cut from previously in paraffin wax embedded lung tissue. Thereafter, a histochemical investigation was done using antibodies directed against 8-OHdG and γ-H2AX, respectively. The antibody binding was visualized using a chromogen, and the amount of positively stained nuclei per total cell amount was counted. The positive cells were counted within specific lung areas, which were centered around the opening of the terminal bronchioli. Following 28-days of inhalation (pre-study) no increase in positively marked cell nuclei was found after overall 29 and 61days . After the 12-month inhalation of cerium dioxide (main study) elevated γ-H2AX positive nuclei were detected. A possible reason of this elevation might be a cerium dixode inhalation-induced directly or via the provoked inflammation indirectly generated cellular apoptosis or senescence. Furthermore, the cells might have been activated to be able to cope with possible DNA double-strand breaks.

Work package 3:

The lungs of the 500 rats from the two years and the two years plus a 6-month recovery period carcinogenicity study were histopathologically investigated. An extended histopathology was performed consisting of step sections of 500 µm to detect even small tumors and pre-neoplastic lesions. More than 60 slices per lung and animal and in total more than 35000 step sections were made.

At both time points investigated no significant amount of tumors within the CeO_2-exposed animals were detected. Within these groups only three benign tumors (bronchiolo-alveolar adenomas) were found after two years (one animal in dose group 0.1, 0.3, and 1 mg/m³, respectively) and the two years plus a 6-month recovery, respectively (one animal in dose group 0.3, 1, and 3 mg/m³, respectively). This type of tumor occurs not uncommonly within this rat strain and at this age and can also be seen in untreated animals. Even within the high dose group, which is already within the so called "overload" condition (Morrow, 1988; Schwotzer, 2017), only one adenoma was found. Furthermore, no increased amount of pre-neoplastic lesions (bronchiolo-alveolar hyperplasia with atypia) was seen. Hyperplasias without atypia were found to occur dose-dependently and were significantly increased at both time points in the high-dose animals compared to the negative control group. A CeO_2-induced specific (micro)granulomatous inflammation was observed. A granulomatous inflammation is a specific chronic inflammation, in which macrophages and multinucleated giant cells predominate. These cells could further arrange themselves in a specific way and form so called (micro)granulomas. Further lesions were dose-dependent and represent lesions which were commonly found following chronic inhalation of poorly soluble particles with low toxicity (PSP particles) in "overload" condition:

1. Accumulation of particle-laden macrophages (alveolar/interstitial, bronchus-associated lymphoid tissue [BALT]),
2. Inflammatory cell infiltration (mainly mononuclear cells, few granulocytes),
3. Alveolar bronchiolization (bronchiolo-alveolar hyperplasia, bronchiolar type),
4. Minimal to slight interstitial and pleural fibrosis,
5. Alveolar lipoproteinosis, and
6. Cholesterin granuloma.

The occurrence and severity of these lesions showed a pronounced individual variation in all test groups. This was especially true within the low dose groups. An inflammatory reaction to CeO_2, which persisted until 30 months was already observed in the lowest dose group (0.1 mg/m³).

Though a dose-dependent increase of the genotoxicity marker γ-H2AX was found following 12-months inhalation, no consecutive tumor formation was detected.

Zusammenfassung

Während zur akuten und subakuten Toxizität von Nanomaterialien zunehmend Daten zur Verfügung stehen, gibt es erhebliche Datenlücken zu Langzeiteffekten von Nanomaterialien. In Bezug auf die inhalative Exposition wurden bisher lediglich mit nano-Titandioxid und Industrieruß chronische Studien durchgeführt. Hier wurden bei hoher Belastung Lungenentzündung und Tumorbildung festgestellt. Im tumorerzeugenden Dosisbereich traten darüber hinaus DNS-Schädigungen im Lungengewebe auf. Es ist gegenwärtig strittig, welche Prozesse ursächlich für die nachgewiesenen Tumoren sind. In diesem Zusammenhang steht auch die Frage, ob und welche Wirkungen von Nanomaterialien bei chronischer Exposition im umweltrelevanten Niedrigdosisbereich zu erwarten sind. Zudem sind die systemische Verteilung von Nanomaterialien nach chronischer Inhalation und die Geschwindigkeit der Ausscheidung weitgehend unbekannt und müssen ebenfalls untersucht werden.

Unter der Schirmherrschaft von und mittels Kofinanzierung durch das Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit wurde deshalb ein Kooperationsprojekt mit der BASF und den Bundesoberbehörden Umweltbundesamt, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin sowie Bundesinstitut für Risikobewertung zur Durchführung und Auswertung einer chronischen Inhalationsstudie mit Nanomaterialien gestartet. Ziel dieser Studie war es, fundierte Aussagen über die Langzeitwirkung ausgewählter Nanomaterialien ableiten zu können. Ein besonderer Fokus lag dabei auf der Untersuchung von Wirkungen im Bereich niedriger Belastungen, die für eine Umwelt-oder Arbeitsplatzexposition am ehesten relevant sind. Als Prüfsubstanz wurde nano-Cerdioxid (CeO_2) festgelegt, da dieses Material kommerziell relevant ist (Verwendung als UV-Absorber in Lacken und Plastik, Polier- und Schleifmittel in der Halbleitertechnik, Kraftstoffadditiv), bisher aber nur in toxikologischen Kurzzeitstudien geprüft wurde. Die Testsubstanz, NM212, wurde – da die Langzeitstudie großer Mengen bedurfte – direkt von dem Hersteller bezogen. Die Charakterisierung der Testsubstanzen NM212 ist verfügbar (Link: EU SCIENCE HUB).

Die über 24 Monate angelegte Inhalationsstudie mit zusätzlicher 6-monatiger Recoveryphase wurde mit dem nanoskaligen CeO_2 (NM212) an weiblichen Ratten vom Stamm Han: WIST (100/Gruppe) mit 4 verschiedenen Dosen (0,1; 0,3; 1; 3 mg/m^3) nach den Prüfvorgaben der OECD (OECD Richtlinie 453) zur Ermittlung der chronischen Toxizität und eventueller Tumorerzeugung (Kanzerogenität) bei der BASF durchgeführt. Es gab eine erweiterte histopathologische Untersuchung der Lunge am Fraunhofer Institut für Toxikologie und Experimentelle Medizin ITEM. Außerdem erfolgte eine histochemische Untersuchung des Lungengewebes auf zwei verschiedene Gentoxizitätsmarker nach 12-monatiger Inhalation. Initial wurde zunächst eine Vorstudie zur Dosisfindung mit höheren Dosen durchgeführt (geringe Dosis: 0,5 mg/m^3, mittlere Dosis: 5 mg/m^3, hohe Dosis: 25 mg/m^3), wobei das Institut auch involviert war, um den Cer-gehalt in der Lunge und den assoziierten Lymphknoten zu bestimmen. Diese verschiedenen Aufgaben wurden in 3 Arbeitspaketen am Fraunhofer Institut für Toxikologie und Experimentelle Medizin ITEM, Hannover, bearbeitet.

Arbeitspaket 1:

In diesem Arbeitspacket wurde der Cer-Gehalt in der Lunge und den lungen-assoziierten Lymphknoten (LALN) aus der Vorstudie mittels ICP-MS bestimmt. Der analytische Auftrag beinhaltete die Bestimmung von Cer nach 28-tägiger Inhalation (Retention und Clearance) in Proben aus der Vorstudie, welche zur Dosisfindung durchgeführt wurde.

Zur Probenvorbereitung waren die Arbeitsschritte Gefriertrocknung, Plasmaveraschung und Mikrowellen-aufschluss sowie eine Verdünnung der jeweiligen Aufschlusslösung erforderlich. Bei den Lungen war in der Dosisgruppe 1 (0,5 mg/m^3, Niedrigdosis) innerhalb des Prüfzeitraums ein deutlicher Clearance-Effekt zu beobachten. Die Gruppen 2 (5 mg/m^3, Mitteldosis) und 3 (25 mg/m^3, Hochdosis) zeigten hingegen keine ausgeprägte Abnahme der ursprünglichen Lungenbeladung. Es bestand eine lineare Korrelation zwischen der Expositionsdosis und dem CeO_2-Gehalt in der Lunge. Es gab weiterhin Hinweise für einen „Partikel-Overload“ ab einer Dosis von > 0,5 mg/m^3.

Bei den LALN war Cer ebenfalls in allen Dosisgruppen nachweisbar, in den Dosisgruppen 2 und 3 war ein signifikanter, dosis- und zeitabhängiger Anstieg des CeO_2 zu beobachten. In der Kontrollgruppe war Cer weder in Lungen noch LALN über den gesamten beobachteten Zeitraum (signifikant) nachweisbar, der Messwert lag jeweils unterhalb der definierten Bestimmungsgrenze.

Arbeitspaket 2:

Eine histochemische Untersuchung des Lungengewebes wurde mit den Markern 8-OHdG und γ-H2AX durchgeführt. Dafür wurden vom zuvor in Paraffinblöcken eingebetteten Lungengewebe 3µm dünne Gewebeschnitte angefertigt. Diese wurden anschließend im Rahmen der histochemischen Untersuchung mit einem Antikörper behandelt, der entsprechend gegen 8-OHdG beziehungsweise γ-H2AX gerichtet war. Diese Reaktion wurde mittels eines Farbstoffes sichtbar gemacht. Anschließend wurde die Anzahl positiv-angefärbter Zellkerne im Verhältnis zu der Gesamtzellzahl ermittelt. Dies erfolgte in bestimmten Arealen der Lunge, welche in der Nähe der Öffnung der sogenannten terminalen Bronchioli lagen. Nach 28-tägiger Inhalation (Vorstudie) konnte nach insgesamt 29 und 61 Tagen keine Erhöhung der Anzahl positiv markierter Zellkerne festgestellt werden. Nach einer 12-monatigen CeO_2 Inhalation (Hauptstudie) wurde eine Erhöhung der γ-H2AX positiven Zellen beobachtet. Eine mögliche Ursache dieser Erhöhung liegt in einer durch Cerdioxid direkt oder indirekt über die hervorgerufene Entzündung verursachten Apoptose oder Seneszenz der Zellen. Weiterhin könnte es zu einer Aktivierung der Zellen gekommen sein, um die Bereitschaft der Zellen, auf mögliche Doppelstrangbrüche zu reagieren, zu erhöhen.

Arbeitspaket 3:

Im Rahmen der 24- und 30-Monate (24 Monate Exposition plus 6 Monate Recovery) Kanzerogenitätsstudie wurden von den 500 Ratten die Lungen histopathologisch ausgewertet. Dabei wurden Stufenschnitte des Lungengewebes im Abstand von 500 µm angefertigt, um auch kleinste Tumoren und Präneoplasien zu detektieren. Dies resultierte darin, dass mehr als 60 Stufenschnitte pro Lunge und insgesamt mehr als 35000 Stufenschnittpräparate hergestellt wurden.

Zu beiden Untersuchungszeitpunkten gab es keine signifikante Anzahl an Tumoren in den CeO_2 Expositionsgruppen. Es wurden in diesen Gruppen jeweils 3 (gutartige) bronchiolo-alveoläre Adenome nach 24 und nach 30 Monaten gefunden (je ein Tier in der Dosisgruppe 0,1; 0,3 und 1mg/m^3 nach 24 Monaten und je ein Tier in der Dosisgruppe 0,3; 1 und 3mg/m^3 nach 30 Monaten). Dieser Tumortyp ist nicht ungewöhnlich für Ratten dieses Stamms und Alters und tritt gelegentlich auch bei unbehandelten Kontrolltieren auf. Sogar in der höchsten CeO_2 Dosisgruppe (3 mg/m^3), die bereits im sog. "Overload" Bereich liegt (Morrow, 1988; Schwotzer, 2017), konnte nur ein Adenom gefunden werden. Weiterhin wurden keine erhöhten Raten an Tumorvorstufen, sogenannten Präneoplasien (bronchiolo-alveoläre Hyperplasien mit Atypien), beobachtet. Hyperplasien ohne zelluläre Atypien waren hingegen dosisabhängig und in der Hochdosis zu beiden Zeitpunkten signifikant erhöht. Es fand sich eine CeO_2 induzierte spezifische (mikro)granulomatöse Entzündung. Eine granulomatöse Entzündung ist eine spezielle länger andauernde Entzündung, welche sich durch hauptsächlich auftretende Makrophagen und mehrkernige Riesenzellen auszeichnet. Diese können sich zudem in einer speziellen Form zusammenlagern und dadurch (Mikro)granulome bilden.

Weitere dosisabhängige Befunde waren solche, die auch für schwer lösliche Partikel mit geringer Toxizität (PSP) nach chronischer Exposition, insbesondere im "Overload" Bereich charakteristisch sind:

1. Ansammlung partikelbeladener Makrophagen (alveolär/interstitiell, Bronchus-assoziiertes lymphatisches Gewebe [BALT]),
2. Entzündungszellinfiltrate (hauptsächlich mononukleäre Zellen, wenige Granulozyten),
3. Alveoläre Bronchiolisation (bronchiolo-alveoläre Hyperplasie, bronchiolärer Typ),
4. Minimale bis geringgradige interstitielle und pleurale Fibrose,
5. Alveoläre Lipoproteinose und

6. Cholesteringranulome.

Das Auftreten und der Schweregrad dieser Befunde zeigten über alle Gruppen hinweg eine rattenstammspezifische, ausgeprägte interindividuelle Variabilität. Insbesondere wurden ausgeprägte individuelle Unterschiede in der Schwere der Veränderungen in den niedrigsten Dosisgruppen gefunden. Eine entzündliche Reaktion, welche bis zum Ende der Untersuchung, das heißt auch bis zum Ende der Recoveryphase, bestand, wurde auch in der niedrigsten Dosisgruppe gefunden. Obwohl eine Dosisabhängige Erhöhung des Gentoxizitätsmarkers γ-H2AX nach 12-monatiger Inhalation festgestellt wurde, wurde keine daraus folgende Tumorbildung gefunden.

1 Introduction

Though the amount of information about acute and subacute toxicity of nanomaterials is constantly growing, data about the long-term effects of nanomaterials is still fragmentary. Studies dealing with the chronic inhalation of nanomaterials were only performed using nano-titanium dioxide or carbon black. These studies showed that high doses lead to inflammation in the lung and tumor induction. Furthermore, DNA damages were detectable when using dosages, which resulted in tumor formation later on. However, the processes which are causative for the tumor induction are still not known. If nanomaterials cause effects when inhaled in low doses, is also still not known. Furthermore, the systemic distribution and the excretion of nanomaterials following chronic inhalation is mostly unknown.

To fully reveal relevant human health hazards of nano-CeO_2, putative lung carcinogenicity and putative systemic effects of low-dose life-time inhalation exposure, BASF Experimental Toxicology performed a long-term inhalation study co-financed by and in cooperation with the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, the German Environment Agency, the Federal Institute for Occupational Safety and Health, the German Federal Institute for Risk Assessment and the EU commission (Project NanoReg, FP 7/2007-2013, grant agreement number 310584). The goal of this study is to draw conclusions about the long-term effects of this selected nanomaterial after inhalation. A specific focus was laid on the investigation of low dose effects, which may gain environmental and work place relevance in case of exposure. Nano-cerium dioxide (CeO_2) was chosen as test material, since CeO_2 has a commercial relevance. It is used as ultraviolet absorber in dyes and plastic, as part of polishing and grinding material for silicon wafers, which are required by the electronical industry for ultra modern chip systems and solar cells, as well as as fuel additive. However, it has only been tested in short-term toxicity studies. The test material, NM212, was sourced – due to the large quantities which were required for the Long-term study - directly from the respective producers. The characterization of the test material NM212 is available (link: EU SCIENCE HUB).

This whole-body inhalation study was performed according to OECD test guideline no. 453 with several protocol extensions. Female rats from the strain Han: WIST (100/group) were exposed to nano-CeO_2 (NM212) at four different dosages (0.1; 0.3; 1; 3 mg/m^3) for two years and for two years plus a 6-month recovery period. A control group was exposed to clean air. The in-life part of the study was performed at BASF, Ludwigshafen. The German Federal Institute for Risk Assessment investigated the lung burden at different time points. Furthermore, an extended histopathological investigation of the lungs after two years as well as two years plus a 6-month recovery was done at the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover. In addition, a histochemical investigation of two different genotoxicity markers within the lung was performed after 12 months of exposure at ITEM. The institute was also involved in the work of the pre-study in preparation of the main study, so that the work has been divided into three different work packages.

2 Work package 1

2.1 Pre-study: Measurement of cerium dioxide retention in lung and LALN

2.1.1 Introduction to analysis

The analysis consisted of the measurement of cerium (Ce) within organic material (lung and lung-associated lymph nodes [LALN]) following inhalation (retention and clearance) by rats within the 28-days BASF CeO_2 study (BASF pre-study: 46I0661/10I151).

The quantitative measurement of the cerium content within the specimens was done using the inductively coupled plasma mass spectrometry (ICP-MS).

The preparation of the specimens for this analysis involved freeze drying, plasma combustion and decomposition using microwaves and finally dilution of the digestion solution.

2.1.2 General specimen assignment

To specifically assign the specimens to the respective study and animals, all specimens got a study specific identification number (BASF), which was used for all preparative steps as well as the analysis.

The following table (table 1) gives an overview of the tissue specimens, which were processed at Fraunhofer ITEM (lungs and LALN have the same identification number).

Table 1: Specimens selected for the ICP-MS analysis

Day (d)	**Group 0** Clean air (control) **0 mg/m³**	**Group 1** CeO_2 (low dose) **0.5 mg/m³**	**Group 2** CeO_2 (mid dose) **5 mg/m³**	**Group 3** CeO_2 (high dose) **25 mg/m³**
d28	0/6	1/36	2/066	3/96
d30	0/12	1/42	2/072	3/102
d36	0/13	1/43	2/073	3/103
d62	0/24	1/54	2/084	3/114
d92	0/25	1/55	2/085	3/115
d156	0/26	1/56	2/086	3/116

2.1.3 Preparation of the specimens for the analysis

2.1.3.1 Freeze drying (Freeze dryer BETA 1-8; Christ; Osterode / Harz)

Initially a freeze drying of the tissue was conducted on aluminum plates for 6 hours at a pressure of 0.37 mbar. The weight of the tissue was determined prior and after the freeze drying process.

2.1.3.2 Plasma combustion (Plasmasystem 200-G, Technics Plasma GmbH; Kirchheim)

The freeze dried material was subsequently plasma combusted overnight for more than 12 hours (cool plasma conditions, 400 W, ca 1 mbar O_2), homogenized, and stored in appropriate containers. The weight of the ashes of the specimens is normally lower than 5 % of the initial wet weight. This parameter can be used as quality control of the combustion process.

2.1.3.3 Decomposition using microwaves (Microwave mls 1200 mega; MLS GmbH Leutkirch)

Following freeze drying and plasma combustion the material was further processed using micro waves. The complete decomposition of the material depends on the use of sulfuric acid (96%, suprapur).

The workflow of the decomposition using micro waves is as follows:

1. Pre-warming with 250 watt for 5 min
2. Break for 1 min
3. Heating with 500 watt for 5 min
4. Break for 1 min
5. Heating with 400 watt for 8 min

6. Break for 1 min
7. Heating with 400 watt for 8 min
8. Ventilation/cooling for 15min

Following cooling-down of the material it was transferred to a 25 mL volumetric flask, and ultrapure water was added ad 25 mL. The specimens were stored under cool conditions within a 50 mL plastic container. To avoid possible carryover of analytes, the specimens were processed starting with the control group material followed by the 0.5 mg/m^3, 5 mg/m^3, and 25 mg/m^3 CeO_2 exposed group material.

2.1.4 Parameters of ICP-MS analysis

Quantification was done using the Quadrupol ICP-MS-System, X-Series II (ThermoFisher)

Apparatuses Parameters: Argon plasma (power: 1400 W), Xt-Interface (matrixtolerant), Konic spray chamber, (Peltier cooling; quarz, 3°C)

Nebulizer: Meinhard

Optimization of signal:Optimizing of parameters such as ion optic, torch position and nebulizers pressure was done using the autotune-sequence combined with a 10 parts per billion multi element standard. Oxides and doubly charged ions should not exceed the determined level (cerium dioxide amu 156, <2%, Ce^{++} amu 70, < 3%) and the measurements should have a variation in the reproducibility for cerium, indium und lutetium of lower than 2%.

Screening: Pre analysis of the specimens to determine the necessary level of calibration and detect possible impurities.

Calibration (general): Standard calibration in parts per billion; depending on the investigated element, calibration in the respective acidic matrix.

Quantification: Peak jump mode (CPM: 5, sweeps: 100)

Target: Cerium (amu Ce 140, 142)

Internal standard: Indium (amu In 115) 10 ppb; Lutetium (amu Lu 175) 10 ppb

Assurance of data quality

a) Repetition: Every specimen was measured 5 times.

b) Control samples: To determine the validity of the measurements, the content of elements of some randomly selected specimens (selected by the Quality Assurance [QA] unit of the Fraunhofer ITEM) was additionally checked by the standard addition method (specimen plus element standards of defined concentration) to compensate for influence in the measurements like matrix effects. Three standard concentrations were added per each specimen.

The dilution of the original specimen and the concentration of the standards depended on the expected amount of the element within the specimen.

The variations of these measurements were lower than 3 percent, not showing any trend. The mean of variation was lower than 0.5 %.

c) QC-specimens: As standard material containing certified cerium amounts SRM BCR-667 estuarine sediment was used for the lung specimens and SRM BCR-670 aquatic plant for the LALN. These were measured in parallel. The variation between the measured amounts compared to the expected amount in the material was lower than 5 %. Therefore, the measured data were not affected by contamination or unexpected disturbances during the measurement process.

2.1.5 Validation of the method

For validation of the parameters and as QC-specimens to determine the amount of cerium within the specimen, standard reference material containing certified amount of cerium was used. Depending on the amount of the analyte two reference specimens were available with different cerium content:

BCR-667: Estuarine sediment - trace elements 50 - 60 µg/g

BCR-670: Aquatic plant (Lemna minor) – trace elements 0.95 – 1.05 µg/g

From each BCR-standard three samples were weighted and quantified multiple times.

2.1.6 Calibration

The measurement of the cerium content using ICP-MS was done using the standard calibration function. The calibration was performed with seven cerium standards against the zero amount solution (VBW, Blank). For quantification the main isotopes cerium 140 (88.48 % relative occurrence) and cerium 142 (11.07 % rel. occurrence) were used, which should lead to the same results when interpolating the data over the internal standards indium 115 and lutetium 175.

2.1.6.1 Standard calibration

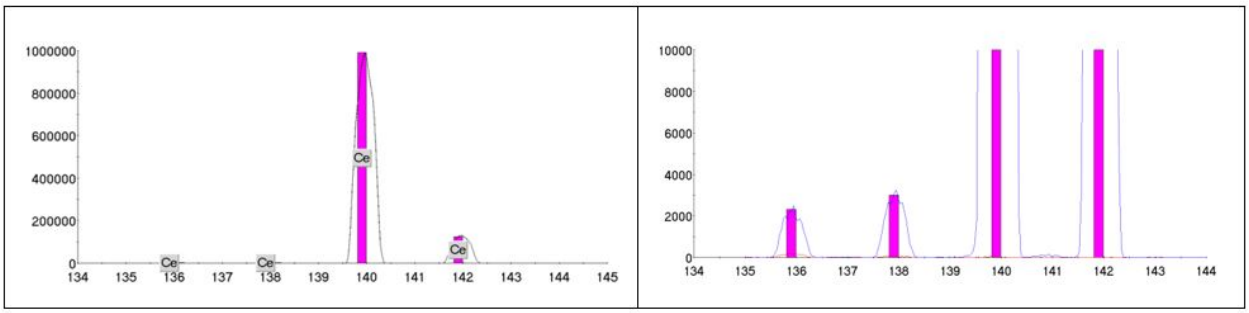
For calibration standards were measured having between 10 to 250 ppb cerium (1% sulfuric acid). As blank value an acid blank (1% sulfuric acid) was included.

2.1.6.2 Spectra of the survey scan

The survey scan spectra (figure 1 and 2) covering the relevant measurement area were without any perturbations. Potential influencing elements were not detectable during the measurement of the specimens. However, generating the survey scan (survey scan in the mass range 110 to 120 amu indium, 134 to 144 cerium and 170 to 180 lutetium) during the measurement process is very helpful to exclude possible influences on the ICP-measurement such as organic material.

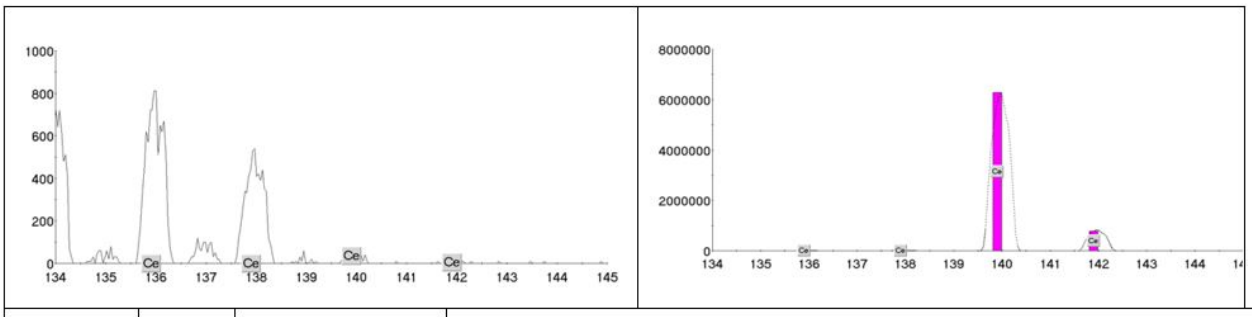
Figure 1: Survey scan spectra of cerium

Calibration standard **cerium** (1704 **25 ppb Ce,** 1% H_2SO_4)



Blank value **cerium** (1704 **blank**, 1% H_2SO_4)

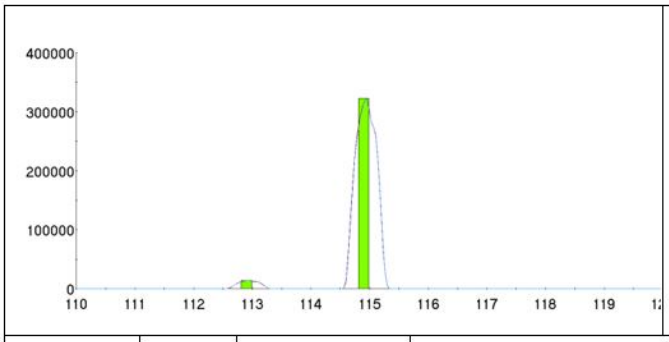
Real specimen **cerium** (1704 **lung** d28 2/66 FD100)



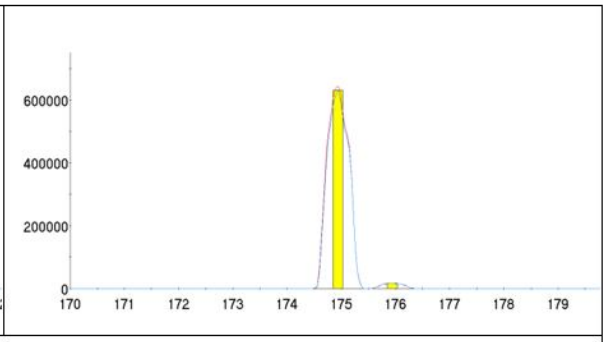
Isotope	amu	occurence (%)	possible contamination
136Ce	135.9	0.193	136Xe, **136Ba**, 16O + 120Sn, 40Ar + 96Mo, 17O H + 119Sn, 1H + 135Ba
138Ce	137.9	0.250	**138Ba**, 17O H + 121Sb, 40Ar + 98Mo, 12C + 126Te, 1H + 137Ba
140Ce	139.9	88.48	1H + 139La, 17O H + 123Sb, 12C + 128Te, 14N + 126Te, 40Ar + 100Ru
142Ce	141.9	11.07	142Nd, 1H + 141Pr, 12C + 130Te, 14N + 128Te, 40Ar + 102Ru

Figure 2: Survey scan spectra of indium and lutetium

Survey spektrum **Indium** (10 ppb)



Survey spektrum **Lutetium** (10 ppb)



Isotope	amu	occurence (%)	possible contamination
113In	112.9	4.16	113Cd, 14N + 99Tc, 1H + 112Cd, 12C + 101Ru, 17O H + 96Mo
115In	114.9	95.84	115Sn, 16O + 99Tc, 40Ar + 75As, 12C + 103Rh, 1H + 114Cd
175Lu	174.9	97.4	16O + 159Tb, 1H + 174Yb, 17O H + 158Gd, 12C + 163Dy, 14N + 161Dy
176Lu	175.9	2.6	176Yb, 176Hf, 17O H + 159Tb, 1H + 175Lu,14N + 162Dy, 40Ar + 136Ba

2.1.7 Limit of quantification

The limit of quantification was defined as the lowest concentration used during establishment of the calibration curves. Therefore, different limits of quantification might be set depending on calibration requirements.

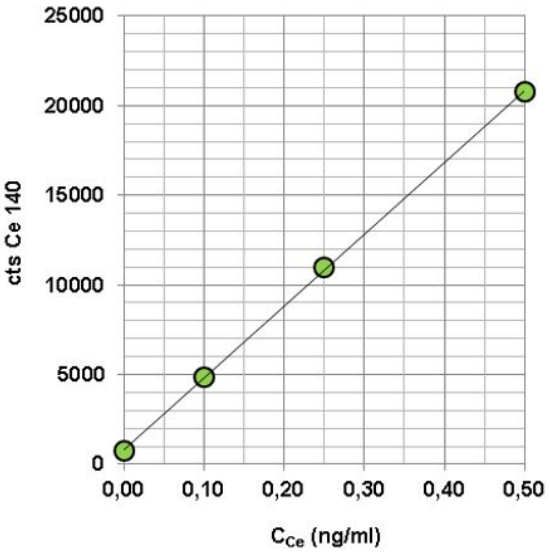
2.1.7.1 Lung tissue

With the controls and the expected low content of cerium therein a standard addition of 0.1, 0.25 and 0.50 ng Ce/ml specimen was done and the following specific limit of quantification (LOQ) was established for the lung according to DIN 32645:

Figure 3: Calibration standard of cerium in the lung

Calibration-(addition)steps

No.	Calibra-tor	Data
	(ng/ml)	cts Ce 140
1	0.000	728.75
2	**0.100**	4865.18
3	0.250	10959.31
4	0.500	20785.45



Analytic limit of detection according to DIN 32645 (DIN-TEST®)

Limit of detection: 0.008 ng/ml

Limit of quantification: 0.099 ng/ml

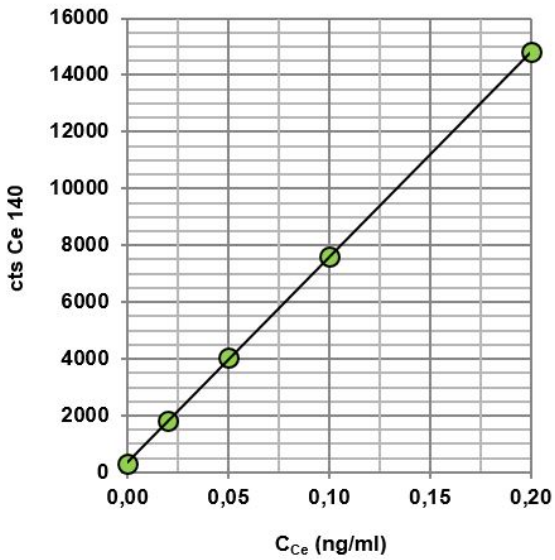
2.1.7.2 LALN

With the controls and the expected low content of cerium therein a standard addition of 0.02, 0.05, 0.10 and 0.20 ng Ce/ml specimen was done and the following specific limit of quantification (LOQ) was established for the LALN according to DIN 32645:

Figure 4: Calibration standard of cerium in the LALN

Calibration-(addition)steps

No.	Calibrator (ng/ml)	Data cts Ce 140
1	0.000	314.010
2	**0.020**	1813.480
3	0.050	4035.420
4	0.100	7627.880
5	0.200	14815.720



Analytic limit of detection according to DIN 32645 (DIN-TEST®)

Limit of detection: 0.001 ng/ml

Limit of quantification: 0.015 ng/ml

Having a volume of **25 ml** specimen (following decomposition) and a dilution factor of 10 times during the ICP-MS analysis, the background level of cerium was established as
< 0.03 µg Ce/lung and < 0.006 µg Ce/LALN.

2.1.8 Measurement of retention

The results of the quantitative measurement of cerium within the organic specimens (lung and LALN) of rats following inhalation (retention and clearance) during the 28-days BASF CeO_2 study (BASF pre-study number: 46I0661/10I151; ITEM study number: 05G12036) will be presented within the following paragraph. All data are mean data based on 4 independent measurements of 5 specimens each.

2.1.8.1 Lung

The measurements of the lung specimens of the control animals (clean air exposed group) showed a value of approximately 0.02 ng cerium per ml diluted specimen from the lung (figure 5). Therefore, the background level is lower than the defined limit of quantification of 0.1 ng cerium per ml specimen. However, it is higher than the defined limit of detection (LOD), which is 0.01 ng cerium per ml specimen.

Figure 5: Cerium content measurements in the lungs of the control group

Group 0: Clean air - 0 mg/m³ (control)

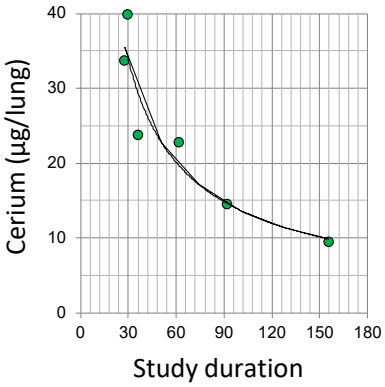
Group	Animal	Day	Lung wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
0	6	28	0.92	< 0.03		
0	12	30	1.06	< 0.03		
0	13	36	1.04	< 0.03		
0	24	62	0.97	< 0.03		
0	25	92	0.91	< 0.03		
0	26	156	0.95	< 0.03		

Within the dose group 1 (low dose) a clearance was visible within the time line investigated. In contrast, groups 2 (mid dose) and 3 (high dose) did not show a significant reduction of the initial cerium content within the lung (figure 6).

Figure 6: Cerium content measurements in the lungs of the groups 1 to 3

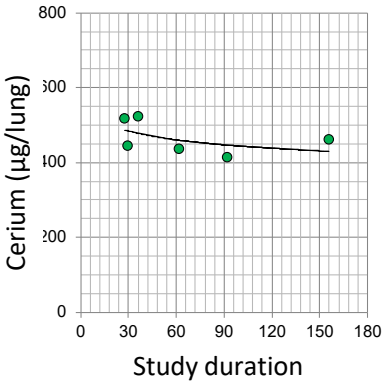
Group 1: CeO_2 - 0.5 mg/m³ (low dose)

Group	Animal	Day	Lung wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
1	36	28	0.93	33.7	0.7	2.0
1	42	30	1.03	39.9	0.8	2.1
1	43	36	0.97	23.7	0.5	2.2
1	54	62	1.00	22.7	0.5	2.2
1	55	92	0.96	14.5	0.3	2.3
1	56	156	1.13	9.5	0.2	2.5



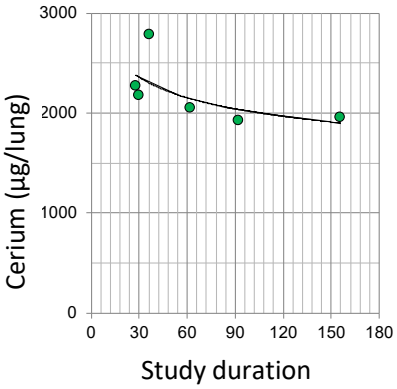
Group 2: CeO_2 - 5 mg/m³ (mid dose)

Group	Animal	Day	Lung wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
2	66	28	1.20	517	3	0.6
2	72	30	0.97	444	2	0.5
2	73	36	1.12	521	4	0.8
2	84	62	1.07	436	4	0.9
2	85	92	0.99	414	3	0.7
2	86	156	1.12	460	1	0.2



Group 3: CeO_2 - 25 mg/m³ (high dose)

Group	Animal	Day	Lung wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
3	96	28	1.16	2268	5	0.2
3	102	30	1.23	2174	6	0.3
3	103	36	1.31	2789	35	1.3
3	114	62	1.15	2056	3	0.1
3	115	92	1.13	1930	3	0.2
3	116	156	1.20	1962	9	0.5



2.1.8.2 LALN

The measurements of the LALN specimens of the control animals (clean air exposed group) showed a value of lower than 0.006 ng cerium per ml diluted specimen from the LALN (figure 7). Therefore, the measurement staid below the defined limit of quantification.

Figure 7: Cerium content measurements in the LALN of the control group

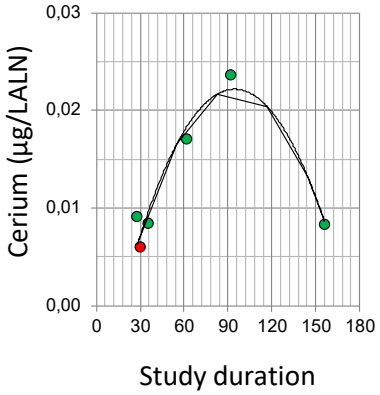
Group 0: Clean air - 0 mg/m³ (control)						
Group	Animal	Day	LALN wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
0	6	28	0.056	< 0.006		
0	12	30	0.038	< 0.006		
0	13	36	0.035	< 0.006		
0	24	62	0.040	< 0.006		
0	25	92	0.042	< 0.006		
0	26	156	0.021	< 0.006		

Within the dose group 1 (low dose) the initial increase of cerium was followed by a clearance within the time line investigated. In contrast, groups 2 (mid dose) and 3 (high dose) revealed a significant increase of cerium content within the lung-associated lymph nodes (figure 8).

Figure 8: Cerium content measurements in the LALN of the groups 1 to 3

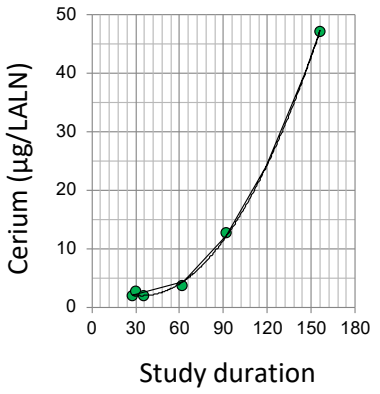
Group 1: CeO_2 - 0.5 mg/m³ (low dose)

Group	Animal	Day	LALN wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
1	36	28	0.059	0.009	0.0001	0.8
1	42	30	0.050	0.006		
1	43	36	0.062	0.008	0.0001	0.9
1	54	62	0.040	0.017	0.0001	0.5
1	55	92	0.032	0.024	0.0003	1.4
1	56	156	0.012	0.008	0.0003	3.4



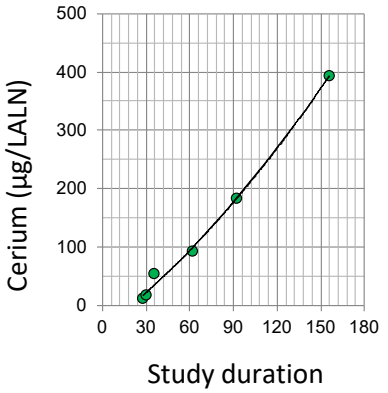
Group 2: CeO_2 - 5 mg/m³ (mid dose)

Group	Animal	Day	LALN wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
2	66	28	0.054	1.9	0.02	0.9
2	72	30	0.031	2.5	0.01	0.5
2	73	36	0.040	1.9	0.01	0.6
2	84	62	0.060	3.6	0.01	0.3
2	85	92	0.054	12.7	0.05	0.4
2	86	156	0.045	47.1	0.25	0.5



Group 3: CeO_2 - 25 mg/m³ (high dose)

Group	Animal	Day	LALN wet weight	Cerium		
				Mean	Asd	Rsd
			g	(µg/organ)		(%)
3	96	28	0.050	10.9	0.1	0.7
3	102	30	0.055	17.1	0.0	0.3
3	103	36	0.086	53.6	0.1	0.3
3	114	62	0.080	93.4	0.5	0.6
3	115	92	0.060	183	0.8	0.4
3	116	156	0.093	394	2.5	0.6



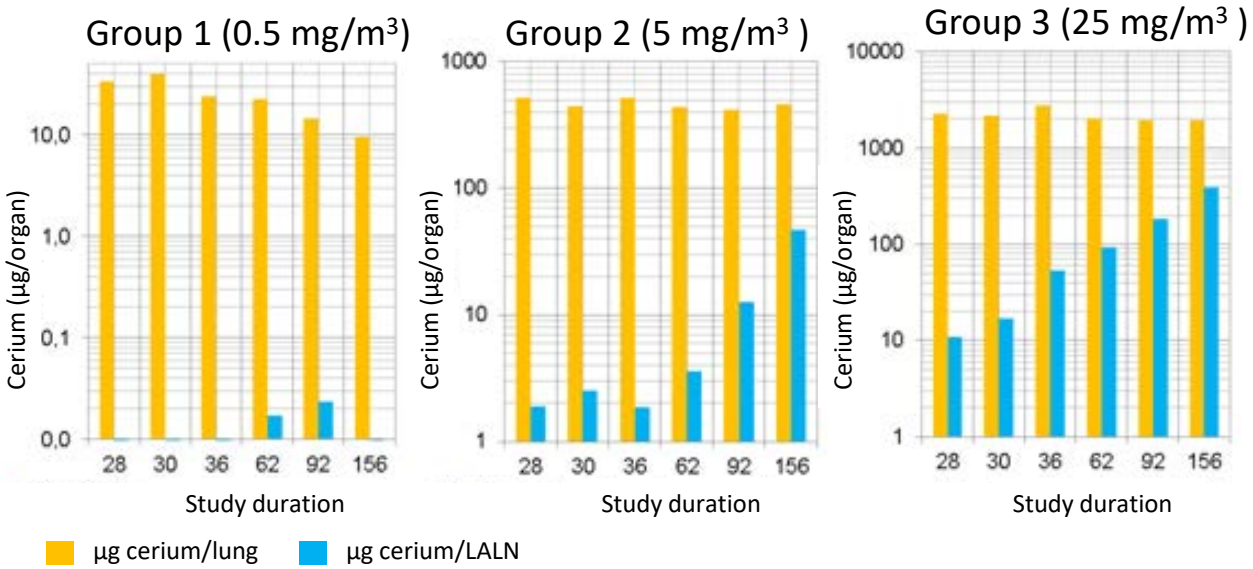
2.1.9 Summary of retention measurements in lungs and LALN

The lungs of group 1 (low dose) revealed an obvious clearance within the time period investigated. In contrast, groups 2 (mid dose) and 3 (high dose) did not show a significant reduction of the initial cerium content within the lungs. There is a linear correlation of the exposure dose and the cerium content in the lungs. Furthermore, there is evidence of a particle overload beginning at a concentration of > 0.5 mg / m^3.

Within the LALN cerium was detectable in all cerium exposed groups (groups 1 to 3). Furthermore, groups 2 and 3 showed a significant dose- and time-dependent increase of cerium within the LALN.

Within the control group 0 (clean air control) no cerium was detectable within the lung or LALN at any time investigated. The measured values were below the defined limit of quantification.

Figure 9: Cerium content within the lungs and LALN



3 Work package 2

3.1 Histochemical investigation of lung tissue for genotoxicity following 28 days (pre-study) and 12 months (main study) inhalation of nano-CeO_2

3.1.1 Specimens for the histochemical analysis

The specimens for this investigations after 28 days (table 2) and 12 months exposure (table 3) were acquired from BASF SE from the following 2 studies: BASF pre-study, number: 46I0661/10I151 and BASF main study, number: 81I0661/I10I170.

Table 2: Overview of specimens used for the genotoxicity investigation (pre-study, 28 days exposure)

Time point of investigation	Group	Material	Concentration (mg/m^3)	Amount of lung tissue
29 and 61 days	0	Clean air	0	5
29 and 61 days	1	CeO_2	0.5	5
29 and 61 days	2	CeO_2	5	5
29 and 61 days	3	CeO_2	25	5

Table 3: Overview of specimens used for the genotoxicity investigation (main study, 12 months exposure)

Time point of investigation	Group	Material	Concentration (mg/m^3)	Amount of lung tissue
12 months	40	Clean air	0	10
12 months	41	CeO_2	0.1	10
12 months	42	CeO_2	0.3	10
12 months	43	CeO_2	1	10
12 months	44	CeO_2	3	10

Rat lungs from 5 or 10 animals of the negative control (clean air) and the CeO_2-exposure groups were fixed by instillation using 4% formaldehyde solution following sacrifice of the animals, transferred to

70% ethanol after 24 to 48 h and embedded in paraplast. After acquiring slices for routine histopathological investigation at BASF SE, blocks of the remaining lung tissue were sent to Fraunhofer ITEM for further immunohistological investigation of genotoxicity markers.

3.1.2 Histochemical investigation of lung tissue for genotoxicity

3.1.2.1 Selection of lung tissue blocks

The analyses were done in the left main lung lobe (lobus sinister, dorsal part) by evaluating 20 lung areas per lung and animal. Every animal was investigated accordingly.

3.1.2.2 Preparation of lung slices and immunhistochemical detection of genotoxicity markers γ-H2AX and 8-OHdG

For the immunhistological staining 3 µm thin slices were prepared on Superfrost plus® glas slides. Thereafter, they were deparaffinized and an appropriate antigen retrieval was done for the respective fixation and immunohistology marker (according to Fraunhofer ITEM specific standard operation procedure no. 050520). The immunhistological staining was conducted using a biotinylated secondary antibody and a chromogen. The first immunohistochemistry consisted of a primary antibody directed against phosphorylated H2AX (γ-H2AX; mouse monoclonal antibody to γ-H2AX [phospho S139] "DNA double-strand break marker [ab26350]") (Abcam, Cambridge, UK) to detect possible genotoxicity like double strand breaks.

For the second immunohistochemistry an antibody directed against 8-Hydroxy-2'-desoxyguanosine (8-OHdG) was used (mouse monoclonal antibody [N45.1] to 8-hydroxy-2'-deoxyguanosine [ab48508]) (Abcam, Cambridge, UK) to visualize possible genotoxicity like premutagenic oxidative base modifications.

Slides of both immunhistochemical stainings were counterstained using hematoxylin and cover slipped. The aforementioned two markers, antibodies were selected for this investigation since they turned out to be the most sensitive in a previous study by Rittinghausen and coworkers (Rittinghausen et al., 2013).

3.1.3 Image analysis and quantification

Image analysis of immunhistologically stained lung slices was conducted using digital photographs (Zeiss MIRAX-Scanner and -Viewer), which were analyzed with an image analysis software (Zeiss Axiovision 4.8.2). From each lung lobe used 5 bronchioles were selected and digital photographs were taken above, below, left and right from the lung tissue next to the bronchiole (20 areas per lung). Within the digital photograph the regions of interest (ROI) were defined and the amount of γ-H2AX or 8-OHdG positive nuclei was determined. Cells within the alveolar region such as macrophages were excluded from counting. The threshold for 8-OHdG and γ-H2AX positive cells was set as 206-250 (hue, H), 72-156 (lightness, L), and 56-132 (saturation, S). For negative cells the threshold was 174-205 (hue, H), 78-165 (lightness, L), and 50-97 (saturation, S) within the HLS color space.

For initial validation of the digital image analysis 5 photographs (8-OHdG and γ-H2AX) were assessed manually and automatically by 2 separate persons individually. The intra- and interpersonal variability was lower than 10 %.

3.1.3.1 Documentation of results

All acquired data of the image analysis was printed out, signed and will be archived. Furthermore, every analyzed photograph as well as the generated data was saved electronically.

3.1.3.2 Statistics of image analysis data

For statistical evaluation the results were exported to excel. The treated animals were compared to the control animals. The analysis was done using the Statistica software (StatSoft, INc. 1984-2010, Tulsa, OK, USA, Release 12).

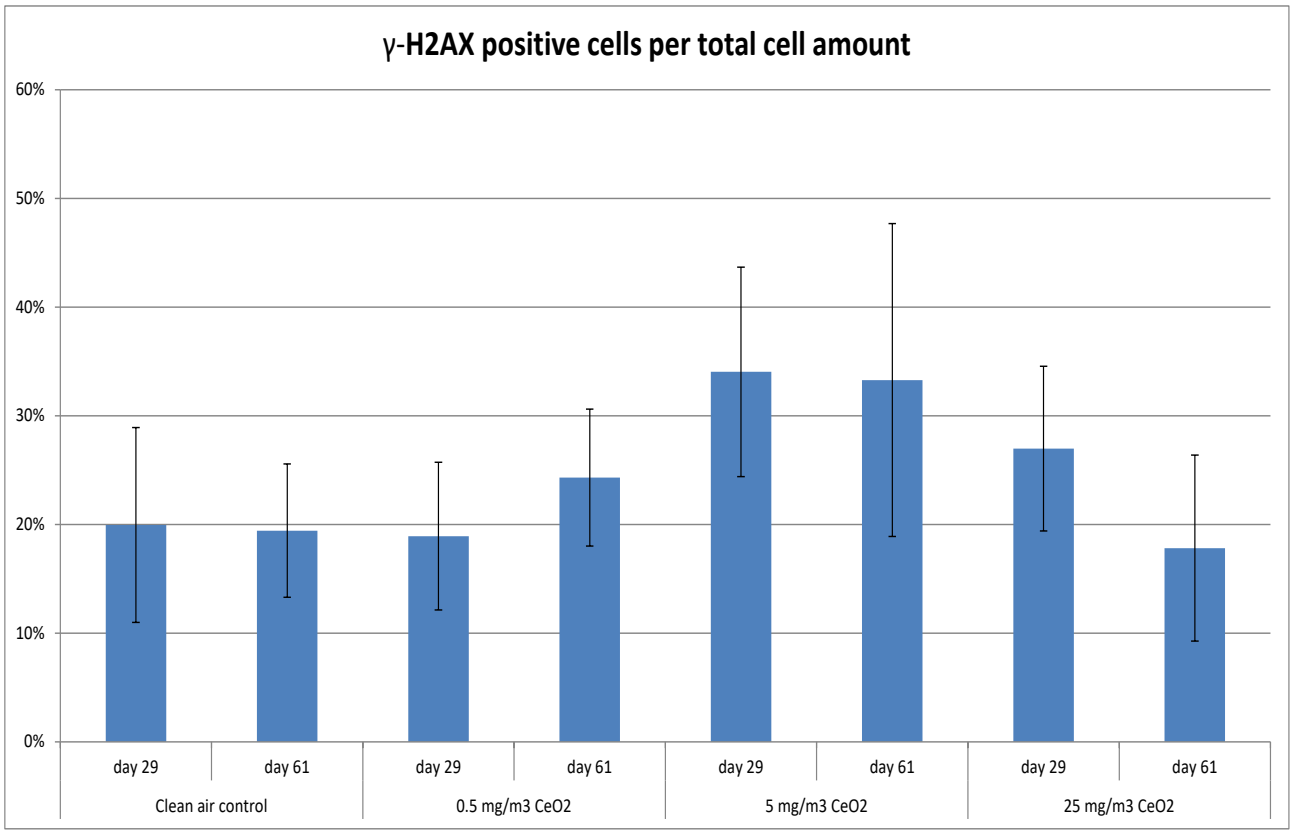
3.1.4 Results of image analysis

3.1.4.1 28 days of exposure

The two factorial analysis of variance (ANOVA) of the γ-H2AX positive cells per total cell amount (figure 10) showed a statistically significant influence of the group (p=0.007879).

The following 2-sided post-hoc Dunnett test showed a significant difference of the mid dose group (group 2; p=0.004566), whereas the high dose group did not reveal any significant difference (p=0.808868). Therefore, no dose-related effect could be established.

Figure 10: γ-H2AX positive cells per total cell amount after 28 days of exposure

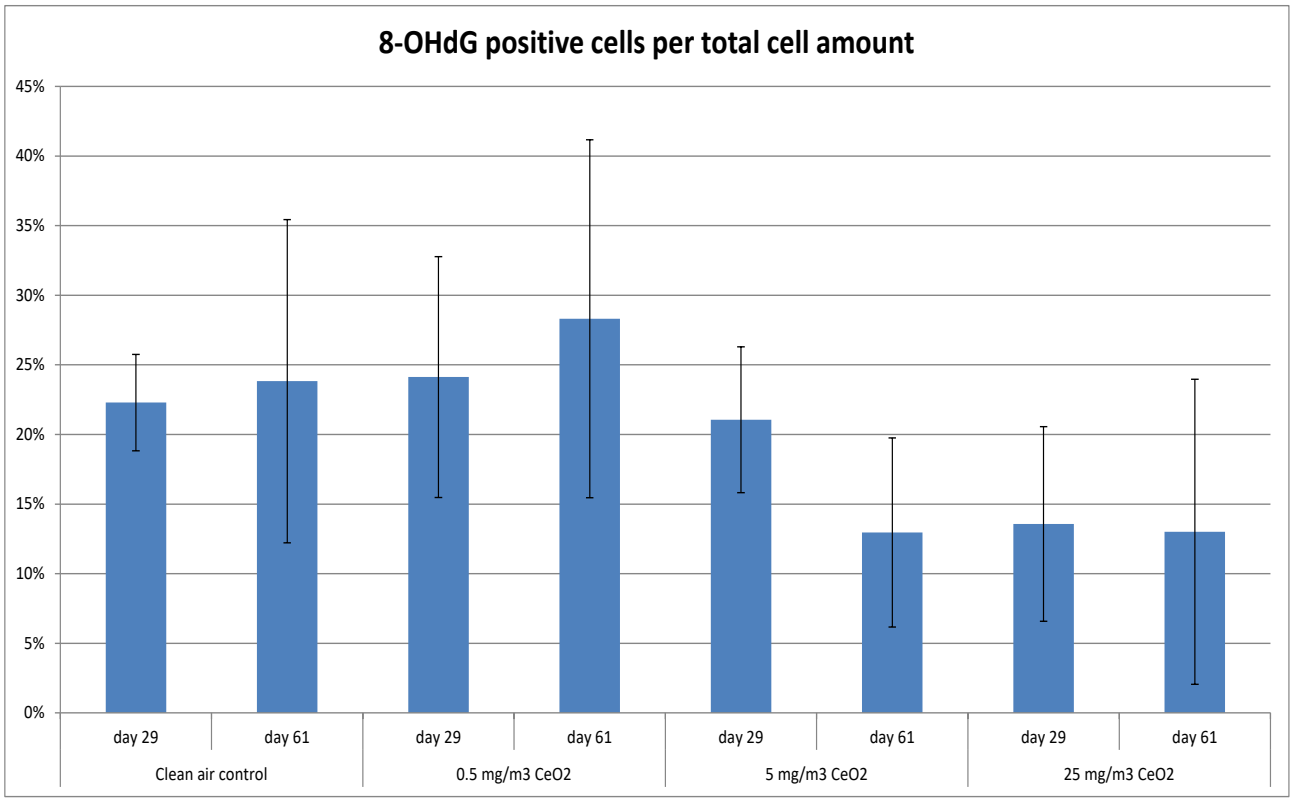


Arithmetic mean and standard deviation

The two factorial analysis of variance (ANOVA) of the 8-OHdG positive cells per total cell amount (figure 11) showed a statistically significant influence of the group (p=0.011461).

The following 2-sided post-hoc Dunnett test showed a significant difference of the high dose group (group 3; p=0.043956). However, the significance is caused by a decline of positive cells as visible in figure 11.

Figure 11: 8-OHdG positive cells per total cell amount after 28 days of exposure



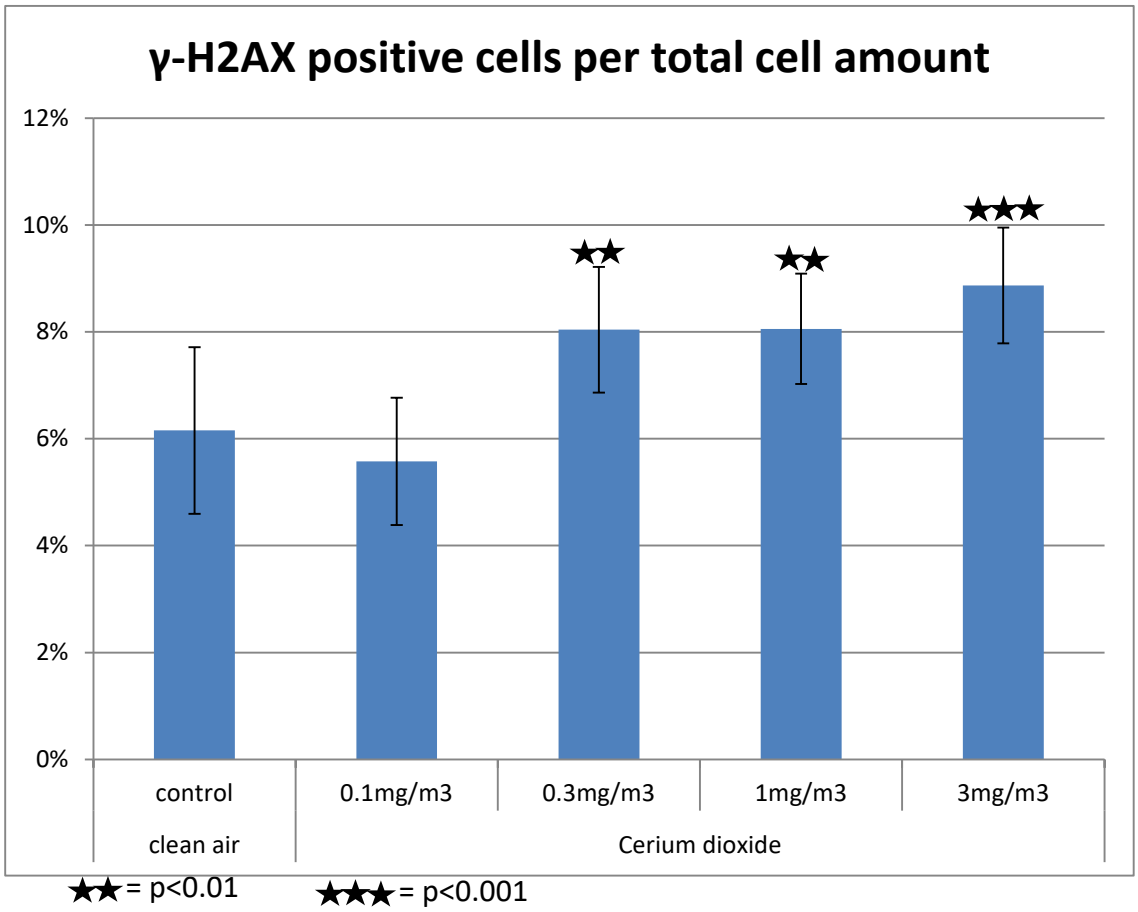
Arithmetic mean and standard deviation

No distinct influence of cerium dioxide particles on genotoxicity could be shown using both immunhistological investigations (8-OHdG and γ-H2AX). Though single significant differences could be shown, no clear conclusion and no dose-dependent effect could be established. A possible influence of different times of fixation and storage of wet tissue prior to embedding could not be ruled out completely.

3.1.4.2 12 months exposure

The one factorial analysis of variance (ANOVA) of the γ-H2AX positive cells per total cell amount (figure 12) showed a statistically significant influence of the group ($p<0.001$). The following 2-sided post-hoc Dunnett test showed a significance of the high dose group (CeO_2-3mg/m³; $p=0.0001$) and both mid dose groups (CeO_2-1mg/m³ and CeO_2-0.3mg/m³; $p=0.0069$ and $p=0.0074$, respectively), whereas the low dose group, CeO_2-0.1mg/m³, did not have a significant difference compared to the clean air control group ($p=0.713$).

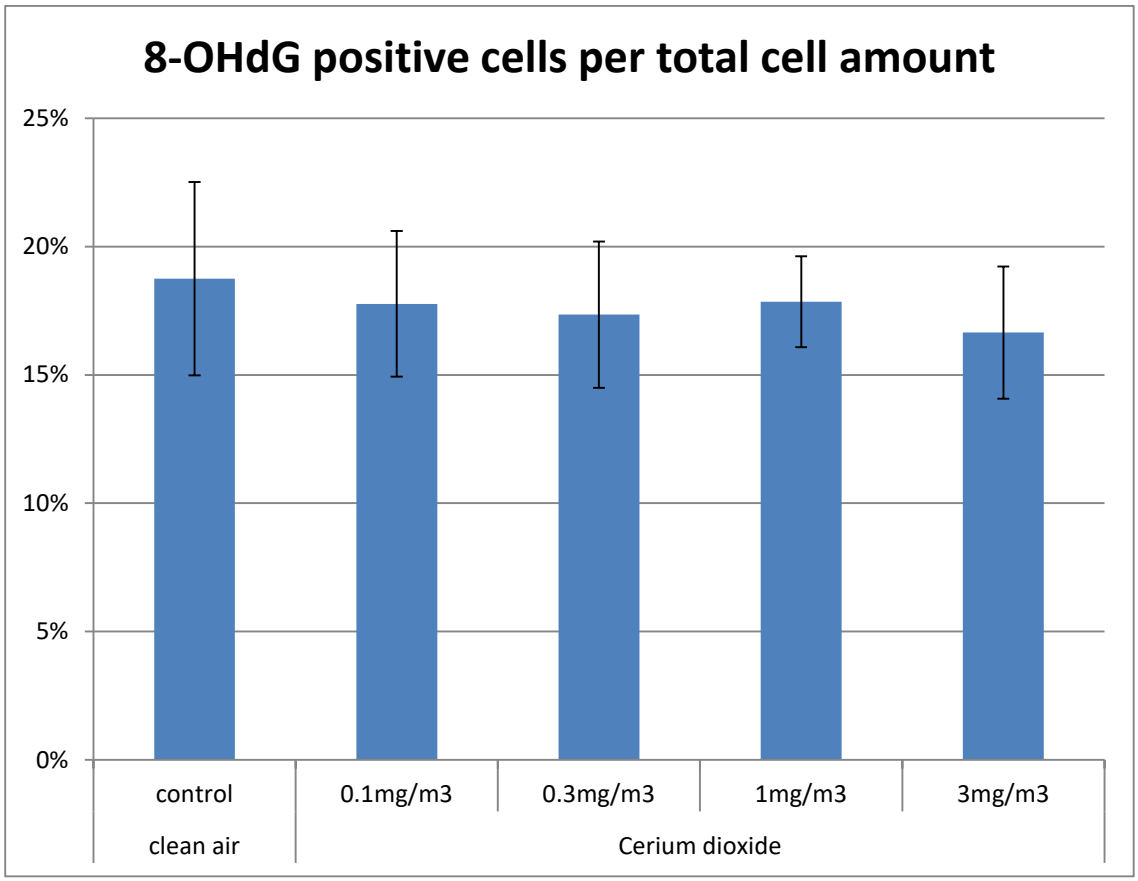
Figure 12: γ-H2AX positive cells per total cell amount after 12 months of exposure



Arithmetic mean and standard deviation

The image analytical quantification of 8-OHdG positive cells per total cell amount (figure 13) did not reveal any significant difference between the different groups (p=1).

Figure 13: 8-OHdG positive cells per total cell amount after 12 months of exposure



Arithmetic mean and standard deviation

In summary, 12 months inhalation of cerium dioxide nanoparticles did not lead to an increase in oxidative damage, specifically investigated by immunhistological staining of 8-OHdG. However, there was a statistically significant increase in γ-H2AX positive cells stained by immunhistochemistry within the mid and high dose groups (0.3, 1 and 3 mg/m³) compared to the clean air control (figure 12). Therefore, a genotoxic activity of cerium dioxide nanoparticles after inhalation could not be ruled out completely. If this increase in γ-H2AX positive cells might be due to a direct genotoxic double strand break of the DNA or an initial increase of γ-H2AX to deal with possible DNA strand breaks could not be identified by this investigation. Another possible cause of this increase might be cerium dioxide evoked cellular apoptosis or senescence.

A possible influence on the immunohistochemistry due to different times of fixation and storage of wet tissue prior to embedding could not be ruled out completely.

4 Work package 3

4.1 Histopathological investigations

4.1.1 Material and methods (carcinogenicity study, 24 months and 30 months)

4.1.1.1 Experimental animals and test group design

The main group animals of the 24 months CeO_2 exposure at BASF were allocated to one control and 5 exposure groups (table 4).

Table 4: Main group animals (carcinogenicity, 24 months)

Test group	Substance	Concentration (mg/m³)	No. of animals	Animal No.
00	Air Control	0	50	1 - 50
01	CeO_2	0.1	50	101 - 150
02	CeO_2	0.3	50	201 - 250
03	CeO_2	1	50	301 - 350
04	CeO_2	3	50	401 - 450

The post-exposure group animals which had a recovery period of 6 months following 24 months CeO_2 exposure at BASF were also allocated to one control and 5 exposure groups (table 5).

Table 5: Post-exposure group animals (carcinogenicity, 30 months)

Test group	Substance	Concentration (mg/m³)	No. of animals	Animal No.
50	Air Control	0	50	51 - 100
51	CeO_2	0.1	50	151 - 200
52	CeO_2	0.3	50	251 - 300
53	CeO_2	1	50	351 - 400
54	CeO_2	3	50	451 - 500

4.1.1.2 Organ/tissue fixation, sample shipment and histotechnical processing

Following sacrifice of the animals after 24 months of CeO_2 exposure at BASF or after the post-exposure period at 30 months, all organs and tissues were fixed and stored in 4% buffered formaldehyde. The lungs were transferred to 70% ethanol following a 24-48h fixation time in formaldehyde. All wet tissues from groups 00-04 and 50-54 together with the individual macroscopic findings were shipped to the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM for histotechnical processing. Histotechnical processing of the organs included trimming according to Ruehl-Fehlert et al. (2003), Kittel et al. (2004), Morawietz et al. (2004) as well as according to internal Standard Operation Procedures.

The investigation of the yellow highlighted organ, the lung, was the purpose of the research project described here, whereas the investigation of the other organs was subject to a separate research project.

Table 6: Organs/tissues of animals sacrificed after 24 months of exposure and after 30 months

Organs	**Test group**	**Test group**	**Test group**	**Test group**	**Test group**
	00 **50**	**01** **51**	**02** **52**	**03** **53**	**04** **54**
1. All gross lesions[1)]	A2	A2	A2	A2	A2
2. Adrenal glands	A1	A1	A1	A1	A1
3. Aorta	A1	A1	A1	A1	A1
4. Bone marrow (femur)	A1	A1	A1	A1	A1
5. Brain	A1	A1	A1	A1	A1
6. Cecum	A1	A1	A1	A1	A1
7. Cervix	A1	A1	A1	A1	A1
8. Colon	A1	A1	A1	A1	A1
9. Duodenum	A1	A1	A1	A1	A1
10. Esophagus	A1	A1	A1	A1	A1
11. Eyes and optic nerve	A1	A1	A1	A1	A1
12. Extraorbital lacrimal glands	A1	A1	A1	A1	A1
13. Femur and knee joint	A1	A1	A1	A1	A1
14. Harderian glands	A1	A1	A1	A1	A1
15. Heart	A1	A1	A1	A1	A1
16. Ileum	A1	A1	A1	A1	A1
17. Jejunum	A1	A1	A1	A1	A1
18. Kidneys	A1	A1	A1	A1	A1
19. Larynx (3 levels)	A1	A1	A1	A1	A1
20. Liver	A1	A1	A1	A1	A1
21. Lungs	**A/T1**	**A/T1**	**A/T1**	**A/T1**	**A/T1**
22. Lymph nodes (tracheobronchial, mediastinal)	A1	A1	A1	A1	A1
23. Lymph nodes (axillary, mesenteric)	A1	A1	A1	A1	A1
24. Mammary gland (females)	A1	A1	A1	A1	A1
25. Nasal cavity (4 levels)	A1	A1	A1	A1	A1

Organs	**Test group**	**Test group**	**Test group**	**Test group**	**Test group**
	00 **50**	**01** **51**	**02** **52**	**03** **53**	**04** **54**
26. Olfactory bulb	A1	A1	A1	A1	
27. Ovaries	A1	A1	A1	A1	A1
28. Oviducts	A1	A1	A1	A1	A1
29. Pancreas	A1	A1	A1	A1	A1
30. Parathyroid glands	A1	A1	A1	A1	A1
31. Pharynx	A1	A1	A1	A1	A1
32. Pituitary gland	A1	A1	A1	A1	A1
33. Rectum	A1	A1	A1	A1	A1
34. Salivary glands (parotid, mandibular, sublingual)	A1	A1	A1	A1	A1
35. Sciatic nerve	A1	A1	A1	A1	A1
36. Spinal cord (cervical, thoracic, lumbar)	A1	A1	A1	A1	A1
37. Spleen	A1	A1	A1	A1	A1
38. Stomach (forestomach, glandular stomach)	A1	A1	A1	A1	A1
39. Sternum with bone marrow	A1	A1	A1	A1	A1
40. Skeletal muscle	A1	A1	A1	A1	A1
41. Skin	A1	A1	A1	A1	A1
42. Teeth	A1	A1	A1	A1	A1
43. Thymus	A1	A1	A1	A1	A1
44. Thyroid glands	A1	A1	A1	A1	A1
45. Tongue	A1	A1	A1	A1	A1
46. Trachea	A1	A1	A1	A1	A1
47. Ureter	A1	A1	A1	A1	A1
48. Urethra	A1	A1	A1	A1	A1
49. Urinary bladder	A1	A1	A1	A1	A1
50. Uterus	A1	A1	A1	A1	A1
51. Vagina	A1	A1	A1	A1	A1

[1)] Gross lesions, which were detected during the necropsy of the animals were processed separately

Methods(scope of examinations:

A= Hematoxylin-Eosin stain

T= Masson-Trichrome stain

1= All animals per test group

2= All animals affected per test group

4.1.1.3 Examination by light microscopy and assessment of findings

Light microscopical examination of all hematoxylin-eosin stained slides and a correlation between gross lesions and histopathological findings was performed by the undersigned Fraunhofer ITEM pathologist (Principal Investigator, PI). All gross lesions were recorded and tabulated at BASF. All macroscopic and microscopic findings were entered into the Fraunhofer ITEM pathology software system (Provantis). Macroscopic findings were correlated to microscopic changes, whenever possible.

Histologic alterations were described, wherever possible, according to their distribution (focal, multifocal, diffuse), severity (grades) and morphologic character.

4.1.1.4 Extended processing of the lungs

After fixation, all 5 lung lobes were separated. The left lobe (lobe 5) and the right caudal lobe (lobe 3) were split in 2 halves which were embedded individually with the ventral surface showing downside. The complete lung tissue was blocked into mega capsules (height: 10 mm) according to the following scheme:

Lobe 5 (left lobe, dorsal half): capsule 8A-D

Lobe 5 (left lobe, ventral half): capsule 8A-V

Lobe 3 (right caudal lobe, dorsal half): capsule 8B-D

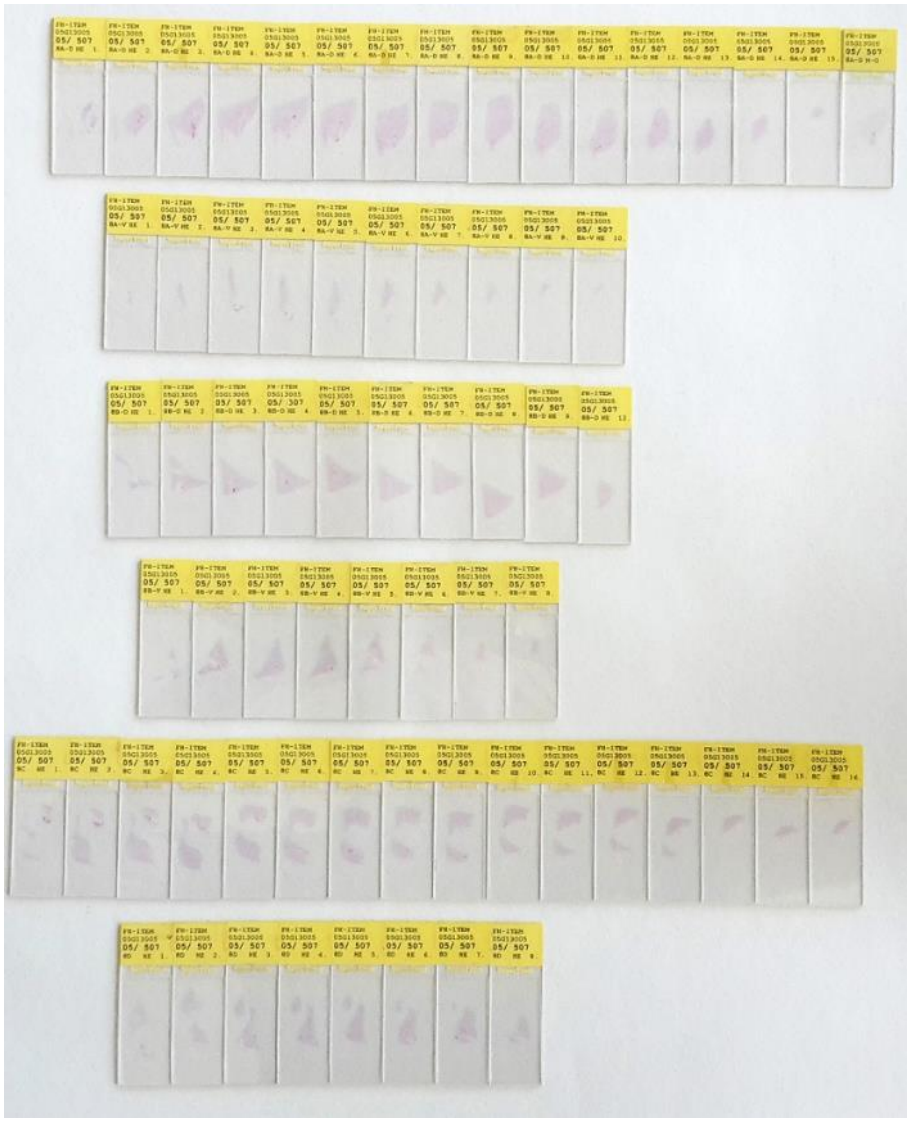
Lobe 3 (right caudal lobe, ventral half): capsule 8B-V

Lobes 1 (right cranial lobe) and 2 (right middle lobe): capsule 8C

Lobe 4 (accessory lobe): capsule 8D

Following dehydration and blocking into paraplast wax, the lung tissues from all rats were step-sectioned at intervals of 500 µm until no lung tissue was left in the blocks. By this method at least 60 step sections of 3 µm thickness per rat lung were obtained (figure 14), H&E-stained and examined under the light microscope. An additional section of the left lung lobe (dorsal half) from all animals was stained with Masson trichrome for assessment of fibrotic changes.

Figure 14: Example of step sections of one rat lung



4.1.1.5 Extended histopathological examination of the lungs

Prior to data entry into the Provantis system, all lesions observed in the lung step sections were first entered animal-wise into a WORD document (figure 15). Following examination of all step sections per lung, the diagnoses were summarized on the last page (5/5) of the WORD document (figure 15), undersigned by the PI and then entered into the Provantis system.

For neoplastic lesions detected in the step sections, the surface area was determined by means of two diameters: longest diameter x mean vertical diameter. Measurements were undertaken using an eyepiece graticule in the microscope (one-centimeter square divided into 100 subsquares). The surface area was given in square millimeters. Surface area x section interval (0.5 mm) = volume of a tumor slice. By adding up all slices determined for a tumor, an approximate value for the whole tumor volume could be calculated (Kolling et al. 2008).

The respective WORD documents from all histologically examined animals are not defined as raw data, but will be archived together with the individual animal data reports which are produced by the Provantis system and signed by the PI as non-GLP documents.

4.1.1.6 Grades and nomenclature used for histopathology

The grades were used for a grading system that takes into consideration either the severity or the number or the size of a microscopic finding (Table 7). The severity of each lesion was graded on a scale of very slight to very severe, indicating the approximate fraction of the organ/tissue or organ structure to be involved.

The nomenclature used was according to INHAND [International Harmonization of Nomenclature and Diagnostic Terms] (Renne et al. 2009*).*

Table 7: Grading system of histopathology

Grade	Severity	Percentage	Number	Size
Grade 1	Very slight (Minimal)	= 1-5%	Very few	Very small
Grade 2	Slight (Mild)	= 6-20%	Few	Small
Grade 3	Moderate	= 21-50%	Moderate number	Moderate size
Grade 4	Severe (Marked)	= 51-74%	Many	Large
Grade 5	Very Severe (Massive)	= 75-100%	Extensive number	Extensive size

Figure 15: Example of WORD table for animal-wise data entry of pulmonary step sections with summary of lesions

DIAGNOSES

05G13005 / BASF-Project-No. 81I0661I10I170 **Dose: 0,1 mg/m³ CeO_2** **Group/Animal No.: 01/0101**

Lung lobe 5 (8 A-D and 8 A-V)	**Level of lobe 5, A-D**													**Level of lobe 5, A-V**								
Non-neoplastic Lesions	**12**	**11**	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
no visible lesion (x)																						na
alv./interst. acc. of particle-laden macrophages	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	1m	
acc. of particle-laden macrophages, BALT				1f	1m	1m	1m	1m	1m	1m	2f	1f	1m	1m		1f	1f	1f			1f	
intra-alveolar particles present (x)	x																					
multinucleated giant cells						1f																
alv./interst. inflammatory cell infiltration		1f				1m	2m	2m	1m	2f	1m	1m		1f		1m	1f		2f		1f	
alv./interst. granulomatous inflammation																						
interstitial fibrosis		1f		1m	1m	1m	2f	2f	2f	3f*	1f	2f			1f	1f						
pleural fibrosis			1f									2f										
alveolar lipoproteinosis																						
cholesterol granuloma																						
bronchial mucous cell hyperplasia																						
bronch.-alv. hyperplasia (at, bt, mt)															1f bt						1f bt	
pleural mononuclear cell infiltration																		1f				
psammoma body																						
osseous metaplasia																			1f			
Pre-neoplastic Lesions / Neoplasms																						
bronch.-alv. hyperplasia with atypia																						
bronchiolo-alveolar adenoma						0.5	3.7	5.3	5.8	6,0	4.2	2.6	2.6	2.6	2.6	0.5						
bronchiolo-alveolar carcinoma																						

*within adenoma

01/0101 – Summary:

Non-neoplastic Lesions:

Very slight multifocal bronch.-alv. hyperplasia, bronchiolar type
Very slight multifocal alv./interst. acc. of particle-laden macrophages
Slight focal acc. of particle-laden macrophages, BALT
(Free) particles present, intra-alveolar/intra-bronchial/-bronchiolar
Very slight multifocal multinucleated giant cells
Slight multifocal alv./interst. inflammatory cell infiltration
Moderate focal interstitial fibrosis: within adenoma
Slight focal pleural fibrosis
Slight focal pleural mononuclear cell infiltration
Very slight focal alveolar hemorrhage

Pre-neoplastic lesions / Neoplasms (incl. volume of tumors):

Bronchiolo-alveolar adenoma (Volume: 18.1 mm³)

Date, Signature (Study Pathologist)

Abbreviations: f = focal, m = multifocal, na = section level not available, bt = bronchiolar type,
1 – 3 = grades (see table 7); the colored numbers show the size of the neoplasm in mm2

4.1.1.7 Data compilation

Macroscopic data were recorded together with the microscopic findings by the undersigned Fraunhofer ITEM pathologist using on-line input into the Provantis computer system (version 8.3; INSTEM Life Science Systems, UK). All macroscopic and microscopic observations are presented for each rat in the 'Individual Animal Reports'. The incidences of macroscopic and microscopic findings are also presented in tabular form. Incidence tables were created by the Provantis computer system and will be archived according to 4.1.3.

4.1.1.8 Statistics of histopathology

The statistical analysis was performed with the Provantis system for each sex using a Chi-squared and 2-sided Fisher's Exact test.

4.1.2 Peer review of histopathological findings

Following the initial examination by the Principal Investigator, an internal pathology peer review of the lungs from selected animals of test groups 00-04 and 50-54, of all neoplastic and pre-neoplastic lesions as well as of the lungs from 10% randomly selected animals of all test groups was performed according to Fraunhofer ITEM SOP 050708 by PD Dr. Susanne Rittinghausen (Fraunhofer ITEM, Hannover, Germany). In addition, a pathology working group was installed thereafter consisting of three internationally recognized experts in this research area and sponsored by the Federal Institute for Occupational Safety and Health. The results of the peer review and the pathology working group are documented in the finding tables and will be archived as raw data.

Results presented in this report reflect the consensus opinion of the study pathologist, the peer review pathologist and the pathology working group.

4.1.3 Archiving

All wet tissues, slides, blocks and data sheets containing the macroscopic findings will be sent back to BASF and will be archived for at least the period of time specified in the GLP principles. The signed final pathology phase report and the signed individual animal reports (raw data) will also be sent to BASF. Copies of the study plan, the final pathology phase report, the histopathology incidence tables and the individual animal reports on macroscopic and microscopic findings are maintained in the histology archive of Fraunhofer ITEM.

4.2 Results of the CeO_2 exposure groups (carcinogenicity, 24 months and 30 months)

4.2.1 Main group animals (carcinogenicity, 24 months)

4.2.1.1 Gross lesions

The **lungs** (Table 8) revealed mainly white or grey foci measuring between 1 and 6 mm in diameter on the surfaces. These were dose-dependently increased in the cerium groups (up to 34/50) and corresponded predominantly with alveolar/interstitial accumulations of particle-laden macrophages. Masses were seen in the lungs of single animals one each of the control group (test group 00) and the CeO_2 mid-dose group (test group 02) and correlated with tumor metastases from primary tumors in other organs.

Discolorations were observed in single females one each of test groups 00 and 01 and correlated with congestion and hemorrhage.

Table 8: Macroscopic findings – lungs (main group animals)

Treatment	**Clean Air**	**CeO_2 0.1 mg/m³**	**CeO_2 0.3 mg/m³**	**CeO_2 1 mg/m³**	**CeO_2 3 mg/m³**
No. of rats	**50**	**50**	**50**	**50**	**50**
Discoloration	1	1			
Focus/Foci	5	11	17	27	34
Mass	1		1		

4.2.1.2 Histopathology

Within the following chapters, the results of the histopathology of the lung of the 24 months group applying the extended histopathology with step sections of the lung are presented.

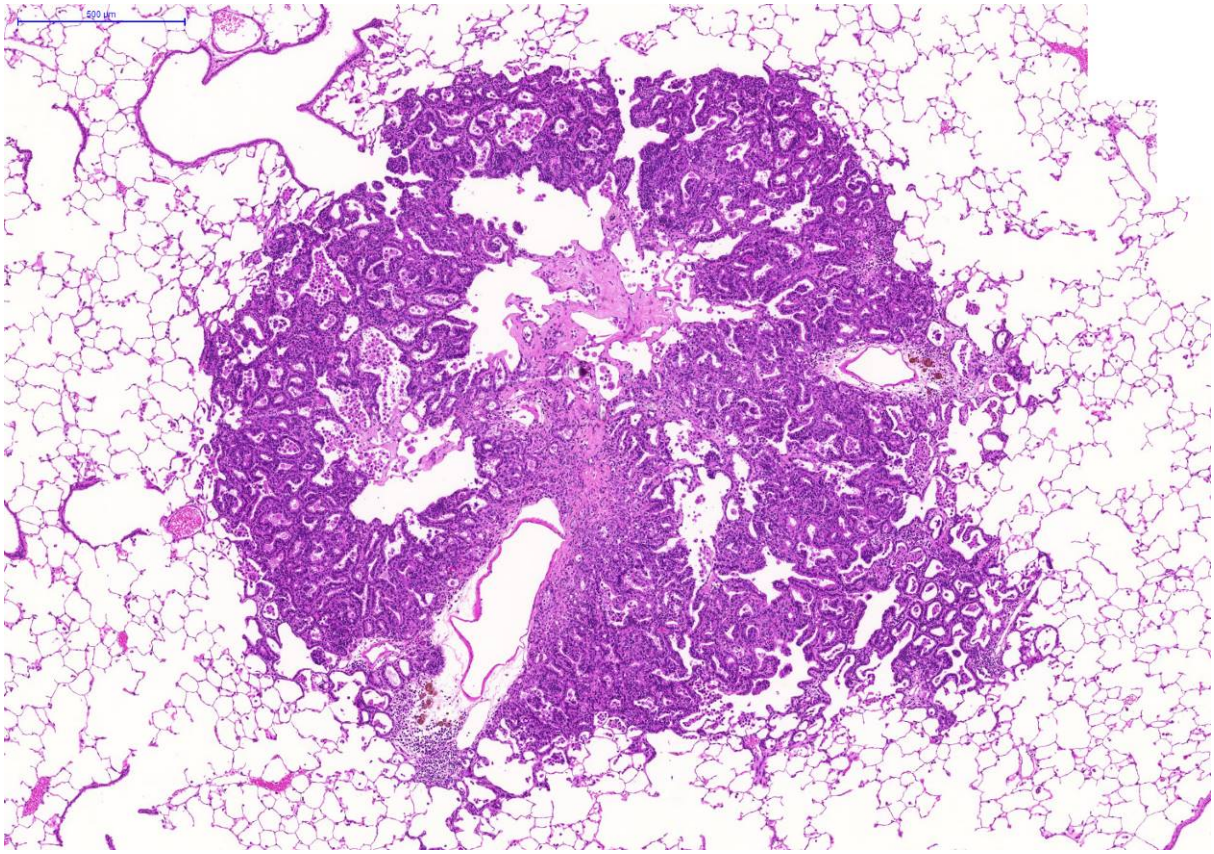
4.2.1.3 Lungs - proliferative lesions

Though only the relevant proliferative lesions are summarized in figure 19, all observed lesions are mentioned within the following chapter.

In single females one each of test group 01 (0.1 mg/m3 CeO2), 02 (0.3 mg/m3 CeO2) and 03 (1 mg/m3 CeO2), a bronchiolo-alveolar adenoma was observed as the only primary lung tumor. Pulmonary metastases from primary tumors in other organs, mainly from the uterus, were observed in 5/50, 1/50, 5/50, 3/50 and 4/50 females of the clean air control (test group 00), the 0.1 mg/m3 (test group 01), 0.3 mg/m3 (test group 02), 1 mg/m3 (test group 03) and 3 mg/m3 (test group 04) CeO2 exposure groups, respectively. These incidences include also lymphoma/leukemia cell infiltration observed in single females each of test group 00, 01, 02 and 04, histiocytic sarcoma cell infiltration in a single rat of test group 00 and tumor cell infiltration from a mediastinal fibrosarcoma in another female control animal.

Bronchiolo-alveolar hyperplasia with atypia, either of the alveolar or mixed type is considered to be a pre-neoplastic lesion. The alveolar type of this focal change was observed in a single female of test group 03 (moderate), at incidences of 2/50 in test group 01 (both severe) and 3/50 in test group 04 (1/50 moderate, 2/50 severe). The mixed type of bronchiolo-alveolar hyperplasia with atypia occurred focally in single rats of test groups 01 (moderate) and 03 (severe), while 2/50 females of test group 04 (1/50 moderate, 1/50 severe) were affected by this change. The combined incidences for both types of this pre-neoplastic lesion were: 0/50, 3/50, 0/50, 2/50 and 5/50 in test groups 00, 01, 02, 03 and 04, respectively.

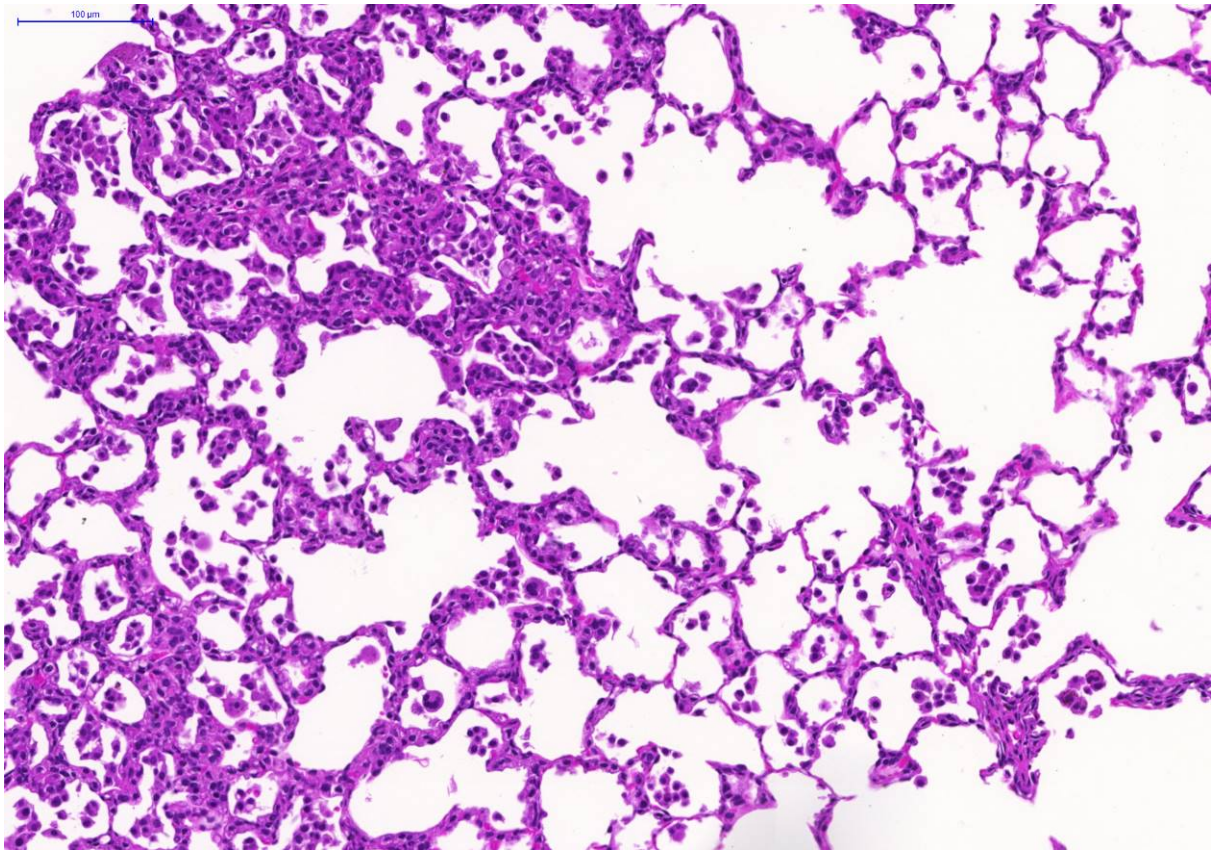
Figure 16: Hematoxylin and eosin stained lung slice showing a bronchiolo-alveolar hyperplasia with atypia



This picture shows a severe focal bronchiolo-alveolar hyperplasia with atypia in a rat lung from the 0.1 mg/m^3 CeO_2 dose group. Centrally there is fibrosis and marked inflammatory cell infiltration of the lesion visible.

Bronchiolo-alveolar hyperplasia (without cellular atypia) **of the alveolar or mixed type** was a common focal or multifocal proliferative change in all test groups. The alveolar type of bronchiolo-alveolar hyperplasia (proliferation of type II pneumocytes) was observed with a dose-dependent increase in incidences and severity grades in the CeO_2 exposure groups: 6/50 in test group 00 (2/50 very slight, 4/50 slight), 5/50 in test group 01 (2/50 very slight, 2/50 slight, 1/50 moderate), 9/50 in test group 02 (2/50 very slight, 7/50 slight), 12/50 in test group 03 (2/50 very slight, 7/50 slight, 3/50 moderate) and 33/50 in test group 04 (9/50 very slight, 20/50 slight, 2/50 moderate, 2/50 severe). The difference between the control group 00 and test group 04 was statistically significant. (Multi)focal **bronchiolo-alveolar hyperplasia of the mixed type** (mixture of type II cell and respiratory epithelial cell proliferation) was diagnosed in 3/50 females of test group 01 (2/50 very slight, 1/50 slight), no females of test group 02, 2/50 rats of test group 03 (1/50 slight, 1/50 severe) and 4/50 females of test group 04 (all slight). The combined incidences for both types of bronchiolo-alveolar hyperplasia were: 6/50, 8/50, 9/50, 14/50 and 37/50 in test groups 00, 01, 02, 03 and 04, respectively.

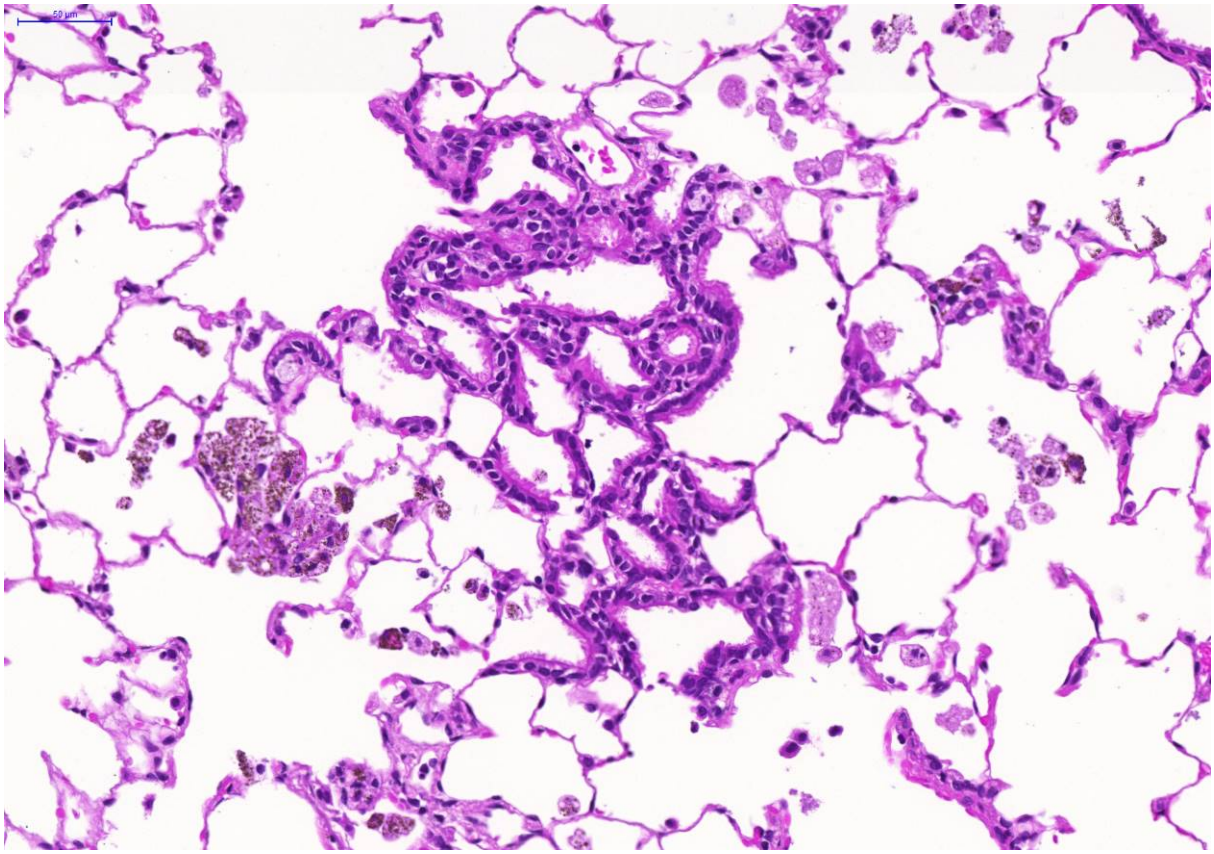
Figure 17: Hematoxylin and eosin stained lung slice with a bronchiolo-alveolar hyperplasia of the alveolar type



This picture shows a bronchiolo-alveolar hyperplasia of the alveolar type in the lung of a rat from the 3 mg/m^3 CeO_2 dose group. The hyperplastic alveolar cells (without atypia) lining the alveoli can easily be seen.

The incidences of (multi)focal **bronchiolo-alveolar hyperplasia of the bronchiolar type** (Synonym: **alveolar bronchiolization**) were significantly increased in all CeO_2 exposure groups as compared to the control group. There was also a dose-dependent increase in the severity grade in the mid- and high-dose CeO_2 exposure groups (test groups 03 and 04). This type of hyperplasia is characterized by extension of respiratory epithelium from the terminal bronchioles into the associated alveolar ducts and alveoli and is interpreted as an attempt to increase the clearance rate of inhaled particles by extension of the so-called „mucociliary escalator". The incidences were 23/50 (21/50 very slight, 2/50 slight), 47/50 (43/50 very slight, 4/50 slight), 45/50 (41/50 very slight, 4/50 slight), 45/50 (29/50 very slight, 16/50 slight) and 49/50 (29/50 very slight, 17/50 slight, 3/50 moderate) in test groups 00, 01, 02, 03 and 04, respectively.

Figure 18: Hematoxylin and eosin stained lung slice with a bronchiolo-alveolar hyperplasia of the alveolar type



This picture shows a slight focal alveolar bronchiolization and accumulation of particle-laden macrophages in the lung of a rat from the 1 mg/m^3 CeO_2 dose group.

The incidences of (multi)focal **bronchial/bronchiolar neuroendocrine cell hyperplasia** were 8/50 (6/50 very slight, 2/50 slight), 16/50 (8/50 very slight, 8/50 slight), 5/50 (4/50 very slight, 1/50 slight), 12/50 (8/50 very slight, 4/50 slight) and 18/50 (10/50 very slight, 7/50 slight, 1/50 moderate) in test groups 00, 01, 02, 03 and 04, respectively. Although the difference between test group 00 and test group 04 was statistically significant, this change is considered to be spontaneous in origin and has not been related to nanoparticle exposure so far. Further hyperplastic/metaplastic lesions in the lungs of the main group animals showed an incidental occurrence and are considered to be unrelated to exposure. These were slight focal **alveolar squamous cell metaplasia** (incidence: 1/50, test groups 01 and 02), (multi)focal very slight to moderate **bronchial/bronchiolar mucous cell hyperplasia** (incidence: 1/50 – 2/50, all test groups), focal very slight **pleural mesothelial hyperplasia** (incidence: 1/50, test group 02), (multi)focal slight **bronchial respiratory epithelial hyperplasia** (incidence: 1/50, test groups 00 and 02) and multifocal slight **bronchial respiratory epithelial squamous cell metaplasia** (incidence: 1/50, test group 02). The latter two lesions in test group 02 (animal no. 213) were related to a foreign body pneumonia caused by aspiration of plant fibers.

Figure 19 Relevant proliferative lesions of the main group animals (24 months)

Treatment No. of animals (♀)	Clean Air 50	CeO_2 0.1 mg/m³ 50	CeO_2 0.3 mg/m³ 50	CeO_2 1 mg/m³ 50	CeO_2 3 mg/m³ 50
Adenoma, bronchiolo-alveolar	**0**	**1**	**1**	**1**	**0**
Metastasis/-es from primary tumors in other organs	**5**	**1**	**5**	**3**	**4**
Hyperplasia, bronchiolo-alveolar, with atypia	**0**	**3**	**0**	**2**	**5**
Mean Score	***0.0***	***0.2***	***0.1***	***0.2***	***0.4***
Hyperplasia, bronchiolo-alveolar, alveolar/mixed type	**6**	**8**	**9**	**14**	**37*****
Mean Score	***0.2***	***0.3***	***0.3***	***0.6***	***1.4***
Alveolar bronchiolization	**23**	**47*****	**45*****	**45*****	**49*****
Mean Score	***0.5***	***1.0***	***1.0***	***1.2***	***1.4***

Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean score describes average score of the animals in the group using scores from 0 up to 5 (very severe)

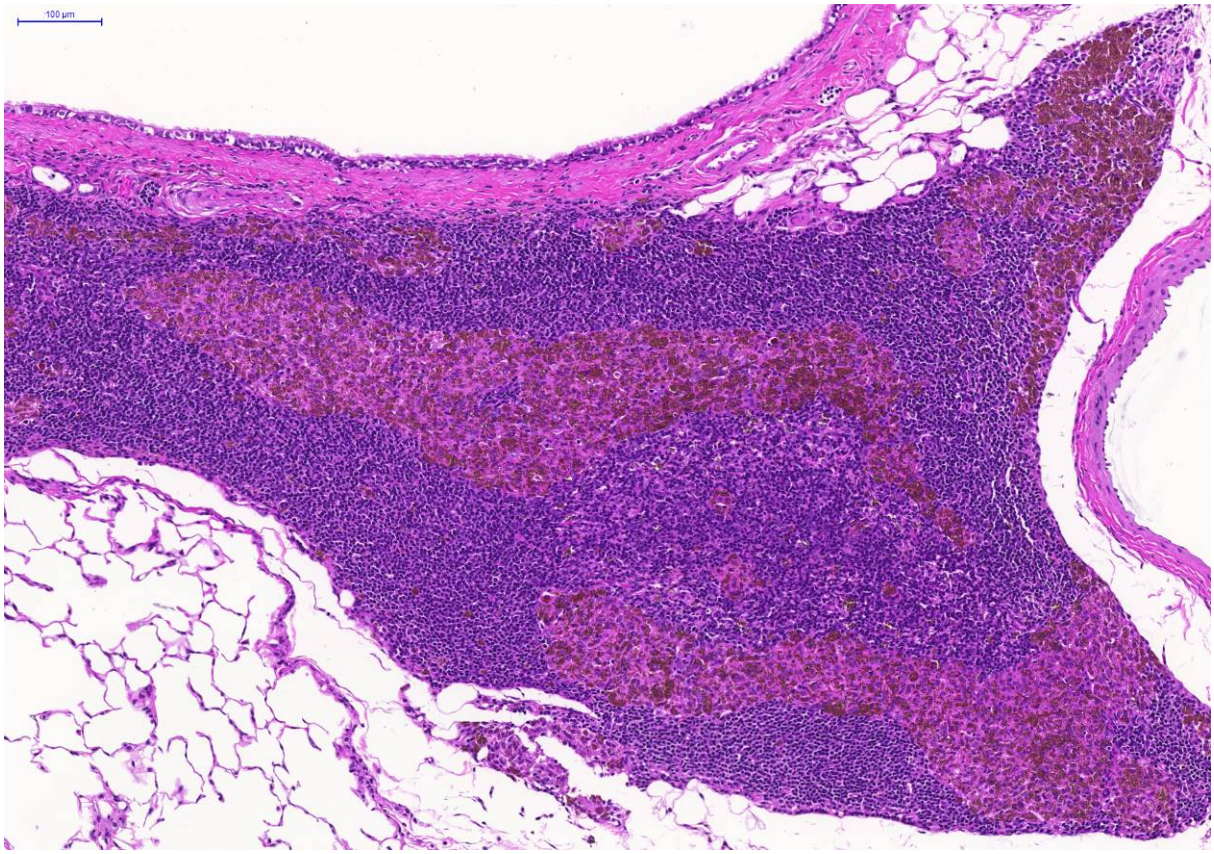
4.2.1.4 Lungs - non-proliferative lesions

Though only the relevant non-proliferative lesions are summarized in figure 23, all observed lesions are mentioned within the following chapter.

Spontaneous (multi)focal alveolar/interstitial accumulations of macrophages (Synonym: macrophage aggregation) are commonly seen in this strain of rats. In the clean air control group (test group 00), 44/50 rats showed this change (31/50 very slight, 11/50 slight, 2/50 moderate). Due to the high prevalence of spontaneous macrophage accumulation in the control group, the incidences of alveolar/interstitial accumulation of particle-laden macrophages in the exposure groups were not significantly different, but the mean grade showed a clear dose-response relationship from very slight to slight and moderate in the high-dose CeO_2 group (test group 04). The incidences were: 50/50 (38/50 very slight, 12/50 slight), 50/50 (21/50 very slight, 27/50 slight, 2/50 moderate), 50/50 (7/50 very slight, 34/50 slight, 9/50 moderate) and 50/50 (46/50 moderate, 4/50 severe) in test groups 01, 02, 03 and 04, respectively.

Comparable frequencies and grades were also observed for (multi)focal **accumulation of particle-laden macrophages in the BALT (bronchus-associated lymphoid tissue**) in the exposure groups: 50/50 (30/50 very slight, 19/50 slight, 1/50 moderate), 49/50 (40/50 slight, 9/50 moderate), 50/50 (4/50 slight, 43/50 moderate, 3/50 severe) and 50/50 (39/50 moderate, 11/50 severe) in test groups 01, 02, 03 and 04, respectively. Deposits of particle-laden macrophages were present not only in alveoli but also in interstitial (intraseptal, peribronchiolar and perivascular) compartments. In addition, agglomerates of CeO_2 particles (**alveolar particles**) lying freely within alveoli were detectable in all CeO_2 exposed animals.

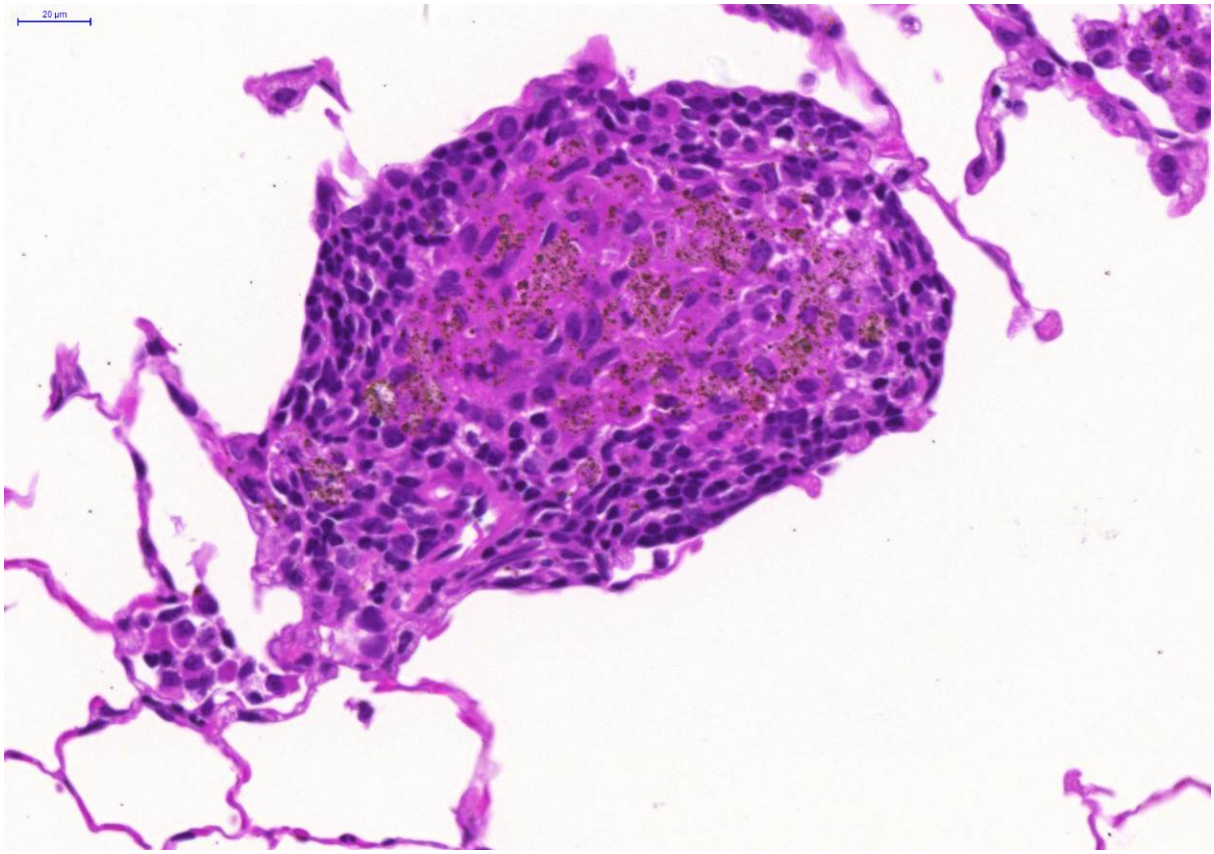
Figure 20: Hematoxylin and eosin stained lung slice with particle-laden macrophages in the BALT



This picture shows an accumulation of particle-laden macrophages in the BALT (bronchus-associated lymphoid tissue) within the lung of a rat from the 3 mg/m^3 CeO_2 dose group.

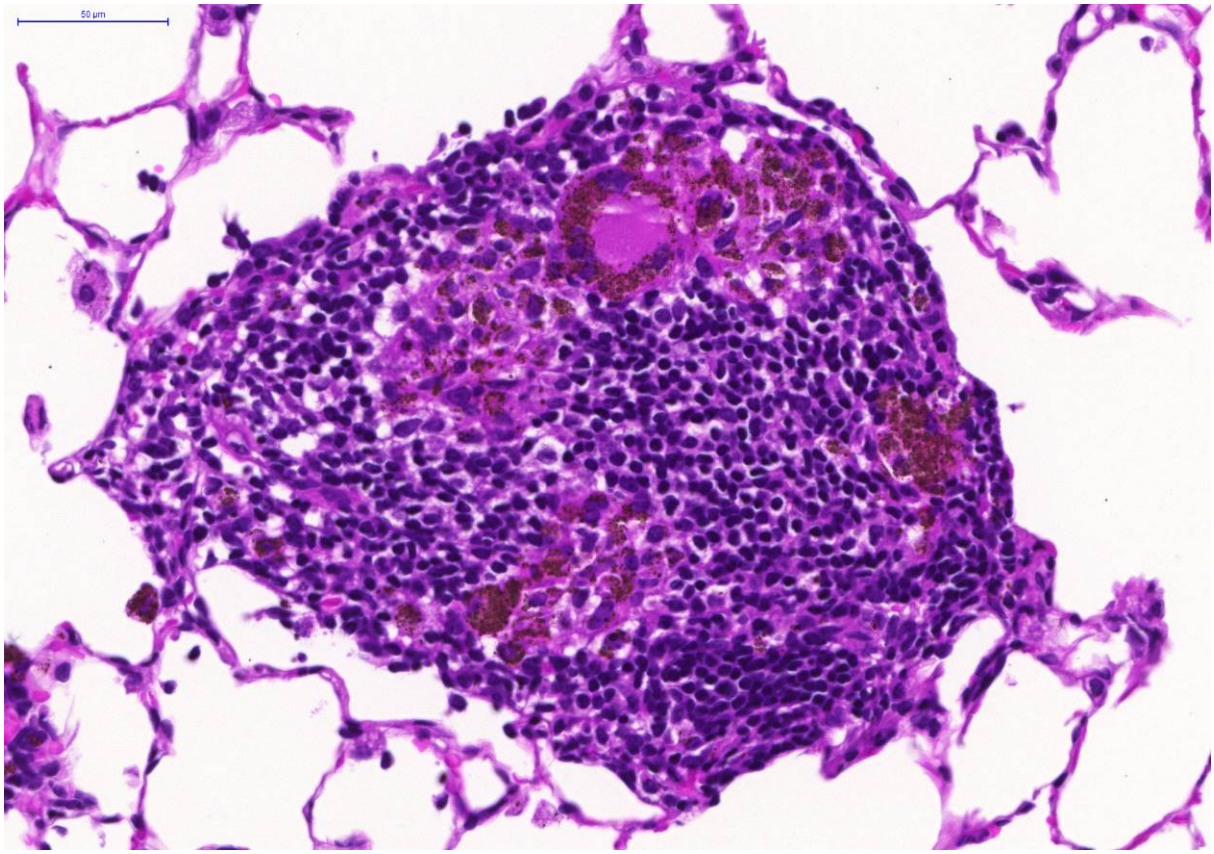
A (multi)focal granulomatous type of inflammation with formation of small (micro)granulomas was specific for CeO_2 and not observed in the control group (test group 00). The term '**alveolar/interstitial granulomatous inflammation**' was used only, if (mixed) inflammatory cell infiltration, syncytial giant cells and interstitial fibrosis were present in conjunction to form a granuloma-like focal lesion. In the CeO_2 exposure groups, there was a significant dose-dependent increase of this change from 11/50 (8/50 very slight, 3/50 slight) in test group 01 and 21/50 (17/50 very slight, 4/50 slight) in test group 02 to 44/50 affected rats each in test group 03 (31/50 very slight, 13/50 slight) and 04 (17/50 very slight, 27/50 slight).

Figure 21: Hematoxylin and eosin stained lung slice with a cerium dioxide-induced micrograniloma



This picture shows a high magnification of a cerium dioxide-induced (micro)granuloma in the lung of a rat from the 0.3 mg/m^3 CeO_2 dose group. There is a rim of lymphocytic cells surrounding particle-laden macrophages in the center of the granuloma.

Figure 22: Hematoxylin and eosin stained lung slice with another example of a cerium dioxide-induced micrograniloma



This picture shows a high magnification of a cerium dioxide-induced (micro)granuloma composed of lymphocytes surrounding particle-laden macrophages and particle-laden syncytial giant cell. The lung is from a rat of the 3 mg/m^3 CeO_2 dose group

Alveolar/interstitial inflammatory cell infiltration was usually associated with macrophage aggregates and affected nearly all animals with a dose-related increase of the mean severity grades in the cerium groups. It has to be emphasized, that the CeO_2-induced inflammatory cell reaction was mainly mononuclear with only few granulocytes present. Another observation was a marked individual variation regarding the intensity of the inflammatory response in animals of all CeO_2 exposure groups, but to some extent also in the control group. About one third of the rats showed a very weak and another third a very intense inflammatory reaction, while the others were somewhere in between. The reason for this variation is unknown and probably due to genetic and other differences in this outbred strain of rats. The incidences of (multi)focal alveolar/interstitial inflammatory cell infiltration were 46/50 (41/50 very slight, 5/50 slight), 49/50 (39/50 very slight, 10/50 slight), 49/50 (35/50 very slight, 14/50 slight), 50/50 (13/50 very slight, 37/50 slight) and 50/50 (3/50 very slight, 42/50 slight, 5/50 moderate) in test groups 00, 01, 02, 03 and 04, respectively. In addition, between 41/50 and 50/50 animals of all test groups showed (multi)focal very slight to slight **pleural mononuclear cell infiltration** with the highest severity grades in the mid- and high-dose cerium groups (test groups 03 and 04).

Syncytial giant cells - mainly particle-laden - were present within the (micro)granulomas as part of the granulomatous inflammation induced by CeO_2 and sometimes within the BALT of the cerium groups. However, they occurred also regularly within cholesterol granulomas (see below) which could be diagnosed in all test groups. The incidences of (multi)focal syncytial giant cells were significantly increased in all CeO_2 exposure groups as compared to the control: 7/50 (all very slight), 29/50 (27/50 very slight, 2/50 slight), 46/50 (43/50 very slight, 3/50 slight), 50/50 (all very slight) and 50/50 (all very slight) in test groups 00, 01, 02, 03 and 04, respectively. The amount of the intracellular particle-

load in both single-nucleated macrophages and multinucleated syncytial giant cells corresponded well to the used CeO_2 exposure dose.

Minimal to slight **interstitial fibrosis** was also seen dose-related in all CeO_2 exposure groups at a significant degree. (Multi)focal interstitial (mainly intraseptal) fibrosis was diagnosed in 39/50 (34/50 very slight, 5/50 slight), 49/50 (40/50 very slight, 8/50 slight, 1/50 moderate), 49/50 (39/50 very slight, 10/50 slight), 50/50 (20/50 very slight, 30/50 slight) and 50/50 (47/50 slight, 3/50 moderate) females of test groups 00, 01, 02, 03 and 04, respectively. In addition, (multi)focal **pleural fibrosis** was observed dose-related in 43/50 (39/50 very slight, 3/50 slight, 1/50 moderate), 45/50 (38/50 very slight, 7/50 slight), 48/50 (39/50 very slight, 9/50 slight), 50/50 (31/50 very slight, 19/50 slight) and 50/50 (23/50 very slight, 27/50 slight) animals of test groups 00, 01, 02, 03 and 04, respectively. The difference between test groups 03 and 04 and the control group 00 was statistically significant.

(Multi)focal **alveolar lipoproteinosis** was another non-proliferative lesion which showed a dose-related increase in the cerium exposure groups. The incidences were: 4/50 (2/50 very slight, 2/50 slight), 4/50 (3/50 very slight, 1/50 slight), 6/50 (all very slight), 28/50 (22/50 very slight, 3/50 slight, 2/50 moderate, 1/50 severe) and 50/50 (6/50 very slight, 35/50 slight, 6/50 moderate, 3/50 severe) in test groups 00, 01, 02, 03 and 04, respectively. A statistically significant increase was observed in test groups 03 and 04. The intra-alveolar lipoproteinaceous material was greyish-granular, eosinophilic or a mixture of both components and often mixed with particle agglomerations reflecting mainly an origin from degenerating particle-laden macrophages. If only greyish-granular lipoproteinaceous material was present in the alveoli, the lipoproteinosis was graded “very slight”.

In areas of pronounced degeneration of (particle-laden) foamy macrophages, development of **cholesterol granulomas** could frequently be observed. The incidence of (multi)focal cholesterol granulomas was very variable in the control and cerium groups: 18/50 (12/50 very slight, 6/50 slight), 7/50 (5/50 very slight, 2/50 slight), 13/50 (6/50 very slight, 7/50 slight), 20/50 (7/50 very slight, 12/50 slight, 1/50 moderate) and 8/50 (2/50 very slight, 3/50 slight, 3/50 moderate) in test groups 00, 01, 02, 03 and 04, respectively. Cholesterol granulomas were mainly observed in subpleural locations and composed of cholesterol crystals surrounded by syncytial giant cells, (mixed) inflammatory cells, interstitial and pleural fibrosis.
Another common finding which showed a statistically significant increase in test groups 03 and 04 was (**chondro-) osseous metaplasia**. This change describes small circumscribed alveolar/interstitial areas, where interstitial (septal) cells have transdifferentiated into bone-forming cells (osteoblasts). (Multi)focal (chondro-) osseous metaplasia was seen in 9/50 (4/50 very slight, 5/50 slight), 16/50 (9/50 very slight, 7/50 slight), 16/50 (9/50 very slight, 7/50 slight), 27/50 (14/50 very slight, 13/50 slight) and 26/50 (10/50 very slight, 16/50 slight) animals of test groups 00, 01, 02, 03 and 04, respectively.
A related change, (multi)focal very slight or slight alveolar/interstitial **psammoma bodies** affected between 2/50 and 7/50 females per test group, but was unrelated to treatment.
(Multi)focal deposits of **intra-alveolar mucus** occurred in areas of more pronounced alveolar bronchiolization (see above) and was observed in a single control animal (test group 00) as well as in 6/50 (4/50 very slight, 2/50 slight) females of test group 04.

A large variety of **incidental pulmonary findings** were also observed in different test groups and included congestion, alveolar/interstitial hemorrhage, agonal blood aspiration, vascular thrombosis, intravascular granulocytic infiltration (granulocytosis), perivascular edema, pulmonary emboli (of foreign material), choristoma (occurrence of striated muscle cells within lung parenchyma), pulmonary cysts, keratin cysts of the BALT, peribronchial fibrosis, acute alveolar/interstitial inflammation, chronic bronchial granulomatous inflammation, foreign body granulomas, alveolar/interstitial necrosis, bronchial respiratory epithelial ulceration, yellow pigment deposits, ectasia of bronchial submucosal glands, and parenchymal collapse (atelectasis). Except thrombosis (incidence up to 6/50 rats per test group), all other findings occurred at frequencies between 1/50 and 2/50 animals per test group. All these findings were considered to be spontaneous in origin and unrelated to CeO_2 exposure.

Figure 23: Relevant non-proliferative lesions of the main group animals (24 months)

Treatment No. of animals (♀)	Clean Air 50	CeO_2 0.1 mg/m³ 50	CeO_2 0.3 mg/m³ 50	CeO_2 1 mg/m³ 50	CeO_2 3 mg/m³ 50
Macrophages, alveolar/interstitial	44	50[1)]	50[1)]	50[1)]	50[1)]
Mean Score	1.2	1.2	1.6	1.7	3.1
Inflammation, granulomatous	0	11***	21***	44***	44***
Mean Score	*0.0*	*0.3*	*0.5*	*1.1*	*1.4*
Infiltration, inflammatory cell	46	49	49	50	50
Mean Score	*1.0*	*1.2*	*1.3*	*1.7*	*2.0*
Fibrosis, interstitial	39	49**	49**	50***	50***
Mean Score	*0.9*	*1.2*	*1.2*	*1.6*	*2.1*
Granuloma(s), cholesterol	18	7*	13	20	8*
Mean Score	*0.5*	*0.2*	*0.4*	*0.7*	*0.3*
Lipoproteinosis, alveolar	4	4	6	28***	50***
Mean Score	*0.1*	*0.1*	*0.1*	*0.7*	*2.1*

[1)] particle-laden; level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean score describes average score of the animals in the group using scores from 0 up to 5 (very severe)

4.2.2 Post-exposure group animals (carcinogenicity, 30 months)

4.2.2.1 Gross lesions

As observed in the main group animals, the **lungs** revealed mainly white or grey foci on the surfaces measuring between 1 and 6 mm in diameter (table 9). These were dose-dependently increased in the cerium groups (up to 29/50) and corresponded predominantly with alveolar/interstitial accumulations of particle-laden macrophages or with metastasis/-es from primary tumors in other organs. A mass was seen in the lungs of a single rat of the CeO_2 low-dose exposure group (test group 51) and correlated also with a tumor metastasis from an extrapulmonary organ.

Discolorations were observed in 2/49 control rats (test group 50) and in a single animal of the CeO_2 low-dose group (test group 51) and correlated with congestion and hemorrhage.

Table 9: Macroscopic findings – lungs (post-exposure group animals)

Treatment	**Clean Air**	**CeO_2 0.1 mg/m³**	**CeO_2 0.3 mg/m³**	**CeO_2 1 mg/m³**	**CeO_2 3 mg/m³**
No. of rats	**49**	**48**	**49**	**49**	**50**
Discoloration	2	1			
Focus/Foci	12	11	17	19	29
Mass		1			

4.2.2.2 Histopathology

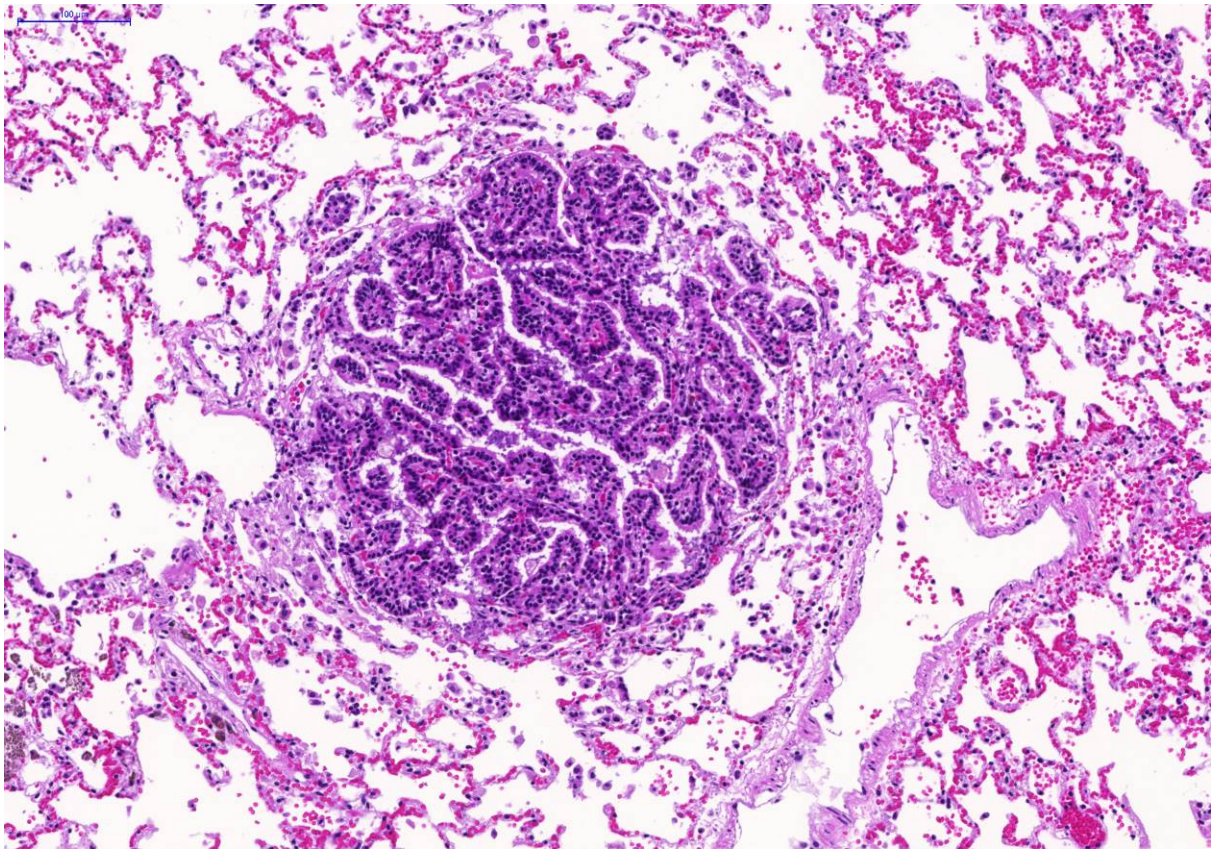
Within the following chapters, the results of the histopathology of the lungs of the 30 months groups applying the extended histopathology with step sections of the lung are presented.

4.2.2.3 Lungs – proliferative lesions

Though only the relevant proliferative lesions are summarized in figure 25, all observed lesions are mentioned within the following chapter.

In single females, one each of test group 52 (0.3 mg/m³ CeO_2), 53 (1 mg/m³ CeO_2) and 54 (3 mg/m³ CeO_2), a bronchiolo-alveolar adenoma was observed as the only **primary lung tumor**.

Figure 24: Hematoxylin and eosin stained lung slice with a small bronchiolo-alveolar adenoma



This picture shows a small bronchiolo-alveolar adenoma in the lung of a rat from the 1 mg/m³ CeO_2 dose group.

Similar to the main group animals, there was no significant difference in the frequency of metastases between the control and CeO_2 exposure groups. Pulmonary **metastases** from primary tumors in other organs, mainly from the uterus, were observed in 11/49, 9/48, 5/49, 8/49 and 4/50 females of the clean air control (test group 50), 0.1 mg/m³ (test group 51), 0.3 mg/m³ (test group 52), 1 mg/m³ (test group 53) and 3 mg/m³ (test group 54) CeO_2 exposure groups, respectively. These incidences include also lymphoma/leukemia cell infiltration observed in 2/50 rats of test group 54 and in single females each of test groups 50, 51, 52 and 53, respectively, as well as tumor cell infiltration from a malignant thymoma or a malignant mesothelioma of the thoracic cavity in 2/48 rats of test group 51.

As a pre-neoplastic lesion, (multi)focal **bronchiolo-alveolar hyperplasia with atypia** of the alveolar type was equally distributed over all test groups and occurred in single females each of test group 50 (moderate) and 52 (severe) as well as in 2/50 females of test group 54 (1/50 slight, 1/50 moderate).

Bronchiolo-alveolar hyperplasias of the alveolar or mixed type (without cellular atypia) were significantly increased only in the high-dose CeO_2 exposure group (test group 54). The alveolar type of hyperplasia (proliferation of type II pneumocytes) was observed (multi)focally in 2/49 (1/49 slight, 1/49 moderate), 5/48 (2/48 very slight, 3/48 slight), 6/49 (4/49 very slight, 2/49 slight), 6/49 (2/49 very slight, 4/49 slight) and 10/50 (5/50 very slight, 5/50 slight) rats of test groups 50, 51, 52, 53 and 54, respectively. (Multi)focal bronchiolo-alveolar hyperplasia of the mixed type (mixture of type II cell and respiratory epithelial cell proliferation) was diagnosed in 2/49 females of test group 50 (both slight), no females of test group 51, 1/49 females in test group 52 (slight), 3/49 females of test group 53 (2/49 very slight, 1/49 slight), in 2/50 rats of test group 54 (both slight). The combined incidences for both types of bronchiolo-alveolar hyperplasia were: 4/49, 5/48, 7/49, 9/49 and 12/50 in test groups 50, 51, 52, 53 and 54, respectively.

Comparable to the main group animals, the incidences of (multi)focal **bronchiolo-alveolar hyperplasia of the bronchiolar type** (Synonym: **alveolar bronchiolization**) were significantly increased in all CeO_2 exposure groups, except test group 51. There was also a dose-dependent increase in the severity grade in the CeO_2 exposure groups. The incidences were 14/49 (11/49 very slight, 3/49 slight), 22/48 (all very slight), 40/49 (39/49 very slight, 1/49 slight), 44/49 (33/49 very slight, 11/49 slight) and 43/50 (26/50 very slight, 16/50 slight, 1/50 moderate) in test groups 50, 51, 52, 53 and 54, respectively.

Regarding (multi)focal **bronchial/bronchiolar neuroendocrine cell hyperplasia,** there were no significant differences between test group 50 and any of the CeO_2 exposure groups, indicating that this change has to be considered as a spontaneous lesion. The incidences were: 8/49 (6/49 very slight, 2/49 slight), 2/48 (1/48 very slight, 1/48 slight), 6/49 (5/49 very slight, 1/49 slight), 7/49 (4/49 very slight, 3/49 slight) and 4/50 (3/50 very slight, 1/50 slight) in test groups 50, 51, 52, 53 and 54, respectively. Further hyperplastic/metaplastic lesions in the lungs of the post-exposure group animals showed an incidental occurrence and are also considered to be unrelated to exposure. These were (multi)focal slight **alveolar squamous cell metaplasia** (incidence: 1/49, test group 50), (multi)focal slight to moderate **bronchial/bronchiolar mucous cell hyperplasia** (incidence: 1/48 – 3/48, all test groups) and (multi)focal slight to moderate **bronchial respiratory epithelial hyperplasia** (incidence: 2/49, test group 50; 1/48, test group 01).

Figure 25: Relevant proliferative lesions of the post-exposure group animals (30 months)

Treatment	Clean Air	CeO_2 0.1 mg/m³	CeO_2 0.3 mg/m³	CeO_2 1 mg/m³	CeO_2 3 mg/m³
No. of animals (♀)	49	48	49	49	50
Adenoma, bronchiolo-alveolar	0	0	1	1	1
Metastasis/-es from primary tumors in other organs	11	9	5	8	4
Hyperplasia, bronchiolo-alveolar, with atypia	1	0	1	0	2
Mean Score	*0.1*	*0.0*	*0.2*	*0.0*	*0.2*
Hyperplasia, bronchiolo-alveolar, alveolar/mixed type	4	5	7	9	12*
Mean Score	*0.1*	*0.2*	*0.2*	*0.3*	*0.4*
Alveolar bronchiolization	14	22	40***	44***	43***
Mean Score	*0.3*	*0.5*	*0.8*	*1.1*	*1.2*

Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean score describes average score of the animals in the group using scores from 0 up to 5 (very severe)

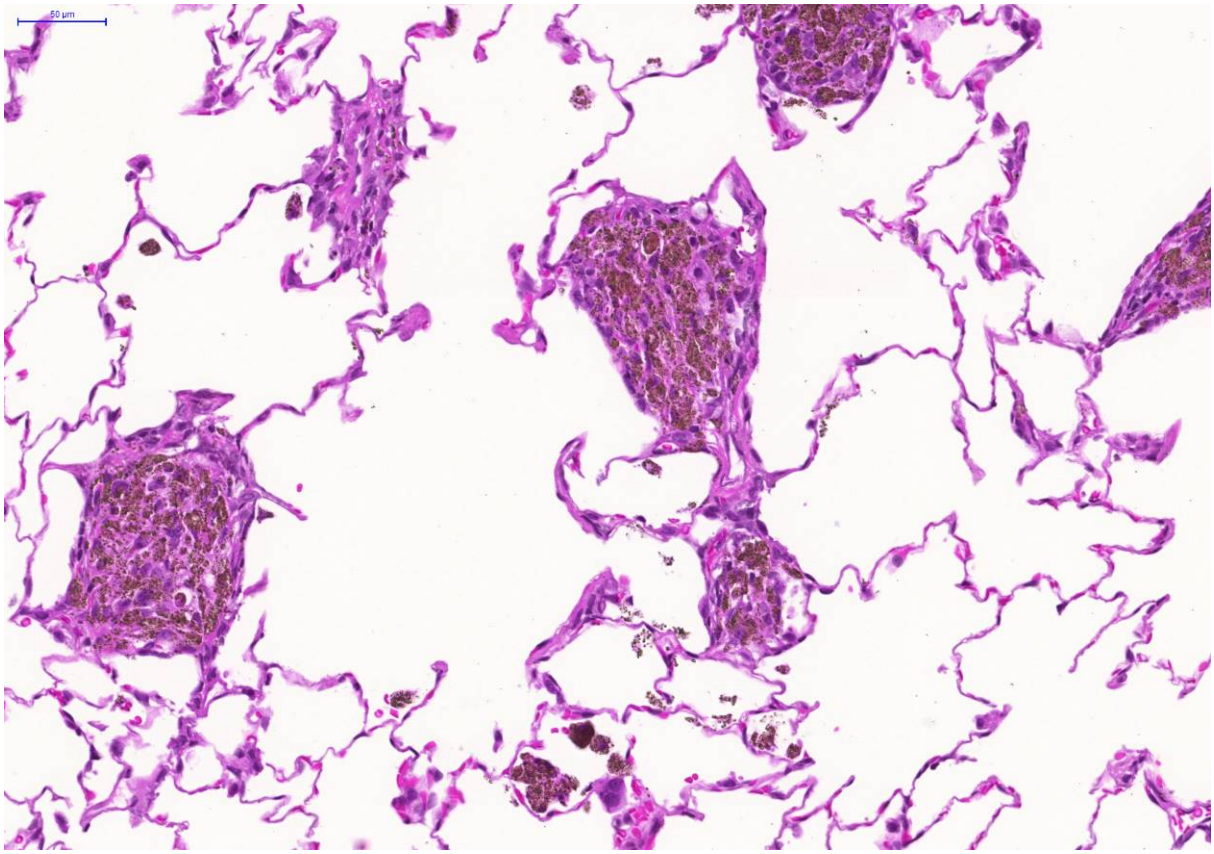
4.2.2.4 Lungs - non-proliferative lesions

Though only the relevant non-proliferative lesions are summarized in figure 29, all observed lesions are mentioned within the following chapter.

The incidences of non-proliferative lung lesions of the post-exposure group animals were also almost the same as observed after 24 months of exposure.

(Multi)focal **alveolar/interstitial accumulations of particle-laden macrophages** in the exposure groups were significantly increased as compared to the still very high control incidence of spontaneous alveolar **macrophage aggregation** and the mean grade again showed a dose-response relationship from very slight to moderate in the high-dose CeO_2 exposure group. The incidences were: 41/49 (21/49 very slight, 16/49 slight, 3/49 moderate), 48/48 (35/48 very slight, 13/48 slight), 49/49 (22/49 very slight, 25/49 slight, 2/49 moderate), 49/49 (4/49 very slight, 31/49 slight, 14/49 moderate) and 50/50 (5/50 slight, 43/50 moderate, 2/50 severe) in test groups 50, 51, 52, 53 and 54, respectively. Comparable frequencies and grades were also observed for (multi)focal **accumulation of particle-laden macrophages in the BALT (bronchus-associated lymphoid tissue):** 48/48 (9/48 very slight, 39/48 slight), 49/49 (3/49 very slight, 33/49 slight, 13/49 moderate), 49/49 (1/49 very slight, 6/49 slight, 42/49 moderate) and 50/50 (1/50 slight, 42/50 moderate, 7/50 severe) in test groups 51, 52, 53 and 54, respectively. Deposits of particle-laden macrophages were present not only in alveoli but also in interstitial (intraseptal, peribronchiolar and perivascular) compartments. In addition, agglomerates of CeO_2 particles (**alveolar particles**) lying freely within alveoli were detectable in nearly all CeO_2-exposed animals.

Figure 26: Hematoxylin and eosin stained lung slice with particle-laden macrophages in the lung interstitium



This picture shows the interstitialization of particle-laden macrophages and a mild interstitial fibrotic response with very few other inflammatory cells in the lung of a rat from the 3 mg/m³ CeO_2 dose group.

The specific (multi)focal **granulomatous inflammation** with formation of small (micro)granulomas was still present in the cerium groups. The term '**alveolar/interstitial granulomatous inflammation**' was used only, if (mixed) inflammatory cell infiltration, syncytial giant cells and interstitial fibrosis were present in conjunction to form a granuloma-like focal lesion. In the CeO_2 exposure groups, there was a statistically significant dose-dependent increase of this change from 5/48 (4/48 very slight, 1/48 slight) in test group 51 to 17/49 (12/49 very slight, 5/49 slight) in test group 52, 18/49 (16/49 very slight, 2/49 slight) in test group 53 and 26/50 (19/50 very slight, 7/50 slight) affected rats in test group 54. In contrast to the 24 months time point, the incidences of (multi)focal or diffuse **alveolar/interstitial inflammatory cell infiltration** in all CeO_2 exposure groups were significantly higher than in the control group. And similar to the main group rats, the CeO_2-induced inflammatory cell reaction was mainly mononuclear with only few granulocytes present and there was also a marked individual variation regarding the intensity of the inflammatory response. The incidences were: 28/49 (21/49 very slight, 6/49 slight), 43/48 (38/48 very slight, 5/48 slight), 49/49 (35/49 very slight, 14/49 slight), 49/49 (25/49 very slight, 23/49 slight, 1/49 moderate) and 50/50 (10/50 very slight, 40/50 slight) in test groups 50, 51, 52, 53 and 54, respectively. In addition, 7/49 (all very slight), 18/48 (all very slight), 40/49 (33/49 very slight, 7/49 slight), 38/49 (30/49 very slight, 8/49 slight) and 38/50 (25/50 very slight, 12/50 slight, 1/50 moderate) females of test groups 50, 51, 52, 53 and 54, respectively, showed (multi)focal **pleural mononuclear cell infiltration** with the highest severity grades in the mid- and high-dose cerium groups (test groups 53 and 54). The difference between the control group (test group 50) and all CeO_2 exposure groups was statistically significant.

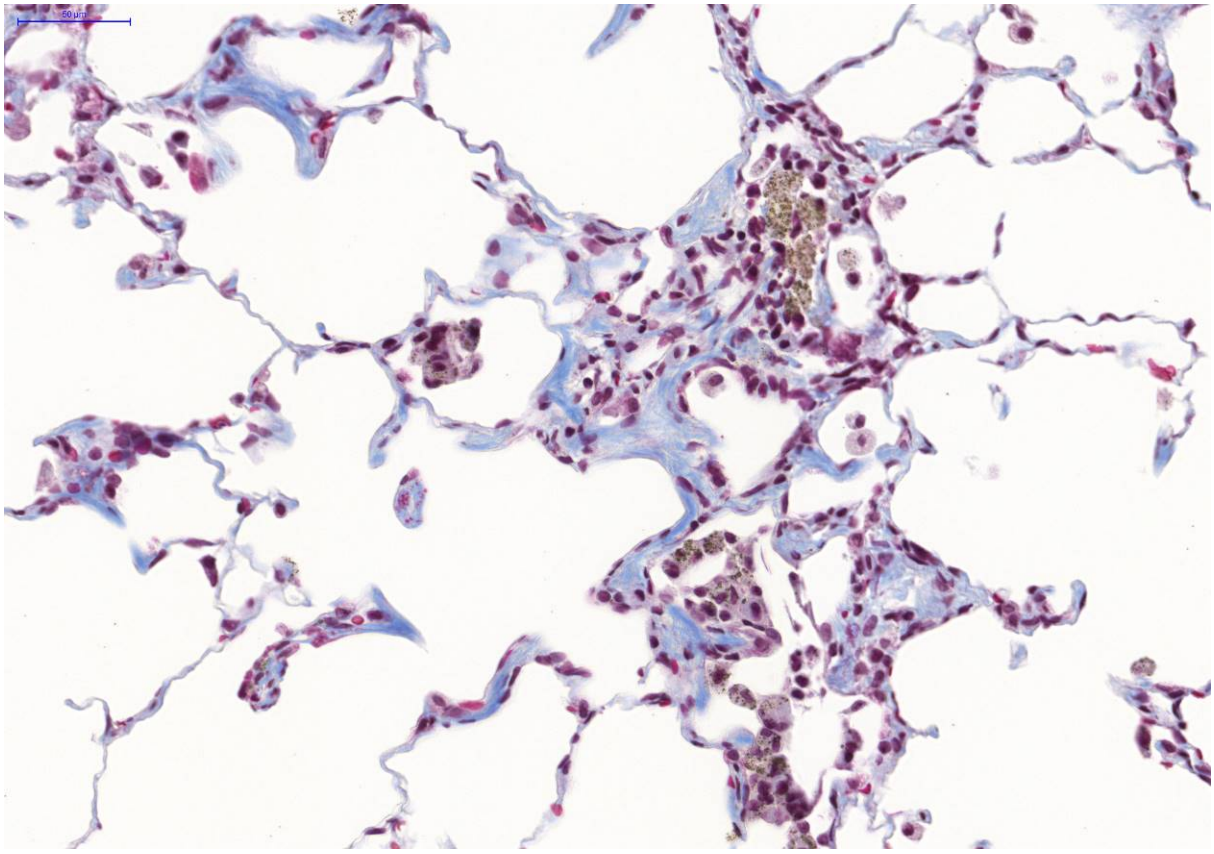
Syncytial giant cells - mainly particle-laden - were present within the (micro)granulomas induced by CeO_2, sometimes within the BALT of the cerium groups and regularly within cholesterol granulomas (see below). With the exception of test group 51, the incidences of (multi)focal syncytial giant cells

were significantly increased in all CeO_2 exposure groups as compared to the control: 7/49 (all very slight), 13/48 (12/48 very slight, 1/48 slight), 28/49 (all very slight), 29/49 (all very slight) and 41/50 (all very slight) in test groups 50, 51, 52, 53 and 54, respectively.

Similar to the main group animals, **interstitial fibrosis** was also seen dose-related in all CeO_2 exposure groups at a statistically significant degree. (Multi)focal interstitial (mainly intraseptal) fibrosis was diagnosed in 24/49 (20/49 very slight, 3/49 slight, 1/49 moderate), 48/48 (39/48 very slight, 9/48 slight), 49/49 (31/49 very slight, 17/49 slight, 1/49 moderate), 49/49 (22/49 very slight, 27/49 slight) and 50/50 (1/50 very slight, 47/50 slight, 2/50 moderate) females of test groups 50, 51, 52, 53 and 54, respectively.

In addition, (multi)focal **pleural fibrosis** was observed dose-related in 31/49 (26/49 very slight, 5/49 slight), 41/48 (39/48 very slight, 2/48 slight), 48/49 (44/49 very slight, 4/49 slight), 47/49 (37/49 very slight, 10/49 slight) and 50/50 (43/50 very slight, 7/50 slight) animals of test groups 50, 51, 52, 53 and 54, respectively. The difference between all CeO_2 exposure groups and the control group was statistically significant.

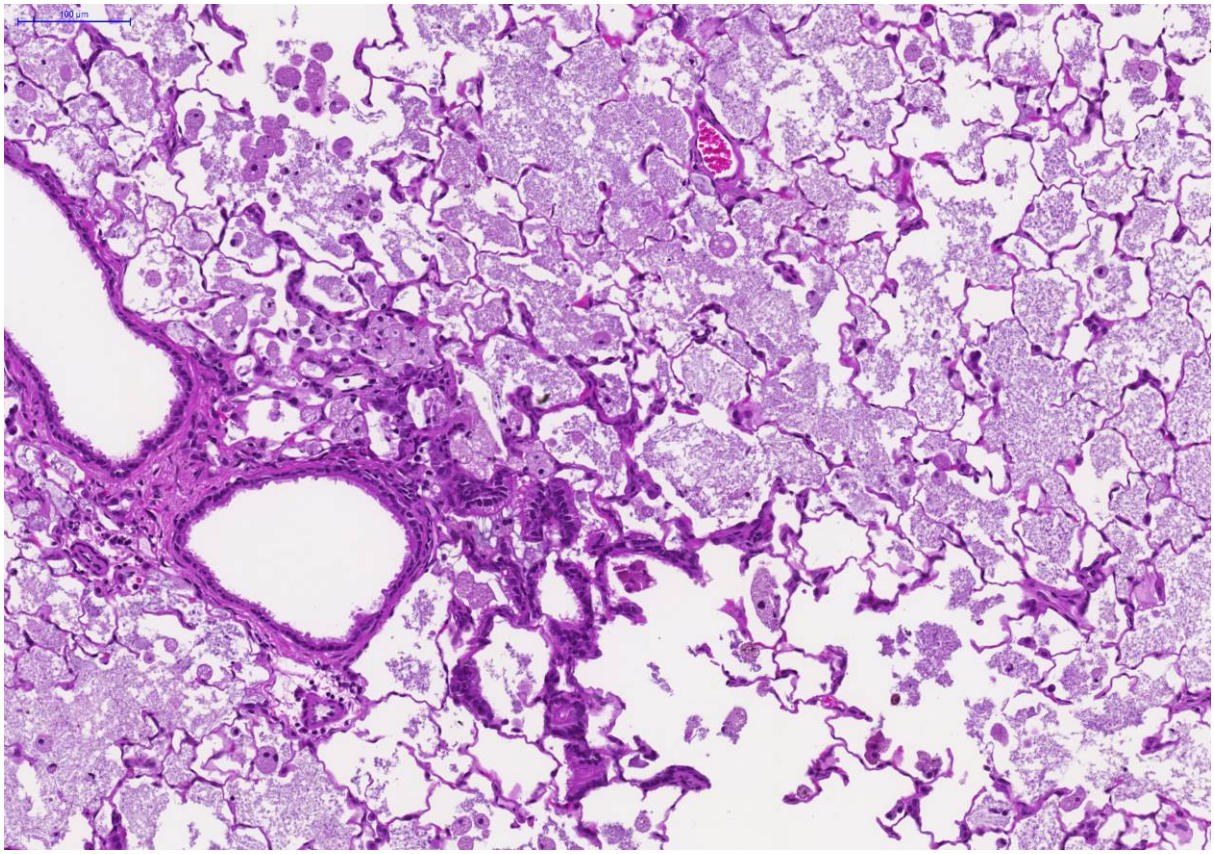
Figure 27: Masson trichrome stained lung slice with a slight fibrosis



This picture shows a Masson trichrome stained section depicting slight multifocal interstitial fibrosis and alveolar/interstitial accumulation of particle-laden macrophages in a lung of rat from the 1 mg/m³ CeO_2 dose group. Masson trichrome staining is specifically used to visualize fibrosis.

And similar to the results in the main group animals, **alveolar lipoproteinosis** was significantly increased only in the mid- and high-dose cerium groups. The incidences were: 3/49 (all very slight), 5/48 (all very slight), 7/49 (4/49 very slight, 3/49 slight), 26/49 (20/49 very slight, 5/49 slight, 1/49 moderate) and 46/50 (19/50 very slight, 19/50 slight, 8/50 moderate) in test groups 50, 51, 52, 53 and 54, respectively. The intra-alveolar lipoproteinaceous material was greyish-granular, eosinophilic or a mixture of both components and often mixed with particle agglomerations reflecting mainly an origin from degenerating particle-laden macrophages. If only greyish-granular lipoproteinaceous material was present in the alveoli, the lipoproteinosis was graded “very slight”.

Figure 28: Hematoxylin and eosin stained lung slice with a lipoproteinosis



This picture shows a rat lung from the 1 mg/m^3 CeO_2 dose group with a focal moderate accumulation of acidophilic, acellular material (lipoproteinosis) within the alveolar region. Due to the granularity of the lipoproteinosis, the lipoproteinaceous material is supposed to be mainly derived from decaying alveolar macrophages. This picture depicts also a slight focal alveolar bronchiolization adjacent to the bronchioles on the left side of the picture.

The frequency of **cholesterol granulomas,** which mainly developed in areas of pronounced degeneration of (particle-laden) foamy macrophages, was insignificantly increased in the mid- and high-dose cerium groups as compared to the control group. (Multi)focal cholesterol granulomas were observed in 7/49 (2/49 very slight, 4/49 slight, 1/49 moderate), 6/48 (3/48 very slight, 3/48 slight), 5/49 (2/49 very slight, 3/49 slight), 14/49 (3/49 very slight, 11/49 slight) and 11/50 (1/50 very slight, 6/50 slight, 4/50 moderate) rats of test groups 50, 51, 52, 53 and 54, respectively. Cholesterol granulomas were mainly observed in subpleural locations and composed of cholesterol crystals surrounded by syncytial giant cells, (mixed) inflammatory cells, interstitial and pleural fibrosis. (Multi)focal deposits of **intra-alveolar mucus** occurred in areas of more pronounced alveolar bronchiolization (see above) and was observed in 2/49 control animals (test group 50; 1/49 very slight, 1/49 slight), 3/49 (2/49 very slight, 1/49 slight) and 6/50 rats (1/50 very slight, 5/50 slight) of the mid- and high-dose CeO_2 exposure groups (test groups 53 and 54), respectively. In contrast to the results in the main group animals, **(chondro-) osseous metaplasias** were less commonly observed and at comparable incidences in all test groups. This change describes small circumscribed alveolar/interstitial areas, where interstitial (septal) cells have transdifferentiated into bone-forming cells (osteoblasts). (Multi)focal (chondro-) osseous metaplasia was seen in 4/49 (2/49 very slight, 2/49 slight), 4/48 (3/48 very slight, 1/48 slight), 5/49 (2/49 very slight, 3/49 slight), 5/49 (3/49 very slight, 2/49 slight) and 6/50 (3/50 very slight, 2/50 slight, 1/50 moderate) animals of test groups 50, 51, 52, 53 and 54, respectively.

A large variety of **incidental pulmonary findings** were also observed in different post-exposure test groups and included congestion, alveolar/interstitial hemorrhage, vascular thrombosis, alveolar emphysema, alveolar foreign bodies, foreign body granuloma, peribronchial fibrosis, alveolar/interstitial

psammoma bodies, alveolar accumulation of pigment-laden macrophages and luminal cellular debris. Except alveolar/interstitial hemorrhage (incidence up to 4/49 rats per test group), all other findings occurred at frequencies between 1/50 and 3/48 animals per test group. All these findings were considered to be spontaneous in origin and unrelated to nanoparticle exposure.

Figure 29: Relevant non-proliferative lesions of the post-exposure group animals (30 months)

Treatment No. of animals (♀)	Clean Air 49	CeO_2 0.1 mg/m³ 48	CeO_2 0.3 mg/m³ 49	CeO_2 1 mg/m³ 49	CeO_2 3 mg/m³ 50
Macrophages, alveolar/interstitial	41	48[1) **]	49[1) **]	49[1) **]	50[1) **]
Mean Score	1.3	1.3	1.6	2.2	2.9
Inflammation, granulomatous	0	5*	17***	18***	26***
Mean Score	*0.0*	*0.1*	*0.4*	*0.4*	*0.7*
Infiltration, inflammatory cell	28	43***	49***	49***	50***
Mean Score	*0.7*	*1.0*	*1.3*	*1.5*	*1.8*
Fibrosis, interstitial	24	48***	49***	49***	50***
Mean Score	*0.6*	*1.2*	*1.4*	*1.6*	*2.0*
Granuloma(s), cholesterol	7	6	5	14	11
Mean Score	*0.3*	*0.2*	*0.2*	*0.5*	*0.5*
Lipoproteinosis, alveolar	3	5	7	26***	46***
Mean Score	*0.1*	*0.1*	*0.2*	*0.7*	*1.6*

[1)] particle-laden; level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Mean score describes average score of the animals in the group using scores from 0 up to 5 (very severe)

4.3 Summary and Conclusion (carcinogenicity study, 24 months and 30 months)

The current carcinogenicity study was histologically evaluated using serial step sections of 500 µm through the entire lung to enhance the detection rate of tumors and pre-neoplastic lesions. By this technique, a total of more than 60 histological lung sections were obtained from each animal as compared to 6 sections with the routine examination method, thus allowing a much more rigorous statistical comparison of findings with concurrent controls. According to Kolling et al. (2008), who employed this technique in the evaluation of another comprehensive rat inhalation carcinogenicity study (supported by the Federal Environmental Agency and Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, Germany, Grant no. 29861273 and 20361215), the detection rate of neoplasms and pre-neoplastic lesions was 2-3 times higher compared to the standard histological evaluation method of the lung. However, the benefit of the step section technique need to be balanced against the much higher financial effort.

Summarizing the results of the carcinogenicity study, there were no significant numbers of neoplasms in both the main (24 months exposure) and post-exposure (24 months exposure + 6 months recovery) CeO2 groups. The observed bronchiolo-alveolar adenomas in the CeO2 exposure groups are not untypical for rats of this strain and age and occasionally are also seen in untreated control animals. Even in the high-dose (3 mg/m3) CeO2 exposure group which has been shown to be in the lung "overload" range (Morrow, 1988; Schwotzer, 2017), only one bronchiolo-alveolar adenoma could be observed in

a single female of the 30 months age group. This shows that the CeO2-induced type of chronic inflammation in the lungs did not automatically lead to tumor formation as described for many other PSP (poorly soluble particles with low toxicity) at clear overload doses such as carbon black, titanium dioxide, talc, and coal dust (s. review by Nikula, 2000). In these rat carcinogenicity studies, increased tumor rates developed under the condition of impaired macrophage-mediated lung clearance (overload) and subsequent chronic inflammation. Besides bronchiolo-alveolar adenomas and adenocarcinomas, squamous cell tumors like (benign) cystic keratinizing epithelioma and (malignant) squamous cell carcinomas were commonly observed in the exposed rat lungs. For the pathogenesis of squamous cell tumors, a continuous progression from slight squamous cell metaplasia to very severe or „exaggerated" squamous cell metaplasia and finally to benign and malignant squamous cell tumors is possible and has been described (Dungworth et al., 1994; Boormann et al., 1996). In the present study, pre-neoplastic squamous cell lesions were completely absent in the CeO2 exposure groups. The observed few slight squamous cell metaplasias were incidental and unrelated to CeO2 exposure. Furthermore, there was also no increase in pre-neoplastic bronchiolo-alveolar hyperplasia with (cytological) atypia in the CeO2 exposure groups after 24 or 30 months. This kind of pre-neoplasia is characterized by usually large (severe and very severe) proliferations of type II alveolar pneumocytes (alveolar type) or type II pneumocytes intermixed with areas of alveolar bronchiolization (mixed type). In addition, these lesions show cytological abnormalities such as anisocytosis, nuclear or cytoplasmic pleomorphism, increased mitotic rate, etc., and are considered to progress to bronchiolo-alveolar adenomas and adenocarcinomas. On the other hand, bronchiolo-alveolar hyperplasias of the alveolar or mixed type (without atypia) showed a dose-dependent increase in the CeO2 exposure groups, especially after 24 months of exposure. In the high-dose CeO2 exposure group where this lesion was significantly increased at both sacrifice dates, there was a marked drop in the incidence after 30 months as compared to 24 months of exposure indicating the possibility of partial reversibility of this type of hyperplasia during the recovery period. The more pronounced hyperplasias, however, might probably also further develop to bronchiolo-alveolar hyperplasias with atypia (= pre-neoplasias).

The current results show that CeO2 induced a specific granulomatous inflammation in the lungs characterized by the formation of microgranulomas and the presence of syncytial giant cells. Many of these microgranulomas appeared as immune granulomas characterized by a rim of lymphocytes. Such granulomas are not seen with other poorly soluble particle with low toxicity (PSP), although the majority of the dose-related significant findings in the cerium groups are also typical for other PSP. These findings include:

1. increase of (particle-laden) macrophages (alveolar/interstitial, bronchus-associated lymphoid tissue [BALT]),
2. inflammatory cell infiltration (mainly mononuclear cells, few granulocytes),
3. alveolar bronchiolization (bronchiolo-alveolar hyperplasia, bronchiolar type),
4. interstitial and pleural fibrosis,
5. alveolar lipoproteinosis, and
6. cholesterol granulomas.

Chronic exposure of rats to high (overload) concentrations of PSP causes progressive histopathologic alterations in the lungs. Incidence and severity of the alterations and their rate of progression are influenced by the inherent toxicity of the particle and dose factors such as exposure concentration or lung burden (Nikula, 2000). In a previous short-term nose-only inhalation toxicity study with 10 mg/m^3 nano-sized TiO_2 particles, which is considered to be in the slight overload range, Eydner and coworkers (2012) observed an inflammatory response characterized by minimal alveolar/interstitial infiltration of mononuclear cells, neutrophilic granulocytes and minimal bronchiolo-alveolar hyperplasia. They could not detect marked differences between lung effects of nanoscale and fine TiO_2 particles and concluded that the observed effects were related to destruction of alveolar macrophages due to their high particle load rather than to any kind of specific TiO_2 particle toxicity. The PSP induced inflammation in the lung usually starts with an initial alveolar increase of particle-laden macrophages, which tend to aggregate in the area of the bronchiolo-alveolar junction. Depending on time, exposure

concentration and particle characteristics, the aggregated macrophages may degenerate with subsequent release of various inflammatory mediators and alveolar accumulation of cellular debris and particles. In these areas, a progressive inflammation, formation of cholesterol crystals, cholesterol granulomas, bronchiolo-alveolar hyperplasias including alveolar bronchiolization, squamous cell metaplasias, alveolar lipoproteinosis and interstitial fibrosis may then develop as secondary alterations. These events and their dependency on the used exposure concentration have been described for PSP at high overload doses such as TiO_2 (Wahrheit et al., 1997; Lee et al., 1986) and carbon black (Driscoll et al., 1996; Elder et al., 2005).

A surprising and unexpected result of the current study was that CeO_2 already in the lowest dose (0.1 mg/m^3) produced inflammatory lung lesions including microgranulomas, which persisted until 30 months. These findings are in compliance with the changes in gene expression of inflammatory mediators in pneumocytes type II after subchronic low dose CeO_2 exposure (Schwotzer et al., 2018). This highly sensitive method predicted the upcoming inflammation at an early stage of exposure and showed that pneumocytes type II contribute to the inflammatory reaction (Schwotzer et al., 2018).

The CeO_2-induced (micro)granulomatous inflammation was somewhat different to the granulomatous inflammation observed in rat lungs after repeated intratracheal instillations of non-biopersistent amorphous SiO_2 (Ernst et al., 2002; Kolling et al., 2008). To compensate the high clearance rate, up to 30 intratracheal instillations of 0.5 mg amorphous SiO_2 (Aerosil® 150) were applied in this study to rats at intervals of 14 days in order to produce a persistent inflammation. The histopathological findings revealed a multifocal granulomatous inflammation, which was characterized by a lack of intraalveolar macrophages and alveolar lipoproteinosis. The granulomas induced with amorphous SiO_2 had a much larger size than the CeO_2-induced (micro)granulomas in the present study and seemed to result from acute epithelial damage at the site of particle deposition with subsequent production of granulation tissue. Due to the fast removal rate of the SiO_2 particles from the deposition site, those granulomas became progressively 'interstitialized' and resolved by leaving focal fibrotic scar tissue (Ernst et al., 2002). However, some reversibility can be assumed during the 6-month post-exposure recovery period for the majority of the CeO_2-induced inflammatory changes in the present study. The CeO_2-induced microgranulomas showed also signs of "scarring" with a diminished proportion of inflammatory cells (mononuclear cells and syncytial giant cells) and appeared as small spots of collagen surrounding CeO_2 particle deposits (aggregations). It has to be emphasized, however, that the observed interstitial and pleural fibrosis in the present study was clearly not a "stand-alone" finding, but always associated with inflammatory changes and macrophage aggregations.

On the other hand, there was a marked individual variation in the inflammatory response of the rats in all test groups, especially in the low CeO_2 doses after 24 months of exposure as well as after 30 months. The reason for this difference in the inflammatory reaction to CeO_2 inhalation is unknown, but is probably due to genetic variability of the rat strain being the outbred strain [Han: WIST].

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